CYTOGENETICAL, MICROMORPHOLOGICAL, MOLECULAR AND PHYTOCHEMICAL CHARACTERIZATION OF SELECTED SPECIES OF THE GENUS *AMARANTHUS* L. (AMARANTHACEAE)

Thesis
submitted to the University of Calicut
for the award of the degree of

DOCTOR OF PHILOSOPHY IN BOTANY

By

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CERTIFICATE

This is to certify that no corrections were reported by both the examiners in the thesis entitled **"Cytogenetical, Micromorphological, Molecular and Phytochemical Characterization of Selected Species of the Genus *Amaranthus* L. (Amaranthaceae)"**, submitted by Mrs. Prajitha V.

Yours truly

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DECLARATION

I, Prajitha V., hereby declare that the thesis entitled **"Cytogenetical, micromorphological, molecular and phytochemical characterization of selected species of the genus *Amaranthus* L. (Amaranthaceae)"** submitted to the University of Calicut, for the award of the degree of DOCTOR OF PHILOSOPHY IN BOTANY is a record of original research work done by me under the supervision and guidance of Dr John E. Thoppil, Professor, Department of Botany, University of Calicut and that it has not formed the basis for the award of any degree or diploma to any candidate of any University.

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Supervising Teacher

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ABBREVIATIONS

µg	-	Microgram
µl	-	Microlitre
µM	-	Micromolar
A	-	Degree of asymmetry of karyotypes
A_1	-	Intrachromosomal asymmetry index
A_2	-	Interchromosomal asymmetry index
AAI	-	American Amaranth Institute
AB	-	*Amaranthus blitum*
AC	-	*Amaranthus caudatus*
AD	-	*Amaranthus dubius*
AFLP	-	Amplified Fragment Length Polymorphism
AH	-	*Amaranthus hybridus* subsp. *hybridus*
AI	-	Asymmetry Index
$AlCl_3$	-	Aluminium chloride
AOAC	-	Association of Official Analytical Chemists
APG	-	Angiosperm Phylogeny Group
approx.	-	Approximately
AR	-	*Amaranthus hybridus* subsp. *cruentus*
As K%	-	Karyotype asymmetry index
AS	-	*Amaranthus spinosus*
AT	-	*Amaranthus tricolor*
AV	-	*Amaranthus viridis*
BIC	-	Bayesian Information Criterion
BLAST	-	Basic Local Alignment Search Tool
BSA	-	Bovine Serum Albumin
C	-	Conserved sites
CAAS	-	Chinese Academy of Agricultural Sciences
cpDNA	-	Chloroplasts DNA
DAPI	-	4',6-diamidino-2-phenylindole

DI	-	Disparity Index
DW	-	Dry Weight
E	-	Equatorial diameter
ESI	-	Electro-Spray Ionization
ETS	-	External Transcribed Spacer
FAO	-	Food and Agriculture Organization of the United Nations
FW	-	Fresh Weight
g	-	Gram
GAE	-	Gallic Acid Equivalents
GC/MS	-	Gas Chromatography-Mass Spectrometry
h	-	Hour
H_2SO_4	-	Sulfuric acid
ha	-	Hectare
HCl	-	Hydrochloric acid
HNO_3	-	Nitric acid
HPLC	-	High Performance Liquid Chromatography
HR-LC/MS	-	High-Resolution Liquid Chromatography-Mass Spectrometry
ICP-AES	-	Inductively Coupled Plasma Atomic Emission Spectrometer
ISSR	-	Inter-simple sequence repeats
ITS	-	Internal Transcribed Spacer
KCl	-	Potassium chloride
Kg	-	Kilogram
KV	-	Kilovolt
m	-	Metre
M	-	Molar
matK	-	Maturase K
MB	-	Megabyte
mg	-	Milligram
min	-	Minute
ML	-	Maximum Likelihood
ml	-	Millilitre

mm	-	Millimeter
MS	-	Mass Spectrometry
Na_2CO_3	-	Sodium carbonate
$NaNO_2$	-	Sodium nitrite
NaOH	-	Sodium hydroxide
NBPGR	-	National Bureau of Plant Genetic Resources
NCRPIS	-	North Central Regional Plant Introduction Station
ndhF	-	NADH dehydrogenase F
ng	-	Nanogram
nm	-	Nanometer
NTS	-	Non-Transcribed Spacer
^oC	-	Degree Celsius
OD	-	Optical Density
P	-	Polar axis length
PBS	-	Phosphate Buffered Saline
PCR	-	Polymerase Chain Reaction
PDB	-	Paradicholoro benzene
Pi	-	Parsimony informative sites
PMC's	-	Pollen Mother Cells
QE	-	Quercetin Equivalent
RAPD	-	Random Amplified Polymorphic DNA
rbcL	-	Ribulose bisphosphate carboxylase/oxygenase
RCL	-	Range of Chromosome Length
RDA	-	Recommended Dietary Allowance
RFLP	-	Restriction Fragment Length Polymorphism
rpm	-	Resolution per minute
RRC	-	Rodale Research Center
RT	-	Room Temperature
S	-	Singleton sites
SD	-	Standard Deviation
SE	-	Standard Error

SEM	-	Scanning Electron Microscopy
SNP	-	Single Nucleotide Polymorphism
TB	-	Terabyte
TCL	-	Total Chromosome Length
TE	-	Tris-EDTA
TF%	-	Total forma percentage
UNSAAC	-	National University of Saint Anthony the Abbot in Cuzco
USDA	-	United States Department of Agriculture
V	-	Variable sites
v/v	-	volume/volume
VC	-	Variation coefficient
WHO	-	World Health Organization

CONTENTS

INTRODUCTION

'Amaranth - thou art immortal and dost never fade, but bloomest for ever in renewed youth'.

Amaranth has always been in the limelight as a crop for farmers and as a topic of interest for researchers. The plant gets its name from the greek word '*amaranthon'* meaning 'unfading' or 'immortal'. Interestingly, this plant has been a favourite of poets too. An early Greek fable counted among Aesop's Fables compares the rose to the amaranth to illustrate the difference in fleeting and everlasting beauty. The beauty of its unfading flowers was even referred by famous poets like John Milton, Francis Thompson, Samuel Taylor Coleridge & John Keats. In the poem "The Hound of Heaven" (1893), Francis Thompson even goes a step further to compare God's love to "an amaranthine weed". No words can express the beauty and popularity of this leafy and grain crop than those mentioned above.

The question why this crop has attained global attention, points to its peculiar properties, mysterious origins and current agricultural development. And, it is increasingly popular with gardeners who find it fascinating for its ease of cultivation, colourful appearance and diverse uses in the kitchen. Owing to its wider adaptability, less production cost and high nutritional value, it played a major role in wiping out malnutrition and hunger amongst the poorest populations in Africa in the past 20 years. It serves as a poor man's vegetable. There are evidences for this plant being grown from prehistoric times. A plant grown as a food crop 1,000 years ago could be one of the answers to the coming dry seasons. It can definitely ensure global health and food security in future. Hence this plant can rightly be considered as a food from the past for the future.

Amaranthaceae is a family of flowering plants commonly known as the 'amaranth family'. According to the gene-based APG system I (1998) and II (2003) the former goosefoot family Chenopodiaceae was merged in Amaranthaceae and now it contains about 165 genera and 2,040 species (Christenhusz & Byng, 2016), making it the most species-rich lineage within its parent order, Caryophyllales (http[1]). This is a widespread and cosmopolitan family that can be found from the tropics to cool temperate regions. The Amaranthaceae is native to tropical and subtropical areas of Central America, Africa and Australia (Kadereit et al., 2003). Amaranthaceae is regarded as closely related to Chenopodiaceae since the early 19th century. They share many features in terms of their morphology, anatomy as well as phytochemistry (Müller & Borsch, 2005). In spite of problems in identifying the distinguishing characters, majority of the authors have treated both families separately. Amaranthaceae is one of the core centrospermous families that comprises mostly herbs, shrubs, rarely lianas and small trees with opposite or alternate exstipulate leaves. The flowers are often small and inconspicuous with scarious tepals; the flowers are bisexual or unisexual and the plants may be monoecious, dioecious or polygamous. Each flower is generally subtended by a ventral bract and two lateral bracteoles. The stamens stand opposite the tepals, hypogynous to perigynous and in majority of the genera the stamens are united below into a cup or tube. Interstaminal appendages, mostly called pseudostaminodia, frequently occur. The anthers are dorsifixed and introrsely dehiscent. The gynoecium comprises two or three carpels united into one pistil. The unilocular ovary contains one (in most genera) or several ovules. The fruit is generally a thin-walled utricle, rarely a berry-like capsule (Anilkumar, 2014).

Amaranthus L. is the type genus of the family Amaranthaceae, first described by Linnaeus having mostly annual, monoecious and dioecious species with worldwide distribution. The total number of species present in

the genus *Amaranthus* is still tentative due to the inadequate scientific research in *Amaranthus* systematics. The phenotypic flexibility exhibited by frequent hybridisation and vast genetic and morphological diversity is the major reason for the existing taxonomic ambiguity. *Amaranthus* is known by different local names in different regions, have many synonyms and thus causes further difficulties in identification. According to Mujica and Jacobsen (1999), Jacobsen et al. (2000), Mujica et al. (2001) and Jacobsen and Mujica (2001), it is estimated that, there are 87 species of *Amaranthus* of which 17 are in Europe, 14 in Australia and 56 in America. Of the American species, 10 are dioecious, only found in North America and 46 are monoecious (Hunziker, 1991). Of the 46 monoecious American amaranth species, 13 are endemic to North America and Mexico, 17 to the Antilles, Central and South America, while 16 being common to America. Brenner et al. (2010) mentioned 60 species and United States Department of Agriculture (USDA) mentioned 86 species in the genus *Amaranthus* (http[2]). According to Sauer (1955), Costea et al. (2001) and Espitia-Rangel et al. (2012) the genus *Amaranthus* consists of about 70 species, of which 40 are native to the Americas and the rest are in the temperate and tropical regions of Australia, Africa, Asia and Europe. According to the report by Sharma et al. (2013) there are 20 species of *Amaranthus* in India.

The species of the genus *Amaranthus* are monoecious or dioecious herbs, usually annual, rarely perennial. Stems erect, ascending, prostrate or diffuse, glabrous to tomentose (trichomes uniseriate filiform, whitish to yellowish), white to red-brownish, often branched (sometimes simple). Leaves alternate, petiolated, with blade linear to ovate, elliptic to deltoid/ rhombic; base cuneate to obtuse; apex acute, obtuse, emarginate or bilobed, sometimes mucronate; margins entire, sometimes undulate; blade glabrous to pubescent (sometimes only on the veins), with trichomes whitish to yellowish, uniseriate, filiform. Inflorescences thyrsoid paraclades arranged in terminal

and/or axillary spike or panicle-like inflorescences or only in axillary glomerules. Bracts 1−5, ovate to lanceolate, with membranous border thinning to apex or abruptly interrupted at the half of the total length, sometimes keeled; apex acute to obtuse. Flowers unisexual, sessile. Staminate flowers with 3−5 free and equal tepals, ovate to lanceolate, usually glabrous; apex usually acute; margin entire; stamens 3−5, anthers tetrasporangiate with 2 lines of dehiscence, filaments free to the base; pseudostaminodia absent. Pistillate flowers with (0−) 2−5 usually free tepals, linear to ovate-lanceolate sometimes spathulate, usually glabrous; apex acute to emarginate (sometimes mucronate); margins entire; one pistil, one ovule, 2-5 stigmas. Fruit dry (dehiscent capsule or indehiscent utricle), globose to ellipsoid, smooth to strongly rugose on the surface, with often persistent styles; seed one, usually lenticular, smooth to reticulate; embryo annular (Iamonico, 2015).

The most frequently used infrageneric classification of *Amaranthus* involves 2 subgenera as suggested by Sauer (1955) and include Acnida (L.) Aellen ex K. R. Robertson which comprises dioecious species and Amaranthus that include monoecious species. Traditionally, the subgenus Amaranthus is divided into two sections, Amaranthus and Blitopsis Dumort. Mosyakian and Robertson (1996) discussed about the infrageneric classification of the genus and elevated the two sections of subgenus Amaranthus to subgeneric rank, subgenus Amaranthus and subgenus Albersia (Kunth) Gren & Godr. (= section Blitopsis). The infrageneric classification of *Amaranthus* is not clearly resolved due to the wide range of morphological variability, large numbers of synonyms and misapplication of names. According to the generally accepted concept, the genus comprises three subgenera subgenus Acnida that includes dioecious species, subgenus Amaranthus and subgenus Albersia. Monoecious grain amaranths are included in the subgenus Amaranthus and vegetable amaranths are included in the subgenus Albersia.

Amaranthus was domesticated in American continent over 4000 years ago by pre-hispanic cultures. It was cultivated in Mexico City valley, together with corn, beans, and pumpkins. It was also cultivated, by the Mayans in Guatemala and by the Incas in South-America. Amaranth leaves were used as vegetable in America, before its domestication (Mujica et al., 1997). Several amaranth species are of paramount importance, therefore, consumers can take advantage of them for various uses, such as flour from seeds, salads from fresh leaves, inflorescences as source of natural red dye and waste products as animal foodstuff (Juan et al., 2007). The three amaranth species, mainly used for seed production are *A. cruentus* L., *A. caudatus* L., and *A. hypochondriacus* L. (Hernandez & Herrerias, 1998). These species produce big inflorescences full of seeds (Morales et al., 2009). *A. cruentus* and *A. hypochondriacus* are native to Mexico and Guatemala. *A. caudatus* is native to the Andean regions of Ecuador, Peru and Bolivia (Sauer, 1976). This species originated as one of the staple foods for the ancient inhabitants of the Andean region. The Aztecs, Incas and Mayans considered amaranth as their staple food together with maize and beans. It is one of the traditional food plants in Africa to wipe out malnutrition among poor segments of the population. It is used to be one of the most important crops in America before Spanish colonialists conquered it and further cultivation of the crop was banned (Kauffman & Weber, 1990). Amaranth was first introduced as an ornamental plant in Europe in the 16th century. Different species of amaranth spread throughout the world during 17th, 18th and 19th century. In India, China and even under the harsh conditions of Himalayas, this plant became an important grain and/or vegetable crop.

After the arrival of the Spanish Conquistadors in Mexico in the early 1500s, amaranth almost disappeared in the Americas as a crop until research began on it in the U. S. in the 1970s. In the meantime, amaranth had spread around the world and became established for food use of the grain or leaves in

places such as Africa, India and Nepal. In the past two decades, amaranth has reiterated to be grown by a much larger number of farmers around the world, in China, Russia, parts of Eastern Europe, South America and is re-emerging as a crop in Mexico. Amaranth was grown as a grain crop in the U. S. in the late 1970s. Still, the valuable characteristics of amaranth grain, combined with its adaptation to a wide range of growing areas, make it a very promising crop for the future (Myers, 2004). At present amaranth is grown in the USA, South America, Africa, India, China and Russia. The Czech Republic is the most important grower in Europe (approx. 250 ha). The largest area of the grain-type amaranth is in China, where 150,000 ha are reportedly grown for forage use (Kauffman & Weber, 1990). *Amaranthus* is the most popular leafy vegetable in India, mostly cultivated in Kerala, Tamilnadu, Karnataka, Maharashtra, Andhra Prasesh and Telangana.

Amaranth is a predominantly self-pollinated crop, with varying amounts of outcrossing (Hauptli & Jain, 1985). By growing amaranth in isolation, it is possible to control the amount of outcrossing for the development of true-to-type lines from segregating accessions within only a few generations of selection. The Rodale Research Center (RRC) began an amaranth research project in 1976. In their 14 years of operations, the RRC breeders assembled a germplasm collection of approximately 1,400 accessions and developed many of the world's most successful grain cultivars. The RRC germplasm collection was donated to the North Central Regional Plant Introduction Station (NCRPIS) in 1990, where it is maintained as part of the USDA National Plant Germplasm Collection. Germplasm enhancement has been conducted at RRC since 1977 using recurrent single plant selection and mass selection. Selections were continued for the development of ideotypes which meet the needs of modern agriculture. The first released line of amaranth named 'MT-3' was announced by the Montana Agricultural Experiment Station (Cramer, 1988). It was selected from a

segregating accession of *A. cruentus* originally collected in Mexico. The germplasm collection at various institutes such as RRC, AAI, CAAS, INIFAP, UNSAAC, NBPGR *etc*. is the backbone of varietal improvement programs which is aimed at developing agronomically acceptable lines using classical plant breeding and selection methods. *Amaranthus* is well suited to *ex-situ* conservation because the seeds are long lived and small. Brenner and Widrlechner (1998) described an efficient protocol for regenerating seeds of *Amaranthus* germplasm and for maintaining genetic integrity *ex-situ*.

The global nutrition transition towards more simplified diets high in energy but poor in micronutrients and functional properties is linked with epidemic increase of non-communicable diseases. For centuries, more than 10000 plant species have been used as food. However, in recent years, it was estimated that 80% of the world's dietary energy supply is obtained from only 12 species (FAO, 2005). Thousands of species and many more varieties fall into a category defined as underutilised or neglected crops. (Global Forum for Underutilized Species, 2009). In a country like South Africa, people are still using a wide variety of plants in their daily lives for food, water, shelter, fuel, medicine and other needs of life (Van Wyk & Gericke, 2000). However, many traditional, native vegetables are underutilised in favour of introduced non-native vegetables. The availability of indigenous vegetables has declined because of excessive cultivation of field crops, chemical elimination of wild vegetables, habitat change and the growing ignorance of the young generation about the existence of these foods (Odhav et al., 2007). Traditional vegetables are readily available in the field and have the potential to contribute to reducing malnutrition in the poor segment of the population by providing resources for food diversification. *Amaranthus* is such an unexploited crop, which shows promising potential as a global resource by supplying nutritious grains as well as tasty leafy vegetables (Saunders & Becker, 1984).

The genus *Amaranthus* has gained much attention for its high economic and nutritional value (Khaing et al., 2013), of which *A. caudatus*, *A. hypochondriacus* and *A. cruentus*, have attained great interest during recent years as agricultural crops in many regions of the world, due to the exceptionally high nutritional value of their seeds and leaves. Hundreds of articles documented the nutritional value, the agronomical potential, genetic resources and breeding of amaranth (Costea et al., 2001). But the evolutionary origin of grain amaranth are still unclear (Costea et al., 2001). Sauer (1967, 1976, 1993) has suggested two possible hypotheses. The monophyletic hypothesis states that the three cultivated species were originated from a single wild progenitor namely *A. hybridus*, followed by subsequent introgressive hybridization with two other wild species in different regions. According to this hypothesis, the first domesticated species was *A. cruentus*, derived from *A. hybridus* in Central America, followed by the domestication of *A. hypochondriacus* by repeated crossing of *A. cruentus* with *A. powellii* in Mexico and of *A. caudatus* by crossing with *A. quitensis*. The polyphyletic hypothesis suggests that each of the three grain species were domesticated separately from a different wild species. In concordance with this hypothesis, *A. hypochondriacus* was domesticated in Mexico from *A. powellii*, *A. cruentus from A. hybridus* in Central America and *A. caudatus* from *A. quitensis* in South America.

According to one school of thought, all the grain amaranths are of the new world origin, but another school of thought suggested that grain amaranths might have been cultivated in South Asia from prehistoric period and probably might have domesticated there. A comparative study of grain amaranths in India and Central America indicated close similarity in species distribution, evolution, variety pattern and cultivation practices (Das, 2016 a).

Grain amaranths are herbaceous annuals with upright growth habit. They are tall (0.5–2 m) and moderately branched from a main stem/unbranched, form dense panicles at the tip of the stem. Vegetable types form flowers and seeds along the stems. They are indeterminate in growth habit, but may set seed at a smaller size during short days. Grain types may produce yields comparable to rice or maize (2,500 kg/ha) (O'Brien & Price, 2008). For cultural purposes grain, vegetable, ornamental and weedy types do have some distinct variations. The leaves and grain of all types are edible and equally nutritious. The seed or grain of the grain type is of a pale colour, varying from off-white to pale pink. The seed of the vegetable, ornamental and weedy types is black and shiny. Both types are edible and may be used as flour sources, but if the black seed types are mixed with the pale types, it is often considered as contamination. *Amaranthus* is a fast growing cereal-like plant with a seed yield of about 3 tons per hectare in 3-4 months when growing in monoculture, and a vegetable yield of 4-5 tons dry matter per hectare after 4 weeks (Grubben & Van Slotten, 1981). Amaranth has a C4 photosynthetic pathway as other cereals, which enables it to be uniquely efficient in utilizing sunlight and nutrients at high temperatures. It is more drought-resistant than maize and thrives 30–35°C temperatures and tolerates poor fertility also. Amaranth can be cultivated in some areas where farmers have limited options, especially in those areas with limited rainfall. The drought tolerant characteristics of amaranth make it a prospective dry land crop for farmers in semi-arid areas (Kauffman & Weber, 1990).

Most of the species from *Amaranthus* are summer annual weeds and are commonly referred to as pigweeds (Bensch et al., 2003). These species have an extended period of germination, rapid growth and high rates of seed production (Bensch et al., 2003) and have been causing problems for farmers since the mid-1990s. This is partially due to the reduction in tillage, reduction in herbicidal use and the evolution of herbicidal resistance in several species

where herbicides have been applied more often (Wetzel et al., 1999). The following 9 species of *Amaranthus* are considered as invasive and noxious weeds in the United States and Canada: *A. albus, A. blitoides, A. hybridus, A. palmeri, A. powellii, A. retroflexus, A. spinosus, A. tuberculatus* and *A. viridis* (USDA, 2009). The species, *A. palmeri* (Palmer amaranth) is among the five top most troublesome weeds in the southeast and has already evolved resistance to dinitroanilines and acetolactate synthase inhibitors and the herbicide glyphosate (Culpepper et al., 2006). Also, this plant can survive in tough conditions. This makes the proper identification of *Amaranthus* species at the seedling stage, which is essential for agriculturalists. Proper herbicide treatment needs to be applied before the species successfully colonizes in the crop field and causes significant yield reductions.

Amaranthus is a plant with high nutritional value, whose nutrients are concentrated in the leaves and the grains. The plant has showed many health benefits, since it contained bioactive phytochemicals and nutraceutical compounds mainly in the seeds and leaves of the plant. The most pronounced functional benefits include pseudocereal, leafy vegetable, essential amino acids, proteins, unsaturated fatty acids, lectins, tocopherols, tocotrienols, phytosterols, squalene, isoprenoid compounds, aliphatic alcohols, terpene alcohols, polyphenols, carotenoids *etc*. Some components have industrial applications in the market sectors of food production (betalains and carotenoids as antioxidants and natural pigments), cosmetics (squalene) and biodegradable utensils (cellulose) (Santiago et al., 2014). As a healthy food, it can be included as the regular food component in the diet either natural or processed form, which will allow complementing a healthy life style. In the food industry, the amaranth is processed as food or used as an ingredient in prepared products such as breads, cereals, crackers, granola, *etc*. (Schnetzler & Breene, 1994). This nutritional value is very balanced and close to the optimum required in the human diet, as described by the World Health

Organization (WHO) and Food and Agriculture Organization of the United Nations (FAO).

Amaranth species are cultivated and consumed as a leaf vegetable in many parts of the world *viz*., throughout India, Nepal, China, Indonesia, Malaysia, Philippines, whole of Central America, Mexico, Southern and Eastern Africa (Peter & Gandhi, 2017). Amaranth leaves can be used both in cooking and for salads and has a delicious, slightly sweet flavour (http[2]). The four species of *Amaranthus* documented as cultivated vegetables are *A. cruentus*, *A. blitum, A. dubius* and *A. tricolor* (Costea, 2003). The plant, *A. tricolor* is a popular leafy vegetable consumed in Asia. *Amaranthus* leaves are good source of protein, dietary fibres, carotenoids, vitamin A, vitamin C, folic acid, thiamine, niacin, riboflavin and some dietary minerals including calcium, iron, potassium, zinc, copper and manganese (Kadoshnikov et al., 2008; Ozbucak et al., 2007). The vegetable *Amaranthus* has been reported to have high concentration of antioxidant components such as vitamin C, vitamin E, carotenoids, polyphenols and other phytochemicals (Hunter & Fletcher, 2002). The flavour of raw and cooked vegetable amaranth was reported as equal to or better than that of spinach or other similar green vegetables (Abbott & Campbell, 1982). Regardless of species, the choice of variety/ cultivar is influenced by individual preference for leaf colour and taste. The plant, *A. tricolor* has various leaf colours such as white (light green), dark green, red, purple and variegated (Palado & Chang, 2003). The two main types grown as vegetables are termed as green amaranth and red amaranth.

The grains of *Amaranthus* were used as important food crop from the ancient middle and South American civilizations. In fact even today, these are important food crops in Latin America. They are grown abundantly in northern India, Manchuria, Southeast Asia and Africa (Kauffman & Weber,

1988; Irving et al., 1981). In botanical terms, grain amaranth is not a true cereal because it is a dicotyledonous plant. Hence they are referred to as 'pseudocereals', as their seeds resemble in function and composition to those of the true cereals. Amaranth seeds are small (1-1.5 mm diameter), they are lenticular in shape and weight per seed is 0.6-1.3 mg (Bressani, 2003). The grain structure of amaranth differ significantly from cereals such as maize and wheat. In amaranth seeds, the embryo or germ, which is circular in shape, surrounds the starch-rich perisperm and together with the seed coat represent the bran fraction, which is relatively rich in fat, protein, vitamins and minerals (Bressani, 2003; Taylor & Parker, 2002). The food value of grain amaranth was recognized by people from Mexico, Peru and Nepal long before any, nutritional analyses had been conducted. Because it is easy to digest, amaranth is traditionally given to those who are recovering from an illness or a fasting period. Grain amaranth is used as an ingredient primarily in bread, pasta, baby's food, instant drinks *etc*. For such purposes seeds were subjected to various processing technologies *viz*., boiling, swelling, flaking, extrusion, puffing, roasting, grinding, sprouting *etc*. The most common product is flour and whole amaranth seeds are used in breads, musli bars, porridges, pastas, biscuits, cookies *etc*. In Mexico, grain amaranth is popped and mixed with sugar solution to make a confection called "alegria" (happiness). A traditional Mexican drink called "atole" is made from milled and roasted amaranth seeds. In India, *A. hypochondriacus* is known as "rajgeera" (the King's grain) and is often popped to be used in confections called "laddoos," which are very similar to Mexican "alegria". In Nepal, amaranth seeds are eaten as gruel called "sattoo" or milled into flour to make chappatis (Singhal & Kulkami, 1988).

Grain amaranth species are cultivated exclusively for seed production in the U.S. and other regions of the world. The attraction of the crop to both earlier civilizations and modern consumers is the highly nutritious golden

seed. The most studied nutritional aspect concerning the food value of grain amaranth is the identification of the limiting amino acids of the protein component which is higher than other cereal grains and has significantly higher lysine content (Bressani, 1989; Lehman, 1989). The main protein fractions in amaranth grain are albumins, globulins and glutelins, which differ in their solubilities (Martínez & Anon, 1996). Amaranth is valued for the positive chemical composition of seed that does not contain gluten/prolamin, a composite of storage proteins, stored together with starch in the endosperm of various grass-related grains. Consumption of gluten rich grains will cause Celiac disease in sensitive individuals. The individuals who are allergic to wheat use substantial quantities of amaranth grain and find that they can substitute amaranth for wheat without an allergic reaction, since amaranth is gluten-free. (Myers, 2004). In a recent study by Kiskini (2007), amaranth based gluten-free bread fortified with iron was successfully formulated. Starch is the major part of carbohydrate in the grain amaranth and starch granules are small (1-3mm), polygonal in shape, with a high swelling power and easily degradable by alpha-amylase (Stone & Lorenz, 1984). Amaranth starch is highly stable during freezing and highly resistant to mechanical stress. There is a distinctive gel characteristic to the starch (Yanez et al., 1986) and waxy and non-waxy starch granules have been identified (Konishi et al., 1985). Interest has been expressed in specialized food and industrial applications for amaranth starch because of its distinctive characteristics. The most intriguing is the microcrystalline starch in amaranth seed, which is about one-tenth the size of corn starch particles. The small size of the starch could be of value in both food and industrial uses.

Amaranth which produces large amount of biomass in a short period of time can be used as a forage crop for domesticated animals. Several cuttings are made per growing season. The forage use of amaranth is established in both the tropic and temperate zones. In many tropical areas, where amaranth

is consumed as a vegetable, amaranth stover is fed to livestock after several harvests. Amaranth forage has succeeded as an animal feed in the temperate zone of China. Farmers in China are reportedly growing over 150,000 acres of amaranth as forage for hogs and cattle. In Peru, the stover is grazed or milled for use as a feed supplement after the seed heads have been hand harvested. In the U. S., animals have the habit of eating wild amaranth, but not as a deliberately managed forage.

Many amaranths have become popular ornamental plants and are found worldwide. Examples include brightly coloured *A*. *tricolor* 'bedding plants' and *A*. *caudatus* 'Love Lies Bleeding' plants with beautiful drooping rope-like inflorescences. The 'prince's feather', *A. hypochondriacus* has deeply-veined lance-shaped leaves, purple on the under face, and deep crimson flowers densely packed on erect spikes (http[2]). The enchanting beauty of the vividly coloured leaves, stems and seed heads in an amaranth field is a sight which evokes emotions that other crops cannot give.

The colour of *Amaranthus* leaf, stalk and inflorescence varies from red to green, and that of the seed ranges from black to white (http[3]). Highly pigmented vegetables or fruits such as the anthocyanin-rich ones usually have higher nutritional value (Li et al., 2012). The visual appearance of most *Amaranthus* plants is bright red-violet, and betalains are generally considered to be responsible for the maroon pigment. However, the similarity in colour may lead to misidentification of the pigments in *Amaranthus* as anthocyanins. Majority of the literatures suggested that betalains to be the pigments in *Amaranthus* plants. The different patterns of pigmentation in amaranth contribute many practical and aesthetic uses (Kigel, 1994). It can be used as a natural dye upon specific extraction (Schnetzler & Breene, 1994). Of the 70 betalain structures identified so far (50 betacyanins and 20 betaxanthines), 16 betacyanins and 3 betaxanthines are present in the family Amaranthaceae (Cai et al., 2005 a, b). The most predominant betalains found in *Amaranthus* were

amaranthine and isoamaranthine (Stintzing et al., 2004). Carotenoids like lutein, *β*-carotene and others are located in the leaves of *Amaranthus* (Segura-Nieto et al., 1994). Each gram of oil extracted from *A. hybridus* L. contains 5 μg of carotenoids (Martirosyan et al., 2007).

Amaranthus species are a rich source of unsaturated fatty acid and linoleic acid is the most predominant unsaturated fatty acid found in the leaves (Segura-Nieto et al., 1994; Morales et al., 1988). Its seed oil has a small amount of linolenic acid followed by oleic acid (León-Camacho et al., 2001). Amaranth grain contains 6 to 10% oil, which is found mostly within the germ. The oil fraction of the amaranth grain have considerably higher amount of squalene when compared to many other cereal or pseudocereal grain sources. Squalene is a precursor of steroids and terpenoids (León-Camacho et al., 2001). It is used as an immune adjuvant, related to the cholesterol biosynthesis, lowers the levels of low-density cholesterol, and promotes the increase of 7α-hydroxylase that breaks the hepatic cholesterol into bile acids (León-Camacho et al., 2001; Qureshi et al., 1996). It is also used in pharmacy and cosmetic preparations for treating skin disorders due to its emollient and moisturizing activity, as well as anti-tiredness and anti-aging agents. The traditional source for squalene is primarily shark and whale liver oil (Catchpole et al., 1997; Jahaniaval et al., 2000), which contain ~ 30-45% of squalene. Use of squalene in cosmetic applications is limited by the uncertainty of its availability as a result of international concern for the protection of marine animals. In addition, the presence of similar compounds, such as cholesterol, which are not recommended for human consumption (Guil-Guerrero et al., 2000), in the oils from marine animal liver can make squalene purification difficult. Hence now *Amaranthus* is considered to be an alternate source for natural squalene.

Tocopherols and tocotrienols (vitamin E isomers) are well-known natural antioxidants, and their presence in oilseeds is often correlated with the relative abundance of unsaturated fatty acids. Besides their known activity as

antioxidants and free radical scavengers, they have also proved to be active against hypercholesterolemic arteriosclerosis (Ozer & Azzi, 2000). Tocopherols and tocotrienols present in different *Amaranthus* species have various bioactivities such as antitumor activity, antioxidant activity, suppression of cholesterol synthesis and serum cholesterol regulatory activity (Schnetzler & Breene, 1994). It reduces the synthesis of low density lipoprotein-cholesterol and regulates the enzyme lipoprotein lipase (León-Camacho et al., 2001; Qureshi et al., 1996). Tocopherols reported in *A. cruentus* oil are α-tocopherol, β-tocopherol and δ-tocopherol (León-Camacho et al., 2001). Tocotrienols reported in *A. cruentus* and *A. hypochondriacus* seeds are β-tocotrienol, γ-tocotrienol and δ-tocotrienol (Lehmann et al., 1994).

Phytosterols are present in high concentration in *Amaranthus* species (Marcone et al., 2003). Martirosyan et al. (2007) reported 20 mg/g of phytosterols in *A. hybridus* oil, while León-Camacho et al. (2001) reported the occurance of phytosterols *viz*., 24-methylenecholesterol, campesterol, stigmasterol, Δ^7-campesterol, clerosterol, β-sitosterol, Δ^5- avenasterol, Δ^7-sigmastenol, Δ^5-avenasterol and traces of cholesterol, bassicasterol, campestanol, $\Delta^{5,23}$-sigmastadienol, sitostanol and $\Delta^{5,24}$-stigmastadienol in *A. cruentus*. Phytosterols have wide use in pharmacy due to its anti-inflammatory, analgesic, antibacterial (clerosterol) activity *etc*. Phytosterols present in the unsaponifiable fraction of plant lipid matrices are known to be the inhibitors of cholesterol absorption as they compete for this substance at the intestinal level (Heinemann et al., 1993). Literatures also reported the occurrence of aliphatic alcohols, methylsterols and terpenic alcohols from *A. cruentus* oil (León-Camacho et al., 2001). Aliphatic alcohols reported were docosanol, tetracosanol, hexacosanol and octacosanol. Taraxerol and 24-methylene-cycloartanol are the observed methylsterols. Terpene alcohols observed are dammaradienol, β-amyrin, gramisterol, cycloartenol and citrostadienol. *Amaranthus* are also found to be the rich source of polyphenols like rutin, nicotiflorin and isoquercitrin (Barba de la Rosa et al., 2009). According to Klimczak et al. (2002) the main phenolic compounds found in

amaranth seeds are caffeic acid, p-hydroxybenzoic acid and ferulic acid. Polyphenol compounds have been extensively studied in the last decade for health promoting properties such as their role in the prevention of degenerative diseases which include cancer and cardiovascular diseases (Scalbert et al., 2005). Studies have shown that the pseudocereal amaranth represent good sources of minerals, natural dyes, vitamins and dietary fibres. Contents of minerals depend on species and growing conditions. Amount of calcium and magnesium are higher than the amounts in other cereals. Seeds are good source of vitamins, mainly vitamin B and E (Berghofer & Schoenlechner, 2002).

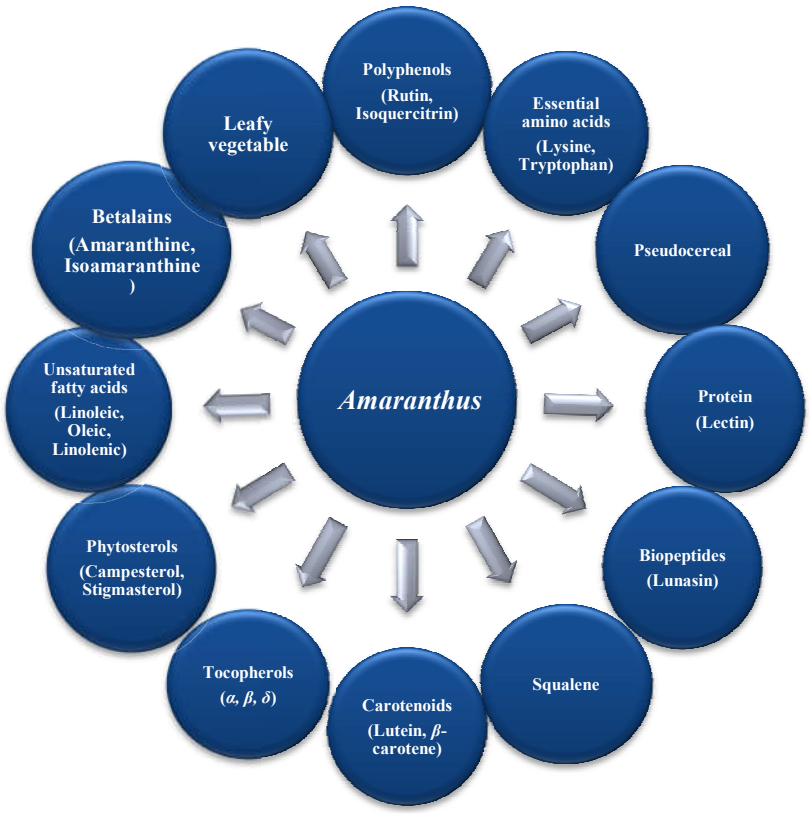
In recent years, *Amaranthus* has emerged as an attractive source of protein due to its high nutritional value. Its seed proteins possess an unusually well-balanced amino acid composition with higher sulphur amino acid content than legume proteins and higher aromatic and hydrophobic amino acid contents than cereals (Gorinstein et al., 2002). Globulins are the most abundant fraction of amaranth protein isolates, their main constituents being the 11S globulin (amaranthin) and the structurally related globulin-P (Quiroga et al., 2007). Bioactive peptides are important components of functional foods, since it is generally acknowledged that specific sequences within the parent food proteins can provide physiological benefits once they are released either by *in vivo* digestion, microbial fermentation or *in vitro* enzymatic hydrolysis (Hartmann & Meisel, 2007). Recently, the presence of encrypted bioactive peptides in several *Amaranthus* seed storage proteins was reported by Silva-Sánchez et al. (2008).

Diverse peptides, which can be *in vitro* or *in vivo* released from animal or plant proteins, have shown potential beneficial effects on health, one of them being the antioxidant capacity (Hartmann & Meisel, 2007; Udenigwe & Aluko, 2012). Some studies have demonstrated that biopeptides can be free radical scavengers, metal transition chelators or lipid peroxidation inhibitors. In addition, some peptides can avoid oxidative damage by induction of the

genes that code for the antioxidant enzymes (Sarmadi & Ismail, 2010). Lunasin is a bioactive peptide located in the amaranth seed protein having a concentration of 11.1 µg/g of extracted protein. Silva-Sánchez et al. (2008) reported the anticarcinogenic properties of lunasin present in *A. hypochondriacus* seed.

The nutritional analysis of amaranth grain was extensively studied rather than leaves. A comparative analysis of the nutritional profile of different amaranth species was scarce, which was attempted in the present study. As a traditional food plant, *Amaranthus* has potential to improve nutrition, boost food security, foster rural development and support sustainable land care (NRC, 2006). Various functional attributes exhibited by *Amaranthus* species are consolidated in Figure 1.

Fig. 1 Functional attributes of *Amaranthus*



The genus *Amaranthus* consists of several important species of vegetable, grain and weeds, all of these species share similar vegetative morphology and high phenotypic plasticity, which makes its identification difficult. The difficulty of taxonomic discrimination is also due to the small size of diagnostic parts especially flowers, broad geographical distribution and frequent interspecific hybradization, which is the reason for many synonyms (Jacobsen & Mujica, 2003). The awareness of phylogenetic relationships among different *Amaranthus* species will provide information useful in breeding programmes and germplasm conservation (Gupta & Gudu, 1991). The independent information from DNA-based topologies can also help in resolving relationships among morphologically intractable groups (Fior et al., 2006) and, along with morphological studies, can help in understanding the taxonomic complexities (Schönenberger & Conti, 2003).

In the last three decades, molecular sequence data have revolutionized the field of systematics by providing additional approaches and giving more confidence to the already published morphology-based systematics or taxonomy. The potential of DNA data to reveal phylogenetic relationships was first discussed in 1965 by Zuckerkandl and Pauling. Earlier systematics studies focused on DNA-DNA hybridization (Bendich & Bolton, 1967). But now the most molecular taxonomy data are generated by PCR amplification and sequencing of nuclear DNA markers (internal transcribed spacers, external transcribed spacers, transposable elements, simple sequence repeats, promoter sequences and single copy nuclear intron sequences), chloroplast (cpDNA) and mitochondrial DNA markers. Soltis and Soltis (1998) have discussed the importance of different DNA marker regions for studying angiosperms at different taxonomic levels. The nuclear and chloroplast regions are found to be more promising than mitochondrial region (Palmer, 1992).

The chloroplast genome is smaller than the nuclear genome and is found in large numbers, making it easy to isolate. The chloroplast genome is divided into protein coding genes, introns and intergenic spacers. Most phylogenetic studies investigating plant evolution utilized chloroplast markers. The cpDNA is successful in such studies because it is structurally stable, non-recombinant and highly conserved in genetic content among closely related species (Downie & Palmer, 1991). Chloroplast protein coding gene substitution rates are generally slower than those of the nuclear genome, making them good markers for high level taxonomic studies (Chase et al., 1993; Soltis & Soltis, 1998). Among the coding genes, rbcL (Chase et al., 1993) was used in the earlier phylogenetic studies, but soon it was followed by other coding genes like ndhF (Olmstead & Palmer, 1994; Clark et al., 1995; Kim & Jansen, 1995), atpB (Hoot et al., 1995; Wolf, 1997) and the more commonly used matK (Johnson & Soltis, 1994; Shaw et al., 2005). Though the coding regions have been used extensively in studies at the family or higher taxonomic levels, the non-coding regions are used more at the lower taxonomic levels, making them more appropriate for species identification (Gielly & Taberlet, 1994).

Most of the studies using nuclear regions have relied on the nuclear ribosomal regions. At higher taxonomic levels, the slowly evolving rRNA genes are used (Soltis & Soltis, 1998; Kuzoff et al., 1998), while at lower taxonomic levels internal transcribed spacers (ITS) and external transcribed spacers (ETS) are more commonly used (Alvarez & Wendel, 2003). In general, the eukaryotic ribosomal RNA genes are part of repeat units that are arranged in tandem and located at chromosomal sites known as nucleolar organizing regions. Each unit consists of a transcribed region having genes for 18S, 5.8S, 26S rRNA, internal transcribed spacer 1 and 2, external transcribed spacers (ETS1 and ETS2) and a non-transcribed spacer (NTS) region.

According to the APG II (2003) system of classification, the plants formerly treated under the family Chenopodiaceae are now categorized under the family Amaranthaceae. Molecular data, in addition to the existing morphological data, supported the combination of the two families into Amaranthaceae. Molecular level support has been provided by several researchers working on different gene regions. For instance, Rettig et al. (1992) reported the phylogenetic relationship between the two species, and based on the nucleotide subunit data of rbcL, supported the placement of Amaranthaceae and Chenopodiaceae as a monophyletic lineage. Similar results were also reported by Downie et al. (1997) by studying the phylogeny based on the sequence of partial chloroplast DNA ORF 2280 homolog.

Despite the increasing attention to *Amaranthus*, the molecular data are scarce. Furthermore, the origin and interrelationship of different species of amaranths has yet to be clearly determined, even though several studies have been carried out on this aspect.

Cytological data can be considered as an excellent and unparalleled source of information for taxonomic and evolutionary studies (Guerra, 2012). The karyotype represents the genome itself and hence, the parameters like environmental conditions, age, developmental phase, *etc*. do not affect its phenotype at metaphase. Moreover, the changes like polyploidy, chromosome inversion and reciprocal translocation have predictable consequences on the fertility of those individuals. Cytotaxonomy relies on the fact that closely related taxa possess more similarity in karyotype than less related ones. Karyosystematics appraises the genetic relationship among species or population and help to comprehend the way it diverged from each other (Guerra, 2008) as well as support the taxonomic studies in a genus (Pedro & Delgado-Salinas, 2009). In karyosystematics, the chromosome number, size, length of arms, position of centromere, secondary constriction and related

parameters have its own significance. The relationship between karyotype evolution and related taxa can be inferred from the variations in the number and position of satellites in karyotypes (Rodrigues et al., 2014). Hence the study of chromosome number along with a precise karyomorphometric analysis of each species can help to solve the taxonomic ambiguity that exists in closely related taxa.

Chromosome morphology is usually studied on the basis of the position of the primary constriction (centromere/kinetochore), which is a principal landmark in the contracted metaphase chromosomes (Abraham & Prasad, 1983). Cytologists have adopted several methods to determine the centromere locations and described the chromosomes as telocentric (centromere at one end of the chromosome), acrocentric (centromere near one end of the chromosome), submetacentric (centromere nearer to one end of the chromosome than the other) and metacentric (centromere at or near the middle of the chromosome). These four categories are not sharply distinct, but grade imperceptibly into each other (Stebbins, 1971). The chromosomes are also described as median, nearly median, nearly submedian, submedian, nearly subterminal, sub terminal, nearly terminal and terminal (Sharma & Sharma, 1962; Sharma & Choudhury, 1964; Adhikary, 1963, 1974; Bose & Flory, 1965). Thus there exists a great deal of confusion in describing a chromosome on the basis of centromere position.

Levan et al. (1964) recognised the location of centromere in median point (M), median region (m), submedian region (sm), subterminal region (st), terminal region (t) and terminal point (T). However, this system did not give due consideration to a number of nomenclatures of centromere locations like nearly submedian and nearly subterminal. The system of Levan et al. (1964) was based on the following aspects: 1) difference (d) between long arm (l) and short arm (s), where the whole chromosome = 10 units; 2) arm ratio (r) as

the ratio of short arm/long arm and 3) centromeric index (i) as 100 s/c in the different regions, where c is the total length of the chromosome and s is the short arm length of the chromosome. Nevertheless, this system gave biased preference to the short arm of the chromosome in determining the arm ratio and the centromeric index. The criterion of difference (d) between long arm (l) and short arm (s) is not always helpful as evident from the study of karyotypes of *Aloe barbadensis* and *Furcraea gigantea* (Abraham & Prasad, 1979, 1980).

Adhikary (1974) proposed a system incorporating all the possible and up-to-date usage of centromere locations with reference to intermediate regions between terminal and median points. He recognised four fixed points and four intermediate regions. The fixed points were median (M), terminal (T), submedian (SM) and subterminal (ST). The intermediate regions were nearly median (nm), nearly terminal (nt), nearly submedian (nsm) and nearly subterminal (nst). This system was based on arm ratios, R1 (short arm/long arm) and R2 (long arm/short arm). Thus the arm ratios from both the arms were given equal importance.

A modification for the above mentioned systems was proposed by Abraham and Prasad (1983) by incorporating all the data from arm ratios and centromeric indices without particular preference to any arm. Four fixed points and six intermediate regions are recognised in each chromosome segment. In the present system due recognition is given to as many criteria as possible for the classification. In addition, this system recognised all the known centromeric locations so far described by cytologists. Also, it maintains the self explanatory terms for nomenclature of chromosomes. This system not only incorporates both the arm ratios and centromeric indices of both arms but also aid in tracing even minor differences in chromosome

morphology. Thus according to this system the chromosomes can be termed effectively (Abraham & Prasad, 1983).

Evaluation of karyotype asymmetry is the major step in the karyotype analysis to reveal cytogenetic features, specifically the cytogenetic similarities and variations among species. There are several methods for evaluating karyotype asymmetry for the elucidation of phylogenetic relationships and taxonomic treatments within a particular group or taxon. Stebbins (1971), who wrote about karyotype asymmetry, belonged to the Russian school of comparative karyotype morphology, led by Levitsky (1931), who developed the concept of its symmetry vs. asymmetry. A symmetrical karyotype is characterised by the predominance of m and sm chromosomes of approximately the same size. Increasing asymmetry can occur either through the shift of centromere position from median/submedian to terminal or subterminal, or through the accumulation of differences in the relative size between the chromosomes of the complement, thus making the karyotype more heterogeneous. A new method for estimating karyotype asymmetry introduced by Zarco (1986), is an attempt to provide an alternative method for measuring the karyotype asymmetry by using quantification and graphic representation. According to him the new method may be useful when studying related taxa which show only slight differences in karyotype asymmetry. In order to reveal the measure of karyotype asymmetry, a new chromosomal parameter of dispersion index was proposed by Lavania and Srivastava (1992) that has the potential to decipher even the minor karyotypic variations.

Chromosome studies are necessary to improve our knowledge on intra and interspecific relationships among taxa. Taxonomic delimitation and application of nomenclature in amaranth are still very tentative due to its morphological variability and frequent hybridization. Meiotic studies could be

of importance in answering evolutionary and agronomic questions. The rate of crossability gives some impression of the possibility of interspecific gene exchange (Greizerstein & Poggio, 1995). The intra and interspecific crossability, chromosome pairing and pollen grain fertility in F1 hybrids are useful parameters in assessing evolutionary relationships (Sax, 1935; De Wet & Harlan, 1972).

Recent reports on the cytogenetic studies to reveal mitotic and meiotic chromosome characterization and comparison of different *Amaranthus* species are less. A comparative analysis will help in establishing both unique and common cytogenetic features of the species studied. Apart from a cytogenetic study (Kolano et al., 2001), detailed karyomorphometric data of *Amaranthus* species was not available which is therefore attempted here. The present study is also aim to reveal the genetic variability and evolutionary status of the species, which are indices of survival of a species to the changing environmental conditions.

Micromorphological characteristics of pollen, seeds, fruits, leaves, stems and roots have played important roles in plant taxonomy, especially at the generic and species levels. Micromorphological studies are sources of useful information relevant to the taxonomic description of species and are also useful in the hands of medicinal plant scientists in comparing and identifying species (Ohikhena et al., 2017). Present day knowledge on classical taxonomy is greatly enhanced by the detailed studies on micromorphology of chromosomes, leaves and pollen grains. Thus the so called micromorphological studies help in solving many intricate taxonomic problems involving proper identification and specification of many plants. Comparative micromorphological studies of fruit, seed and pollen grain characters between *Amaranthus* species have been performed rarely. Many researchers mentioned micromorphology as a valuable source of information

for *Amaranthus* taxonomy and the gathered micromorphological characters can be used for systematic purposes in numerous studies (Srivastava et al., 1977; Chaturvedi et al., 1997; Marcone, 2000; Costea et al., 2001; Franssen et al., 2001; Costea et al., 2006; Zhigila et al., 2014). Parveen et al. (2015) recently proved that seed coat micromorphological studies can be used to recognize even intra-specific variations that exist within the species.

The morphology of the pollen grain is generally a conserved characteristic, which is an excellent means for identification of most species (Iwanami et al., 1988). Palynology means pollen and spore science and it deals chiefly with the walls of pollen grains, not with their live interior. Palynology is either basic or applied. Basic palynology has contacts with morphology, cytology, genetics and other branches of sciences (Erdtman, 1986). Attempts to separate two genera or species on the basis of pollen size indicated consistent average differences between the species. However, considerable overlapping in pollen size ranges suggests that identification by size alone would not be practical with individual pollen grains (Kapp, 1969). The pollen grain wall in most plant species has the same basic structure, although the size, shape and external morphology are species-specific. Pollen grains contain a plasmalemma-encased cytoplasm surrounded by a thin layer of cellulose called intine. Immediately surrounding the intine is a layer of exine. The exine surface often develops various forms of sculpturing and ornamentations, including various types and numbers of apertures. Hence these morphological characters can be used to differentiate species and interspecific hybrids having taxonomic ambiguity. Moreover pollen morphology is not affected by environmental conditions; therefore it has been accepted as a reliable taxonomic marker in the identification of higher plants (Aytug, 1959). In addition, the pollen viability studies are found to be useful tools to guide crosses in breeding programs.

Palynology has become an important tool, supporting the processes related to the analysis and identification of pollen grains. Regardless of the method of collection, the most widely adopted chemical process is acetolysis, as described by Erdtman (1960), which exposes pollen grains to an acidic medium (acetic anhydride and sulfuric acid), destroying its content, allowing a better assessment of the morphological characteristics of the walls of pollen grains and consequently their identification. Other, simpler methods, as proposed much earlier by Wodehouse (1935), had been using only the preparation of fresh pollen grains without eliminating its content and provide rapid morphological results. However, the use of such methods hampers the visualization of exine details, which is necessary for the palynological identification.

Plants are always an interesting subject for scientific researchers, since it is a magic pool of our requirements. Researchers keep on trying to prove the real effectiveness of active principles in many plants and thereby provide solutions for various ailments. The investigation of chemical compounds from natural products is fundamentally important for the development of new drugs, especially in view of the vast worldwide flora (Dontha et al., 2015). Literature indicates that medicinal plants are the backbone of traditional medicine (Fransworth, 1994). Phytochemical characterization can serve as a solution for taxonomic disputes over morphological identification to certain extends, since it gives a proper identity for every plant species. Certain compounds are seen in a single species alone whereas some are commonly seen in all the species. The plants having such unique compounds may have specific bioactivity towards certain aliments, which can be exploited in the field of pharmacology. The proper identification and authentication is essential for medicinal plant materials used for drug preparation. The process of standardization can be achieved by stepwise pharmacognostic studies (Ozarkar, 2005). Correct identification and quality assurance of the starting

materials is an essential prerequisite to ensure reproducible quality of herbal medicine which will contribute to its safety and efficacy. Simple pharmacognostic techniques used in standardization of plant material include its morphological, anatomical and biochemical characteristics (Anonymous, 1998).

Phytochemicals have been used as poison in traditional medicine from the ancient time itself without knowing their cellular actions or mechanisms. The phytochemical category includes compounds recognized as essential nutrients, which are naturally contained in plants and are required for normal physiological functions, so must be obtained from the diet, some are phytotoxins, antinutrients, pro-oxidants *etc*. (http[4]). Traditional medicine has served as a source of alternative medicine, new pharmaceuticals and healthcare products. Medicinal plants are important for pharmacological research and drug development, not only when plant constituents are used directly as therapeutic agents, but also as starting materials for the synthesis of drugs or as models for pharmacologically active compounds (Mukherjee, 2003). The World Health Organization estimates that 80% of the people in developing countries of the world rely on traditional medicine for their primary health care, and about 85% of traditional medicine involved, use the plant extracts. This means that about 3.5 to 4 billion people in the world rely on plants as sources of drugs (Verma & Singh, 2008). Extensive research has demonstrated that certain food components exert beneficial physiological effects on consumer's health, giving rise to the development of functional foods (Council of the European Union Regulation, 2007; Sloan, 1999). Many bioactive compounds (lycopene, polyphenols, unsaturated fatty acids, glucosinolates, proteins, peptides, *etc.*) have been identified as being responsible for different physiological effects.

Recent decades have witnessed a resurgence of interest in *Amaranthus* species as nutraceutical and natural protector against chronic ailments. Extracts of all plant parts of *Amaranthus* seem to have medicinal benefits. Several recent studies have identified many therapeutic constituents from different bioparts of *Amaranthus*. The phytochemical profile of *Amaranthus* comprises essential oils, betalains, phenolic compounds, terpenoids, alkaloids *etc*. Food-based approaches are more practical and sustainable to combat various health or nutritional disorders. Inclusion of these green leafy vegetables in the daily diet will not only add on to the nutritional value of the diet, but also serve as an additional advantage of being functional foods (Nambiar et al., 2010). Researchers have estimated that increase in fruit and vegetable consumption reduces the risk of cancer by 15%, cardiovascular disease by 30% and mortality due to any cause by 20%. This has been confirmed by epidemiological studies (Steimez & Potter, 1996; Rimm et al., 1996). This is often attributed to different antioxidant components in fruits and vegetables such as ascorbic acid, vitamin E, carotenoids, lycopenes, polyphenols and other phytochemicals (Prior & Cao, 2000; Gupta et al., 2005). Studies have shown that these antioxidant components lower the risk of several diseases (Wargovich, 2000; Kahkonen et al., 1999). Special emphasis has been given to antioxidant activity of polyphenols, which have many potent biological properties and beneficial effects on human health (Middleton et al., 2000; Vinson et al., 1995). Green leafy vegetables are rich sources of many nutrients and form a major category of vegetable groups that have been designated as 'nature's anti-aging wonders'.

Many natural products are biologically active and have been used for thousands of years as traditional medicines. Plants are the major contributors of natural products. Over the years, many approaches have been evolved towards the taxonomy of plants. The science of chemotaxonomy or chemical taxonomy is used for the classification of plants on the basis of their chemical

constituents. All the living organisms produce secondary metabolites that are derived from primary metabolites. The chemical structure of the secondary metabolites and their biosynthetic pathways is often specific and restricted to taxonomically related organisms and hence useful in classification. This method of classification is considered better in comparison to traditional method due to the ease of working methodology (Singh, 2016). The findings of chemotaxonomic studies are helpful to taxonomists, phytochemists and pharmacologists to solve various taxonomical problems. The phenolics, alkaloids, terpenoids and non-protein amino acids are the four important and widely exploited groups of compounds utilized for chemotaxonomic classification (Smith, 1976). These groups of compounds exhibit a wide variation in chemical diversity, distribution and function (Smith, 1976; Hegnauer, 1986). The system of chemotaxonomic classification relies on the chemical similarity of taxon (Atal & Kapur, 1982; Rasool et al., 2010).

The genus *Amaranthus*, an emerging food crop in the world is considered as the 'food for future', in the sense that it is the food for the coming dry season. Hence a detailed investigation had been carried out on this plant in the present study. Being a genus under scrutiny for its taxonomic identity, the plant materials chosen are subjected to cytogenetical, micromorphological, molecular and phytochemical characterization studies to gather valuable data on those aspects. Thus the present study of cytogenetical, micromorphological, molecular and phytochemical characterization of selected species of the genus *Amaranthus* aims to fulfil the following objectives:

- Molecular characterization of selected species using rbcL and matK gene sequencing as well as sequencing of ITS region of rDNA.
- Mitotic and meiotic chromosome studies of selected *Amaranthus* species using aceto-orcein and DAPI staining techniques.

- Micromorphological characterization of pollen grain, seed capsule and seed coat using SEM analysis.
- Qualitative phytochemical analysis and estimation of major phytocomponents.
- Screening for volatile and non-volatile components using GC/MS and HR-LC/MS analysis.
- Comparative proximate composition analysis of the selected species.
- Elemental analysis of the selected species using ICP-AES (Inductively Coupled Plasma Atomic Emission Spectrometer).

REVIEW OF LITERATURE

Rational and effective exploration of sustainable plant resources is an important task for ensuring global food security in the future. Humankind had been using more than 10000 edible species; however, today only 150 plant species are commercialized on a significant global scale, 12 of which provide approximately 80% of dietary energy from plants, and over 60% of the global requirement for proteins and calories are met by just 4 species: rice, wheat, maize and potato (FAO, 2005). Therefore, valorisation of valuable, however, sometimes forgotten, crops has been in the focus of many researchers all over the world during the last several decades. *Amaranthus* (Amaranthaceae), collectively known as amaranth, is a cosmopolitan genus of annual or short-lived perennial plants, consisting of more than 70 species (Espitia-Rangel et al., 2012), which according to the uses for human consumption can be divided into grain and vegetable amaranths (Mlakar et al., 2010). Amaranth was an important food crop in the Aztec, Mayan and Incan civilizations; however, its production has declined remarkably, after the collapse of the Central American cultures (Bressani, 2003; Schoenlechner et al., 2008; Alvarez-Jubete et al., 2010 a). In the study of U.S. National Academy of Sciences entitled '*Underexploited Tropical Plants with Promising Economic Value',* performed in 1975, amaranth was elected from among 36 of the world's most promising crops and identified as a major potential crop; since then, extensive research has been carried out in the genus (National Academy of Sciences, 1985).

The number of publications on various aspects of *Amaranthus* species such as cultivation, composition, applications and health effects, has been steadily increasing; several review articles have summarized the output of expanding research (Venskutonis & Kraujalis, 2013). Several articles have

been published, on different aspects of amaranth processing and utilization in Guatemala, Mexico and Peru (Bressani et al., 1992), on germplasm development and agronomic investigations in Mexico (Espitia, 1992), and on the nutritional and antinutritional composition of grain and vegetable amaranth leaves in India (Prakash & Pal, 1991). The plant *A. hypochondriacus* was recently very briefly reviewed together with other traditional plant species as a source of functional components (Rivera et al., 2010). Nutritive value of pseudocereals and their use as functional gluten-free ingredients, including amaranth, was briefly reviewed by Alvarez-Jubete et al. (2010 b). A wider use of ancient grains in a modern Indian/Asian diet to reduce the occurrence of chronic diseases was reviewed by Dixit et al. (2011). Physicochemical and digestibility characteristics of starch present in *A. hypochondriacus* were reviewed by emphasizing the starch after the storage of starchy products, their cooking and the potential of starch from unconventional sources in the production of resistant starch-rich goods using various treatments (Bello-Perez & Paredes-López, 2009). Health-promoting attributes of *A. cruentus* were briefly reviewed by Prokopowicz (2001). A review on nutritional value and utilization of *Amaranthus* species was done by Alegbejo (2013). Venskutonis and Kraujalis (2013) published a complete review, which is focusing mainly on the nutritional composition, properties and uses of *Amaranthus* seeds and vegetables. Most recently, a comprehensive review on morphology, traditional uses, phytochemical constituents and pharmacological activities of *A. spinosus* was made by Kumar et al. (2014 a).

The present review is aimed at providing a comprehensive survey of the published results on the objectives of the present study *viz*., cytogenetical, micromorphological, molecular and phytochemical aspects of selected species of the genus *Amaranthus*. The review is mainly based on articles published

from the year 2000 to 2017; however, some important reports of earlier times are also included in order to provide some valuable information.

1. MOLECULAR CHARACTERIZATION

Amaranthus is a complex genus having taxonomic ambiguity that results from the abundance of morphologically similar species and the occurrence of marked phenotypic plasticity complicating species identification, and presence of putative hybrids further confusing the variation pattern. Uninformative morphological characters and poorly resolved marker dendrograms have led to develop interest in alternative forms of molecular identifications. Chloroplast DNA has sufficient informative gene and intergenic regions to discriminate efficiently between closely related amaranth species. Species discriminations based solely on taxonomic and morphological descriptors are inefficient due to the influences from the environment. Progress to date on whole genome sequencing includes a draft genome assembly of *A. tricolor* with approximately one TB of sequence data generated on the Illumina high throughput platforms. The assembly incorporated 1.5 billion reads, spanning 382 Mb of genomic region, thus representing approximately 80% of the genome (Viljoen, 2015). Previous efforts to address phylogenetic relationships within Amaranthaceae include morphological studies and molecular analyses of chloroplast genes (rbcL, matK, atpB, trnK, intergenic trnL - trnF), nuclear ribosomal RNA gene (ITS1 and ITS2) and variability studies by using RAPD, AFLP, ISSR, PCR-RFLP and Isozyme markers.

For many years, several weedy Amaranthaceae species were identified incorrectly as *A. retroflexus* or *A. hybridus*, and much of the early literature include improperly identified species (Wax, 1995). Many of the species exhibit similar morphological characteristics, especially in the vegetative stage, making it difficult to distinguish between them (Horak et al., 1994).

The great morphological variation within species and the possibility that some of the species may inter cross and form hybrids with intermediate morphological traits (Murray, 1940; Sauer, 1967) add to identification difficulties. Precise identification of the species is important for herbicide selection as well as for biological and ecological studies. The species identification using DNA analysis (Transue et al., 1994) allows precise identification without the variation associated with morphological traits or environmental conditions.

Research on genetic diversity and phylogenetic relationships among cultivated and wild species is needed for a better understanding of crop evolution in *Amaranthus*. In addition, molecular characterization of germplasm aids plant breeders in selecting appropriate materials for further genetic improvement of cultivars and helps to overcome authentication challenges during the formulation of herbal products (Mishra et al., 2016).

In DNA barcoding, a short DNA sequence is used as a molecular marker for identifying the diversity that exists among plant and animal species. An internal transcribed spacer (ITS) region of nuclear ribosomal cistron is the most commonly used sequence locus for plant molecular systematic investigations (Kress et al., 2005). Many chloroplast, mitochondrial and nuclear genes have been utilized for studying sequence variation at genus level. Among these genes, rbcL gene sequence have been analysed by various workers to address plant systematics (Michalowski et al., 1990; Chase et al., 1993; Gielly & Taberlet, 1994). Many studies have substantiated the use of matK gene for resolving phylogenetic relationships in a broad taxonomic range (Johnson & Soltis, 1995; Hilu & Laing, 1997; Meimberg et al., 2000; Hilu et al., 2003). The matK gene has an ideal size, high rate of substitution, large proportion of variation at nucleic acid level at first and second codon position, low transition/transversion ratio and the

presence of mutationally conserved sectors. These features of matK gene are exploited to resolve family and species level relationships. Selvaraj et al. (2008) concluded that the matK gene is a good candidate for DNA barcoding of the plant family Zingiberaceae. The matK-trnK gene complex is commonly used for plant evolution studies and addresses the solution for various taxonomic level disputes (Ito et al., 1999). Polymorphism of chloroplast DNA especially trnK, matK and intergenic trnL - trnF regions has been used to study the phylogeny of various plants (Wolfe et al., 1987).

Molecular data, specifically DNA sequences, have received a great deal of attention as a potential source of "phylogenetically informative" characters that are putatively less ambiguous than non-molecular characters. Chloroplast gene rbcL, which codes for the large subunit of ribulose-1,5-bis-phosphate carboxylase/oxygenase (RuBisCO), was an appropriate locus to use in phylogenetic studies, which began with Ritland and Clegg (1987) and Zurawski and Clegg (1987).

ITS region of rDNA has proven to be a useful source of information for the resolution of phylogenetic relationships at the species level or above in many studies (Baldwin, 1992; Baldwin et al., 1995). Ribosomal DNA directs the synthesis of ribosomal RNA (rRNA). The ribosomes have two subunits: a large subunit encoded by two rRNA genes (5.8S and 26S) and a small subunit encoded by one rRNA gene (18S). The genes occur in tandem repeats and are highly homogeneous (Jorgensen & Cluster, 1988). The three rRNA coding regions are transcribed as a single precursor and then processed into mature rRNA forms. Each gene is separated by several hundred base pairs referred to as the internal transcribed spacer (ITS) regions. The ITS1 region occurs between the 18S and 5.8S genes, and the ITS2 region occurs between the 5.8S and 26S genes (White et al., 1990). These regions are more variable in nucleotide sequence than the rDNA genes, which are highly conserved. The

ITS regions of the nuclear rDNA may vary among species within a genus or among populations (White et al., 1990). Furthermore, 1,000 to 10,000 copies of rDNA are present in each plant cell (Jorgensen & Cluster, 1988), making it easy to detect and amplify. The sequence information of the ITS region can be easily obtained as it is flanked by very conservative sequences based on which universal primers have been developed. Because the ITS region is non coding, it contains a relatively high level of variability among congeneric species.

An ideal DNA barcoding region should be short enough to be amplified from degraded DNA. Han et al. (2013) discussed the possibility of using a short nuclear DNA sequence as a barcode to identify a wide range of medicinal plant species. First, the PCR and sequencing success rates of ITS1 and ITS2 were evaluated. It was based entirely on materials from dry medicinal product and herbarium voucher specimens, including some samples collected 90 years ago. The results showed that ITS2 could recover 91% while ITS1 could recover only 23% efficiency of PCR and sequencing by using one pair of primer. Secondly, 12861 ITS and ITS2 plant sequences were used to compare the identification efficiency of the two regions. Four identification criteria (BLAST, inter- and intradivergence Wilcoxon signed rank tests, and Taxon DNA) were evaluated. Finally their results supported the hypothesis that ITS2 can be used as a minibarcode to effectively identify species in a wide variety of specimens and medicinal materials.

Amaranths are an important group of plants and include grain, vegetable and ornamental types. Despite the economic importance of the amaranths, there is very little information available about the extent and nature of genetic diversity present in the genus at molecular level. Despite the increasing attention to amaranths, the molecular data are scarce (Ranade et al., 1997). In fact, the origin and interrelationship of different species of

amaranths has not been much determined, though several studies have been carried out on this aspect.

The members of the family Amaranthaceae is widely dispersed in temperate and tropical zones (Radford, 1981; Judd et al., 2002). Genera of Amaranthaceae tend to have phenotypic variation partly caused by environmental factors (Xu & Sun, 2001). The phylogeny developed based on morphological features has limitation in data accuracy because of high subjectivity (Stussey, 1990). RAPD is one of the molecular markers that can be used in the study of phylogeny. RAPD is used for polymorphism detection, and it is now possible to survey a large number of loci and ascribes unambiguous taxonomic and genetic relationships among different taxa (Williams et al., 1990; Mondini et al., 2009). Popa et al. (2010) studied genetic diversity of *Amaranthus* species using RAPD markers.

Phylogeny of Amaranthaceae based on morphological and molecular features using RAPD was done by Fatinah et al. (2012). Six genera from the family were subjected to the study. There were 374 bands generated with 18 random primers. The number of monomorphic bands, polymorphic bands and the percentage of polymorphism were 21, 353 and 94.38 % respectively. The higher number and percentage of polymorphic bands revealed genomic DNA variation. This variation is in accordance with phenotypic variation detected in this experiment. Therefore, the study concluded that, based on DNA polymorphism detected by RAPD, Amaranth family can be classified into two sub families namely Amaranthoideae and Gomphrenoideae.

Phylogenetic relationships among selected families of the Caryophyllales including Amaranthaceae were examined by the analysis of nucleotide sequences of the large subunit of rbcL (Rettig et al., 1992). Sequences from 12 Caryophyllalean families were examined using parsimony and bootstrap analysis.

Genetic diversity and relationships of 23 cultivated and wild *Amaranthus* species were examined using isozyme markers. A total of 30 loci encoding 15 enzymes were resolved, and all were polymorphic at the interspecific level. High levels of inter-accessional genetic diversity were found within species, but genetic uniformity was observed within most accessions (Chan & Sun, 1997).

Weedy species of the genus *Amaranthus*, commonly referred to as pigweeds, have increased in frequency and severity over the past few years. Identification of these weeds is difficult because of similar morphological characteristics among species and variation within species. Wetzel et al. (1999) developed a molecular marker identification system utilizing restriction enzyme analysis of amplified ribosomal DNA (rDNA). A set of polymerase chain reaction (PCR) markers was developed to distinguish 10 weedy species of pigweeds. Restriction-site variation, utilizing five endonucleases within the ITS region of the rDNA allowed for the positive identification of eight species and one pair of species. The study revealed the scope of ITS marker for biological and ecological studies on the genus.

Weedy *Amaranthus* species frequently cause economically significant reductions in crop yields. Accurate identification of *Amaranthus* species is important for efficient weed control, but *Amaranthus* species can interbreed, which might cause difficulty when identifying hybrid-derived specimens. To determine which of the several economically important weedy *Amaranthus* species is most genetically similar, and thus most likely to produce viable hybrids, Wassom and Tranel (2005) performed AFLP-based unweighted pair group method with arithmetic mean (UPGMA) analysis on 8 of these species, with 141 specimens representing 98 accessions. The analysis grouped the specimens into four principal clusters composed of palmer amaranth (*A. palmeri)* and spiny amaranth (*A. spinosus*); powell amaranth (*A. powellii*),

redroot pigweed (*A. retroflexus*) and smooth pigweed (*A. hybridus*); waterhemp (*A. tuberculatus*) and sandhills amaranth (*A. arenicola*); and tumble pigweed (*Amaranthus albus*). The cluster analysis provided evidence suggesting hybridization among powell amaranth, redroot pigweed and smooth pigweed. Further investigations using molecular analysis of the ribosomal ITS region from typical plants supported this notion. Three species, palmer amaranth, sandhills amaranth, and waterhemp are dioecious; nevertheless, the palmer amaranth and waterhemp–sandhills amaranth clusters were distinct from each other. The palmer amaranth–spiny amaranth cluster included a cluster of palmer amaranth and two clusters of spiny amaranth, a monoecious species. Thus the dioecious species palmer amaranth and waterhemp may not necessarily hybridize with each other more readily than they would to one or more of the monoecious *Amaranthus* species.

The most economically important group of species in the genus *Amaranthus* is the *A. hybridus* species complex, including three cultivated grain amaranths, and their putative progenitors. Taxonomic confusion exists among the closely related grain amaranths (*A. cruentus, A. caudatus* and *A. hypochondriacus*) and their putative wild progenitor's *viz*., *A. hybridus, A. quitensis* and *A. powellii*. ITS region of nuclear ribosomal DNA, amplified fragment length polymorphism (AFLP) and double-primer fluorescent inter simple sequence repeat (ISSR) were employed to re-examine the taxonomic status and phylogenetic relationships of grain amaranths and their wild relatives in a study by Xu and Sun (2001). Low ITS divergence in these taxa resulted in poorly resolved phylogeny. However, extensive polymorphisms exist at AFLP and ISSR loci both within and among species. In phylogenetic trees based on either AFLP or ISSR or the combined data sets, nearly all intraspecific accessions can be placed in their corresponding species clades, indicating that these taxa are well-separated species. The AFLP trees share many features in common with the ISSR trees, both showing a close

relationship between *A. caudatus* and *A. quitensis*, placing *A. hybridus* in the same clade as all grain amaranths, and indicating that *A. powellii* is the most divergent taxon in the *A. hybridus* species complex. This study has demonstrated that both AFLP and double-primer fluorescent ISSR have a great potential for generating a large number of informative characters for phylogenetic analysis of closely related species, especially when ITS diversity is insufficient.

Molecular phylogenetic studies of the order Caryophyllales including 21 genera from Amaranthaceae family based on nuclear 18s rDNA and plastid rbcL, atpB, and matK DNA sequences were carried out by Cuénoud et al. (2002). To study the inter- and infrafamilial phylogenetic relationships in the order Caryophyllales, 930 base pairs of the matK plastid gene have been sequenced and analyzed for 127 taxa. In addition, these sequences have been combined with the rbcL plastid gene for 53 taxa and with the rbcL and atpB plastid genes as well as the nuclear 18S rDNA for 26 taxa to provide increased support for deeper branches. Most taxa of the order are clearly grouped into two main clades, which are, in turn, divided into well-defined subunits.

Amaranthaceae and Chenopodiaceae together represent the most species-rich monophyletic groups in the angiosperm order Caryophyllales. So far, phylogenetic relationships between Amaranthaceae and Chenopodiaceae have remained unclear. Previous morphological and molecular studies have indicated that most of the currently accepted infrafamilial taxa in Amaranthaceae do not reflect natural groups. A study was conducted by Muller and Borsch (2005) on phylogenetics of Amaranthaceae and Chenopodiaceae based on matK/trnK sequence data evidence from parsimony, likelihood and bayesian analyses. The Amaranthaceae-Chenopodiaceae lineage was resolved as monophyletic, with Achatocarpaceae

and Caryophyllaceae being successive sisters. Within the monophyletic Amaranthaceae, a basal grade of *Bosea* L. (Macaronesian islands, Cyprus, Himalaya), followed by *Charpentiera* Gaudich. (endemic to Hawaii and the Australian Ridge) receives high support. *Celosieae* appear as sister to *Amaranthus* L.

Comparative sequencing of the chloroplast matK coding and non-coding regions was used to examine relationship among the species of Amaranthaceae with emphasis on the West African species and other closely related family such as Chenopodiaceae, Portulacaceae and Caryophyllaceae (Ogundipe & Chase, 2009). Phylogenetic analysis of the matK sequences alone and in combination using maximum parsimony methods produced monophyletic lineage of Amaranthaceae-Chenopodiaceae. The results indicated that a polyphyletic Celosieae as sister to *Amaranthus* lineage. Subfamily Amaranthoideae is paraphyletic to the core Gomphrenoids.

A review pertaining to genetic diversity in the genus *Amaranthus*, from morphology to genomic DNA was made by Sammour et al. (2012), which represented various research aspects of the genus *Amaranthus*. Genetic diversity studies for this genus are essential for providing information for propagation, domestication and breeding programs as well as conservation of genetic resources. Therefore, this review evaluated the genetic diversity between wild and cultivated species and assessed the evolutionary relationships between the cultivated species and their putative species using wide array of available markers. A wide morphological variability between *Amaranthus* species and different accessions of vegetable amaranths was reported. This variability was useful in cultivar improvement for agronomic traits. Isozymes markers showed low heterozygosity in the new world populations of *Amaranthus.* A wide genetic distance was detected between crop and weed species. Alleles at several loci were proved to be diagnostic of

the crop and weed groups. High levels of interspecific and intraspecific variations were found between *Amaranthus* species using isozyme markers. Biochemical and molecular data sets supported a monophyletic origin of grain amaranths, with *A. hybridus* as the common ancestor. The molecular data showed genetic variation among and within the populations of *Amaranthus* species and indicated that genetic diversity within wild was lower than grain species (Sammour et al., 2012).

Park and Nishikawa (2012) developed a PCR–RFLP method to identify the cultivated species of grain amaranth based on variations in the sequences of their starch synthase genes. They sequenced the *SSSI* and *GBSSI* loci in 126 accessions of cultivated grain amaranths collected from diverse locations around the world. They aligned the gene sequences and searched for restriction enzyme cleavage sites specific to each species for use in the PCR–RFLP analysis. The analysis indicated that *Eco*RI would recognize the sequence 5′-GAATT/C-3′ in the *SSSI* gene from *A. caudatus* L., and *Taq*I would recognize the sequence 5′-T/CGA-3′ in the *GBSSI* gene from *A. hypochondriacus* L. The PCR products obtained using gene-specific primers were 423 bp (*SSSI*) and 627 or 635 bp (*GBSSI*) in length. These products were cut with different restriction enzymes resulting in species-specific RFLP patterns that could be used to distinguish among the cultivated grain amaranths. The results clearly showed that *A. caudatus* and *A. hypochondriacus* were easily differentiated at the species level using this method. Accordingly, the PCR–RFLP method targeting amaranth starch synthase genes is simple and rapid, and it will be a useful tool for the identification of cultivated species of grain amaranths.

Fatinah et al. (2013) studied genetic variability of *A. spinosus* species using trnL intron, matK and rbcL gene sequencing. The sequence data obtained were analyzed using MEGA5, Bioedit and DNAsp softwares. The

molecular data had revealed that *A. spinosus* from tropical zone showed higher genetic variability than temperate zone.

The study of Viljoen (2015) was aimed to identify and classify 38 unknown amaranth accessions collected, through groupings with 45 previously positively identified amaranth species. The accessions were first phylogenetically characterized using inter and intraspecies informative chloroplast barcoding genes (matK and rbcL). These two genes revealed poor resolution within and between amaranth species. Using the sequence data from the draft genome assembly, it was possible to extract, assemble and annotate full length chloroplast sequences for each of the 83 amaranth accessions in the germplasm set. Maximum parsimony algorithms were used for the identification and classification of unknown *Amaranthus* species by grouping accessions based on their genetic similarity. A high number of informative sites across the plastid genome revealed more efficient clade representation of the different amaranth species groups.

Grain amaranth is a pseudo-cereal and an ancient crop of Central and South America. Of the three species of grain amaranth, *A. caudatus* is mainly grown in the Andean region. Several models of domestication were proposed including domestication from the wild relatives *viz*., *A. hybridus* or *A. quitensis*. To investigate the domestication history of *A. caudatus* and its relationship to the two wild relatives, Stetter et al. (2015) used genotyping-by-sequencing (GBS) on genotypes of 119 amaranth accessions from the Andean region. They determined the genome sizes of the three species and compared phenotypic variation in two domestication-related traits, seed size and seed color. The results showed that the population genetic analysis based on 9,485 SNPs revealed very little genetic differentiation between the two wild species, suggesting that they are the same species, but a strong differentiation occurs between wild and domesticated amaranths. The plant, *A. caudatus* has a

higher genetic diversity than its wild relatives and about 10% of accessions revealed a strong admixture between the wild and cultivated species suggesting recent gene flow. Genome sizes and seed sizes were not significantly different between wild and domesticated amaranths, although a genetically distinct cluster of Bolivian accessions had significantly larger seeds. The study suggested that grain amaranth is an incompletely domesticated species, either because it was not strongly selected or because high levels of gene flow from its sympatric wild relatives counteract the fixation of key domestication traits in the domesticated *A. caudatus*.

2. CYTOGENETICAL CHARACTERIZATION

The taxonomy of the genus *Amaranthus* has been confused by the extreme range of phenotypic plasticity among species and the possible introgression and hybridization involving weedy and crop species (Hauptli & Jain, 1978). Much of the difficulty in taxonomic discrimination of species within the group may be attributed to attempts at recognizing taxa based on pigmentation or growth forms, which are extremely variable within *Amaranthus* (Sauer, 1967). Taxonomic characterization is very difficult because of the occurrence of many hybrids and broad geographical distribution, which is the reason for many synonyms. Cytogenetic analysis is usually performed to clarify systematic and evolutionary aspects, which offer added information to disputed taxonomic families. It helps for the karyological characterization of the species, increase the knowledge of genetic resources and explore evolutionary trends. To explore the genetic system of any organism, a precise knowledge of its karyotype is essential. Cytogenetics employs number and morphology of chromosomes as the major distinctive characters. Chromosomes are inexplicable part of the genetic system and depict great consistency than any other character (Den Hartog et al., 1979; Iwatsubo & Naruhashi, 1991) which makes it an acceptable

parameter for species identification. The genus *Amaranthus* is characterized by small sized chromosomes with indistinguishable secondary constriction, satellites *etc*. This fact has so far restricted the cytogenetical studies of this crop.

Literature survey revealed that studies on chromosome counts of *Amaranthus* had begun from the 1940s. Murray et al. (1940) studied about the genetics of sex determination in the family Amaranthaceae and reported haploid chromosome number of some species studied. Grant (1959 a) carried out a cytogenetic study in *Amaranthus*, specifically chromosome number and phylogenetic aspects. Their study reported the chromosome numbers of 30 species of *Amaranthus*. Another study by Grant (1959 b) on the cytogenetic details of *Amaranthus*, specifically identified natural interspecific hybridization between *A. dubius* and *A. hybridus*. They had discovered a spontaneous triploid hybrid (2n = 49) between *A. spinosus* L. (2n = 34), a diploid species and *A. dubius* Mart. ex Thellung (2n = 64) which was found to be a tetraploid. The cytogenetic studies in *Amaranthus*, specifically cytological aspects of sex determination in the dioecious species were done in the same year by Grant (1959 c). Cytological observations are included in this report on four dioecious species of *Amaranthus viz., A. arenicola, A. palmeri, A. tamariscinus* and *A. tuberculatus*. All the species except *A. palmeri* showed a diploid chromosome number of 2n = 32, whereas *A. palmeri* revealed a somatic chromosome number of 2n = 34.

Chromosome numbers of nearly 36 species have been studied till now, which showed that the genus is dibasic (x = 8 and 9) and is diploid except for a solitary tetraploid (2n = 64) species, *A. dubius* Mart ex. Thell. (Pal et al., 1982; Madhusoodanan & Nazeer, 1983; Greizerstein & Poggio, 1994; Greizerstein et al., 1997). The two gametic number reported for the genus are n = 16 and 17. A cytogenetic study by Pal et al. (2000) in *A. tenuifolius* Willd.

reported a new gametic chromosome number (n = 14) for the first time, which gives one more secondary basic chromosome number for the genus. A recent study by Srivastava and Roy, (2014) confirmed the above report from the cytogenetic study of *A. blitum*, which also showed the gametic number (n = 14) as it showed a new somatic chromosome number of 2n = 28, previously characterized as 2n = 34. Hence presently the genus have three reported gametic number (x = 14, 16 and 17).

Basic chromosome numbers of x = 16 and x = 17 sometimes occur within the same species of *Amaranthus* (Pal et al., 1982), so it is possible that variable cytotypes of 2n = 32 and 2n = 34 occur in the same species. For example the chromosome number for *A. caudatus, A. hipochondriacus, A. cruentus* and *A. hybridus* is normally 2n = 32, but occasionally it is 34 (National Research Council, 1989), *i.e*. these species are diploids with a basic chromosome number of 16 or 17 (Palomino, 1991). The occurrence of two basic chromosome numbers of x = 16 and 17 in a single species and also the role of aneuploidy in chromosome evolution of the genus *Amaranthus* is a well-known fact (Sammour et al., 2012).

The chromosome analysis of *A. turicensis* hybrid (2n = 34) showed that both parental species (*A. cruentus and A. retroflexus*) should hybridize relatively easily. However, most of the *Amaranthus* hybrids exhibit relatively high level of sterility which was already confirmed by Gupta and Gudu (1991).

Greizerstein and Poggio (1995), studied the meiotic behaviour of 13 spontaneous interspecific F_1 hybrids of *Amaranthus*. The hybrids between species with n = 16 chromosome (*A. caudatus, A. hybridus, A. hypochondriacus, A. mantegazzianus, A. quitensis*) showed normal meiosis with 16 bivalents but varied considerably in pollen stainability (0-55%). Meiotic studies were carried out in four accessions of three grain species *viz*.,

A. cruentus, A. powellii and *A. retroflexus* and their F_1 hybrids to elucidate the genome relationships between the cultivated and wild types and the cytogenetic mechanism involved in speciation. These studies show close genomic homology among all these three species involving certain chromosomal aberrations resulting in their evolution (Pandey, 1999). The relationship between the two basic numbers (n = 16 and n = 17) in the grain species of the genus *Amaranthus* was determined through an interspecific dibasic cross involving white seeded cultivated *A. hypochondriacus* (n = 16) and black seeded wild *A. hybridus* (n = 17, African). In the hybrid at metaphase I, 98 percent of pollen mother cells had 15 bivalents + 1 trivalent; the remaining cells had 16 bivalents + 1 univalent. Chromosome counts in the 55 hybrid plants revealed the distribution of plants with 2n = 32, 33 and 34 in the ratio of 1:2:1, respectively. The origin of n = 17 from n = 16 through primary trisomy is suggested (Pal et al., 1982). Cytogenetic analysis of 12 populations of 10 *Amaranthus* plants (2n = 32 and 34) showed a normal meiosis forming bivalents in metaphase of meiosis I **(**Sheidai & Mohammadzadeh, 2008**)**. A post pachytene diffuse stage occurred in all the species possibly as a means of adaptation to adverse environmental conditions. ANOVA test revealed significant differences in relative cytogenetic characteristics including chiasma frequency and distribution as well as chromosome pairing among the studied species, indicating their genomic differences.

There are several reports on the chromosome counts and features of different *Amaranthus* species for a long period back. But recent reports on mitotic and meiotic chromosome characterization are insufficient. The species of which the chromosome counts were previously identified includes *A. albus, A. arenicola, A. australis, A. blitoides, A. blitum, A. bouchonii, A. caturtus, A. caudatus, A. cruentus, A. dubius, A. edulis, A. giganteus, A. gracilis, A. graecizans, A. hybridus, A. hypochondriacus, A. leucocarpus, A. lividus, A.*

*mangostanus, A. mantegazzianus, A. palmeri, A. paniculatus, A. polygamous, A. polygonoides, A. powellii, A. quitensis, A. retroflexus, A. roxburghianus , A. spinosus, A. tamariscinus, A. tenuifolius, A. tricolor, A. tuberculatus, A. turicensis***,** *A. viridis along with A. caudatus* var. *leucospermus, A. caudatus* cultivar Kiwicha 3, *A. caudatus* cultivar Kiwicha Molinere, *A. hybridus* var. *frumentaceous* and *A. hybridus* var. *paniculatus*. Although there is more number of species to the credit, most of them are same with synonymised names. A consolidated data is provided with the reported chromosome numbers of *Amaranthus* species in Table 1.

Table 1. Previously reported chromosome counts in different species of the genus *Amaranthus*

Sl. No.	Species	Haploid count (n)	Diploid count (2n)	References
1	*A. albus*	-	32	(Madhusoodanan & Nazeer, 1983; Mulligan, 1984; Song et al., 2001)
2	*A. albus*	16	-	(Sheidai & Mohammadzadeh, 2008)
3	*A. albus*	-	34	(Sharma & Banik, 1965)
4	*A. arenicola*	-	32	(Grant, 1959 c)
5	*A. australis*	-	32	(Grant, 1959 c)
6	*A. blitoides*	16	-	(Sheidai & Mohammadzadeh, 2008)
7	*A. blitoides*	-	32	(Song et al., 2001)
8	*A. blitum*	17	34	(Behera & Patnaik, 1974; Sheidai & Mohammadzadeh, 2008)
9	*A. blitum*	14	28	(Srivastava & Roy, 2014)
10	*A. bouchonii*	-	32	(Hügin, 1987; Greizerstein et al., 1997)
11	*A. caturtus*	-	32	(Behera & Patnaik, 1974)
12	*A. caudatus*	16/17	32/34	(National Research Council, 1989; Palomino, 1991; Pal & Khoshoo, 1977)
13	*A. caudatus*	-	32	(Greizerstein & Poggio, 1994; Bonasora et al., 2013; Song et al., 2001; Prajitha & Thoppil, 2017 a)
14	*A. caudatus*	16	-	(Behera & Patnaik, 1974)
15	*A. caudatus* var. *leucospermus*	16	-	(Murray, 1940)
16	*A. caudatus* cultivar Kiwicha 3	-	34	(Małuszyńska et al., 2001)
17	*A. caudatus* cultivar Kiwicha Molinere	-	32	(Małuszyńska et al., 2001)
18	*A. cruentus*	16/17	32	(National Research Council, 1989; Palomino, 1991)
19	*A. cruentus*	16	-	(Sheidai & ohammadzadeh, 2008)
20	*A. cruentus*	17	-	(Gupta & Gudu, 1991)
21	*A. cruentus*	17	34	(Pandey, 1999; Greizerstein & Poggio, 1994;

				Bonasora et al., 2013; Palomino, 1991; Prajitha & Thoppil, 2017 a; Song et al., 2001; Lanta et al., 2003)
22	*A. dubius*	-	64 (4x)	(Behera, 1982)
23	*A. dubius*	32	-	(Ugborogho & Oyelana, 1992)
24	*A. edulis*	16	32	(Pal & Khoshoo, 1977; Covas, 1950)
25	*A. giganteus*	-	32	(Behera & Patnaik, 1974)
26	*A. gracilis*	17	-	(Behera & Patnaik, 1974)
27	*A. graecizans*	-	34	(Madhusoodanan & Nazeer, 1983)
28	*A. graecizans*	16	-	(Sheidai & Mohammadzadeh, 2008)
29	*A. hybridus*	16	-	(Murray, 1940; Ugborogho & Oyelana, 1992)
30	*A. hybridus*	16/17	32	(National Research Council, 1989; Palomino, 1991; Queiros, 1989; Greizerstein et al., 1997; Song et al., 2001)
31	*A. hybridus*	17	34	(Pal et al., 1982; Pal & Pandley, 1989; Sheidai & Mohammadzadeh, 2008)
32	*A. hybridus* var. *frumentaceous*	16	-	(Behera & Patnaik, 1974)
33	*A. hybridus* var. *paniculatus*	16	-	(Behera & Patnaik, 1974)
34	*A. hypochondriacus*	16/17	32	(National Research Council, 1989; Palomino, 1991; Greizerstein & Poggio, 1994; Bonasora et al., 2013; Song et al., 2001)
35	*A. hypochondriacus*	16	-	(Gupta & Gudu, 1991; Pal & Khoshoo, 1974; Pal et al., 1982)
36	*A. hypochondriacus*	-	34	(Palomino, 1991)
37	*A. leucocarpus*	-	32	(Grant, 1959 a)
38	*A. lividus*	17	-	(Sauer, 1967)
39	*A. lividus*	-	34	(Madhusoodanan & Nazeer, 1983; Song et al., 2001)
40	*A. mangostanus*	17	-	(Behera & Patnaik, 1974)
41	*A. mantegazzianus*	-	32	(Greizerstein & Poggio, 1994; Bonasora et al., 2013)
42	*A. palmeri*	-	34	(Grant, 1959 a, c; Gaines et

				al., 2012)
43	*A. palmeri*	-	32	(Rayburn et al., 2005)
44	*A. paniculatus*	-	32	(Song et al., 2001)
45	*A. polygamous*	17	34	(Desai, 1971)
46	*A. polygonoides*	-	34	(Song et al., 2001)
47	*A. powellii*	-	32	(Hügin, 1987)
48	*A. powellii*	17	34	(Pandey, 1999; Pal & Khoshoo, 1974; Kiehn et al., 1991; Greizerstein et al., 1997; Sheidai & Mohammadzadeh, 2008)
49	*A. quitensis*	-	32	(Grant, 1959 a; Greizerstein et al., 1997)
50	*A. retroflexus*	17	-	(Murray, 1940; Sheidai & Mohammadzadeh, 2008)
51	*A. retroflexus*	-	34	(Song et al., 2001; Lanta et al., 2003)
52	*A. retroflexus*	17	34	(Pandey, 1999)
53	*A. roxburghianus*	-	34	(Song et al., 2001)
54	*A. spinosus*	17	-	(Murray, 1940; Behera & Patnaik, 1974; Ugborogho & Oyelana, 1992; Sheidai & Mohammadzadeh, 2008)
55	*A. spinosus*	-	34	(Paiva & Leitao, 1989; Greizerstein et al., 1997; Song et al., 2001)
56	*A. tamariscinus*	-	32	(Grant, 1959 c)
57	*A. tenuifolius*	14	28	(Pal et al., 2000)
58	*A. tricolor*	17	-	(Behera & Patnaik, 1974)
59	*A. tricolor*	-	34	(Madhusoodanan & Nazeer, 1983; Song et al., 2001)
60	*A. tuberculatus*	-	32	(Grant, 1959 c)
61	*A. turicensis*		34	(Gupta & Gudu,1991)
62	*A. viridis*	17	-	(Ugborogho & Oyelana, 1992; Sheidai & Mohammadzadeh, 2008)
63	*A. viridis*	-	34	(Madhusoodanan & Nazeer, 1983; Song et al., 2001)

3. MICROMORPHOLOGICAL CHARACTERIZATION

The trend of plant identification on the basis of phenotypic characters alone was changed and now scientists believe that detailed micromorphological studies can provide more accurate basis for the proper identification of the plant species and to resolve any taxonomic ambiguity that exist.

Because of the vast genetic and morphological diversity within the genus *Amaranthus*, species identification is difficult. The presence of flower is required to identify most species and even then, identification to the species level is sometimes difficult. In addition, interspecific hybridization between *Amaranthus* species causes further difficulties in identification, as these hybrids can exhibit characteristics of both parents (Horak et al., 1994). Correct identification of *Amaranthus* species is also necessary for effective weed management practices (Mayo et al., 1995).

The study of pollen as a heredity component has a predominant role in crop improvement programmes. Pollen based research has great scope in this country because of the tremendous potential of palynology in future agricultural research. So workers will have to fall back upon the researches on palynology because of the obvious importance of pollen as miniature life embodying all essential qualities of the male parent (http[5]). Pollen grains have several morphological characters on the exine, which are of diagnostic importance (Edeoga et al., 1998; Edeoga & Ikem, 2002; Mbagwu & Edeoga, 2006; Mbagwu et al., 2008) and they are species specific. In modern systematics, pollen morphology has been extremely useful in clarifying systematic relationships within plant taxa. Moreover, palynology of the species and subspecies can have taxonomic value as supporting evidence to morphological and phylogenetic traits. Also, studies on pollen grains morphology is considered as the basic necessity for palynology due to its

fundamental value in the recognition and identification of pollen grains found in various conditions (Arora & Modi, 2008). Iwanami et al. (1988) considered that the morphology of the pollen grain is generally a conserved characteristic, which is an excellent means for identification of most species. Pandey and Misra (2009) and Zhigila et al. (2014), suggested that pollen morphological characters play a major role in solving taxonomic problems. Palynological characters have been able to reposition several disputed genera and interpret problems related to the origin and evolution of many taxa (Nair, 1980) and provide classification of angiosperms (Cronquist, 1981). Pollen morphology descriptions were made using light microscopy and scanning electron microscopy (SEM), whereas the viability analysis was performed by *in vitro* germination and histochemical analysis/staining (Lugol's solution, 2,3,5-triphenyltetrazolium chloride, acetocarmine *etc*.), that will support the breeding programmes of species by increasing the understanding of their morphology and pollen grain viability.

Studies showed that the genus *Amaranthus* is a problematic genus from a taxonomic point of view (Brenan, 1961; Robertson, 1981). As a result, infrageneric classification of *Amaranthus* species in sections or subgenera is always controversial or in correction. Yet, many classification schemes have been proposed for the genus and its species have been classified in several subgenera (Sauer, 1955; Mosyakin & Robertson, 1996) or subgenera with sections (Carretero, 1979; Hugin, 1987; Klopper & Robel, 1989). But the distinctions between some sections are not yet clear.

Erdtman (1966) defined the *Amaranthus* pollen grain type, which is also present in other Amaranthaceae genera and even in several other centrospermous families (Nowicke, 1994). The pollen grain is pantoporate, apolar, small (Dimeter = 18-28 μm). Generally it has more than 18 sunken pores, uniformly distributed and having the apertural membrane granulate.

Tsukada (1967) was the first who distinguished Chenopodiaceae from Amaranthaceae by using electron microscopic studies based on various palynological parameters including apertures and exine sculpture, number of pores, exine perforations, form, sizes and number of spinules. Nowicke and Skvarla (1979) compared the Chenopodiaceae with Aizoaceae, Amaranthaceae, Basellaceae, Cactaceae, Halophytaceae, Nyctaginaceae, Phytolaccaceae, Portulacaceae and Caryophyllaceae. According to them ultrastructure of pollen grain of Chenopodiaceae resembles Amaranthaceae in several aspects such as thickened tecta, the aperture structure with reduced pointed flecks of exine underlaid by lamellar plates and a thickened amorphous endexine of pollen. Number of pores has been used as a diagnostic systematic feature by many authors, especially for families with a polyporate type of pollen grain like Chenopodiaceae.

A pollen morphological study of *A. spinosus, A. dubius* and their hybrids has been carried out by Srivastava et al. (1977). The study observed three pollen types namely, Type A: micrograins, Type B: grains with smaller pores and Type C: grains with larger pores. Type B is characteristic of *A. spinosus*, Type C of *A. dubius* and the Type A micrograins were observed in the hybrids. Pollen size, range and frequency of the various morphotypes serve to throw light on the biosystematics of the plants studied.

In earlier studies, the cytological status of a plant has been observed to find expression in various features of pollen morphology such as pollen size, number, configuration and orientation of apertures and exine surface ornamentation. A specific trend of pollen morphological variation has been visualized in earlier cytopalynological studies in various taxa (Ravikumar & Nair, 1986 a, b; Chaturvedi et al., 1990) from the basic pollen type at the lower ploidy level to the higher ploidy level. For example, in the plant taxa with 3-colpate pollen at the diploid level, 4-colpate pollen grains have been

observed at the tetraploid level and spiraperturate pollen at the hexaploid level.

Cytopalynological studies in the species, cytotypes, interspecific hybrids and amphiploids of *Amaranthus* L. and *Chenopodium* L. have been carried out using light microscopy and SEM by Chaturvedi et al. (1997). It has been observed that in the cytotypes, the pollen grains at the lower ploidy level have a narrow size range. The size range widens with the increase of the ploidy level. The exine surface ornamentation is more prominent in the polyploids than in the diploids. The triploid hybrid shows a high degree of variability with regard to pollen size and exine surface ornamentation. Such pollen characters may be used as markers in the biosystematic delimitation of cytotaxa occurring in nature. Pollen grains in both the genera are pantoporate with spinulose (granulose) exine surface ornamentation as observed under SEM.

Pollen morphological study of the varieties of *A. spinosus*, *A. hybridus*, *A. viridis* and *A. dubius* by Ugborogho and Oyelana (1992) revealed that the measurements of the pollen grains have some overlaps with the size distribution ranging from 23.52-35.28 μm. The species are highly fertile with over 85% pollen stainability except *A. dubius* and *A. hybridus* green variety that had 65.8% and 72% respectively. A survey of pollen morphology in Amaranthaceae (Borsch, 1998) revealed a remarkable diversity and many clearly distinguishable pollen types. It also led to the recognition of metareticulate pollen (Borsch & Barthlott, 1998) and suggested the utility of pollen characters for reconstructing phylogenetic relationships in the family.

A study pertaining to the seed structure and localization of seed reserves of *A. hypochondriacus* was done by Coimbra and Salema (1994). Seed characteristics were examined by using light microscopy. The seeds

were found to be cream to golden coloured, lenticular in shape and approximately 1 mm in diameter.

The seed micromorphological studies of a threatened amaranth species, *A. pumilus* (Seabeach amaranth) was done by Marcone (2000). This is the first report of the characterization of this species having large seeds (largest of all studied amaranth species) (Weakley et al., 1996). The study compares the results with those of the more commonly cultivated grain amaranth, namely *A. hypochondriacus* (Plainsman). Physical characterization and comparison of the seeds of *A. hypochondriacus* (AH) and *A. pumilus* (AP) indicate the existence of major distinguishable differences between them, in terms of shape, size and overall colour. Scanning electron microscopy revealed that AP seeds were of much larger size (1.6-fold) and possessed more of an elongated lenticular shape than those of AH. These observed morphological differences were further seen in the almost 2.63-fold higher seed weight of AP, making these seeds the largest among amaranths ever studied and, therefore, potentially more desirable due to higher biomass content. Colorimetric analysis revealed that AP seeds were sufficiently darker as well as more reddish brown in colour than those of its counterpart (AH). Although pale seeds are typically more desirable (Lehman, 1996), examination of AP seed meal upon mechanical grinding clearly revealed that the darker pigments of AP seeds were confined to its seed coat due to the substantially lighter overall meal colour.

Costea et al. (2001) made an attempt on the revision of *A. hybridus* species complex and studied micromorphological aspects of fruit pericarp, seeds and pollen grains of the species using SEM analysis. According to them variation in *Amaranthus* would improve if more care were taken during the preparation of specimens. Plants should preferably be collected when well developed fruits and seeds are present. In addition to improve chances of

correct identification, a range of individuals from the same population and a range of flowers, fruits and seeds belonging to the same plant should be collected and examined. The general characters of the seeds are examined and the transverse dehiscence versus indehiscence was found to be a constant characteristics useful for species separation. In addition, evaluation of fruit shape, size and the pattern of pericarp surface was also undertaken. The diagnostic features of the seeds considered are seed shape, size, colour, appearance of seed base, shape of the seed margin and the sculptures on the seed surface. SEM characters of seeds refer to the ornamentation of the exotesta in the marginal zone of the seeds.

Franssen et al. (2001) examined pollen morphological variation among *Amaranthus* species and interspecific hybrids. Ten weedy *Amaranthus* species, a cultivated grain amaranth species and several putative hybrids resulting from interspecific mating between common waterhemp and palmer amaranth were grown in a greenhouse. Mature pollen was collected, viewed and photographed with a SEM. The pollen grains were spherical shaped with polypantoporate, or golf ball-like aperture arrangement. Differences were observed between the monoecious and dioecious *Amaranthus* species. Pollen grains of the dioecious species had a greater number of apertures on the visible surface. One exception to these trends was the dioecious species, palmer amaranth, whose pollen was similar to that of the monoecious species, spiny amaranth. However, pollen grain diameters did not differ between the monoecious and dioecious plants. Significant differences also were noted between the pollen from the putative common waterhemp × palmer amaranth hybrids and the parental-type pollen grains. Pollen of the hybrids was similar in size to the maternal parent but had an aperture number that was intermediate between parents. This indicates that pollen characteristics may be controlled by the female parent.

Enormous morphological and genetic variations exhibited by the species in the genus results in taxonomic ambiguity at the basic morphologic level. Although basic morphological criteria can be applied to herbarium specimens or germplasm collections for quick taxonomic identification, the morphological data alone can be misleading. To ascertain the taxonomic identity of a species, along with morphological details, a combination of molecular and micromorphological analysis can provide more reliable data for germplasm identification than each method used alone (Costea et al., 2006).

To ascertain the taxonomic identity of the 'Morelos' accessions of *Amaranthus* from Mexico and their hypothesized species affiliation to *A. caudatus* or *A. cruentus*, Costea et al. (2006) conducted a comparative analysis of phylogenetic relationships among these taxa/accessions using amplified fragment length polymorphism (AFLP) and micromorphology methods. Based on AFLP data, all the controversial 'Morelos' accessions can be consistently placed into a single *A. cruentus* species clade, which is clearly separated from the *A. caudatus* species clade. The AFLP-based phylogenetic relationship of 'Morelos' and delimitation of *A. cruentus* and *A. caudatus* are further supported by micromorphology. The basic morphology without SEM and micromorphology using SEM of fruits, seeds, perianth and pollen was studied on the accessions collected. Although the basic morphology of the 'Morelos' plants shows characteristics that are somewhat intermediate between *A. cruentus* and *A. caudatus*, the micromorphology of the perianth, pollen, fruit and seed support the classification of these accessions under *A. cruentus*. Both micromorphology (Costea et al., 2001) and AFLP (Xu & Sun, 2001) have also been successfully used before to separate the species of *A. hybridus* complex, the phylogenetic group in which the cultivated grain amaranth species have evolved.

Fresh flowers of five species of *Amaranthus* were studied and documented in detail using light microscope to examine their morphology and pollen characteristics in relation to taxonomy by Zhigila et al. (2014). The species studied are *A. spinosus*, *A. viridis*, *A. hybridus*, *A. australis* and *A. tricolor*. The species showed close relationship in their pollen surface characteristics with some slight variations in psilate, micropitted or microrugulate ornamentations. The microscopic study of the pollens revealed the presence of 5 types of pollen apertures which include dicolpate, monolete, inaperaturate, periporate and triporate types. The analysis of mean polar and equatorial measurement i.e. the pollen sizes showed that the largest pollen was recorded in *A. hybridus* (61.95 ± 2.40 μm) and the smallest pollen was recorded in *A. spinosus* (23.70 ± 0.65 μm). The mean density of pollens was found to be highest in *A. australis* (12.55 ± 1.13 mm^2) and the lowest mean density was recorded in *A. hybridus* (1.29 ± 0.05 mm^2). Two diagnostic shapes were noticed in the samples studied, which are spherical grains in *A. spinosus* and *A. viridis* and oval grains in *A. hybridus*, *A. australis* and *A. tricolor*. The study revealed that floral and palynological characters are of taxonomic significance in the genus.

Parveen et al. (2015) conducted a study to find out the intra-species variation in seed coat micromorphology of *A. hybridus* by SEM analysis**.** Seed character is an important part of any crop plant. The seeds of *A. hybridus* are small in size and not possible to differentiate easily with naked eyes. The micromorphological typology of seed surfaces was investigated in eight accessions of *A. hybridus*. The aim of their study was to explore the seed characters which are useful as a tool for identifying crop species and variations among those accessions. Morphological studies showed that seeds were either pyriform or sub-pyriform or sub-spherical in shape. Pleurogram was found on the seed surface in some accessions. The ornamentations of exo-testa were found to be either polygonal or reticular or crosslinked type or

spindle shaped or scalariform or undulated lump with tertiary depositions or slits.

In a recent study by Talebi et al. (2016) to solve taxonomical problems and to aid in the identification of Iranian *Amaranthus* taxa, the palynological characteristics of seven taxa from two subgenera (*Amaranthus* and *Albersia*) were examined. Pollen grains were investigated with light microscopy and SEM using the prolonged acetolysis procedure. Twelve quantitative and qualitative characteristics were studied. Results showed that all the studied taxa had polypantoporate pollen and surface sculpturing was seen as scabrate, while its aggregation differed between samples. Statistical analysis showed that some quantitative morphological features were more valuable in the identification of studied taxa. The studied taxa were separated from each other using multivariate analysis. Their study outcome together with the results of previous investigations confirmed that the infraspecific classification of *A. blitum* requires change and it is advisable to return to the previous taxonomical status of the genus.

4. PHYTOCHEMICAL CHARACTERIZATION

A wide variety of indigenous and minor crops have been utilized for daily consumption since ancient times. They are not only important ingredients of unique gastronomic dishes but also traditional functional food to maintain wellness (Kazuhiko et al., 2002). In order to elucidate such a phenomenon, as well as seek highly effective plants, a number of plant extracts and isolated compounds have been tested for their bioactivity on various *in vitro* model systems. Information on the biological functions and active constituents of each plant species may contribute to the improvement of food habits and public health in tropical countries. Furthermore, it is expected that the wide use and extension in the utilization of such local

agricultural products would increase and stabilize the income of farmers in the rural areas (Kazuhiko et al., 2002).

Medicinal plants have attracted considerable global interest in recent years. Investigation of traditional medicine is very important for the welfare of rural and tribal communities for the treatment of conventional illness. The extensive survey of literature revealed that *Amaranthus* species are important medicinal plants with diverse pharmacological spectrum. Several pharmacological studies have been carried out with extracts of the different parts of the plants and are widely used in traditional medicinal system of India and has been reported to possess various bioactivities *viz*., antidiabetic, antipyretic, anti-inflammatory, antioxidant, hepatoprotective, antimalarial, antibacterial, antimicrobial, antidiuretic, antiviral *etc*. The whole parts of the plants are known to contain medicinally active constituents, which enlightens the enormous scope for future research in various treatments and for the discovery of safer drugs.

Amaranthus has been reported as one of the many vegetables rich in bioactive phytocomponents such as phenols, flavonoids, terpenoids, alkaloids, tannins, carotenoids, betacyanins, anthocyanins, saponins, amino acids, proteins, minerals, carbohydrates such as sugars and starch, lipids *etc*. Adequate literature is available on the phytochemical aspects of different *Amaranthus* species, but a comparative study pertaining to the phytochemical relatedness of different species are scarce. The reported phytochemical studies in different *Amaranthus* species are mentioned here onwards.

First report pertaining to the chemical characterization of a threatened *Amaranthus* species namely *A. pumilus* (Seabeach amaranth) was made by Marcone (2000). They studied about the seed characteristics using SEM, proximate analysis of seeds, elemental analysis, fractionation of amaranth

proteins, amino acids, free carbohydrates, fatty acids, triglycerides, squalene and vitamin E content and found to have promising potential.

Occurrence of tannins, phlobatannins, alkaloids, coumarins, cardiac glycosides, terpenes, phenylpropanes, organic acids, flavonoids, isoflavonoids and saponins in *A. hybridus* was previously reported by Evarando et al. (2005) and Matasyoh et al. (2009).

Preliminary phytochemical profiling on hexane, ethyl acetate, dichloromethane and methanol leaf extracts of *A. hybridus, A. spinosus* and *A. caudatus* was done by Maiyo et al. (2010). Results from the study indicated that the leaf extract contained varied types of pharmacologically active compounds. The commonly identified components in the 3 species included flavonoids, steroids, terpenoids and cardiac glycosides. In addition tannins and phlobatanins were also present in *A. caudatus* while saponin was present in *A. spinosus* alone.

Rao et al. (2010) conducted the chemical analysis of various extracts *viz*., petroleum ether, benzene, acetone, chloroform, ethanol and water of *A. tricolor* and found that carbohydrates were present in ethanol and aqueous extracts; glycosides, phenolic compounds, flavonoids and saponins were present in chloroform, ethanol and aqueous extracts; steroids were present in petroleum ether, benzene, acetone and chloroform extracts. Steroidal glycosides, carbohydrates, saponins and flavonoids were analysed for all the extracts by TLC and the results showed that carbohydrates, saponins and flavonoids were not detected in petroleum ether and benzene extracts. Acetone, chloroform and ethanol extracts were found to contain saponins and flavonoids. Carbohydrates were present in acetone and ethanolic extracts. Steroidal glycosides were found in petroleum ether and benzene extracts. The total polyphenolic content of the ethanolic and aqueous extracts was also estimated by UV spectrophotometric method and water extract showed the

presence of significant amounts of phenolic compounds compared to the standard and ethanolic extract.

Microchemical screening tests of the different parts of *A. viridis viz*., flower, leaf, stem and root was done by Khan et al. (2011). The results showed the occurrence of alkaloid, saponin, starch, fat, protein and cellulose in all the parts, mucilage and calcium oxalate in the stem alone and anthraquinone derivatives and lignin in stem and roots of the plant. Tannin was not detected in any parts of the plant studied.

Phytochemical investigation of methanolic and petroleum ether extracts of *A. spinosus* revealed the presence of flavonoids, phytosterols, glycosides, tannins, phenolic compounds, carbohydrates, terpenoids and amino acids (Mishra et al., 2007; Kumar et al., 2010 a; Mathur et al., 2010; Baral et al., 2011; Kumar et al., 2014 a). The methanol and aqueous extracts showed maximum phytochemicals like alkaloids, glycosides, steroids, flavonoids, saponin, tannin, phenolic compounds, terpenoids, carbohydrates *etc*. (Sangameswaran & Ramdas, 2010; Balakrishnan et al., 2011).

A preliminary assessment of the phytochemical and nutraceutical value of *A. cruentus* and *A. hybridus* in hydroacetonic (HAE), methanolic (ME) and aqueous extracts (AE) from the aerial parts was described by Nana et al. (2012). Phytochemical analyses revealed the presence of polyphenols, tannins, flavonoids, steroids, terpenoids, saponins and betalains. HAE extracts have shown the most diversity for secondary metabolites. The TLC analyses of flavonoids from HAE extracts showed the presence of rutin and other unidentified compounds.

Qualitative phytochemical screening of the leaf and stem extracts (acetone, benzene, methanol and cow urine) of *A. spinosus* revealed the presence of alkaloids, glycosides, terpenoids, steroids, flavonoids, tannins,

saponins, phenols and reducing sugars (Pannu et al., 2013). Methanol extract of whole plants showed the presence of flavonoids and tannins while saponins and phenols were absent. Acetone and benzene extracts showed the presence of only few phytochemicals while cow urine extracts failed to show any phytoconstituents. The presence of alkaloids, flavonoids, steroids, glycosides, anthraquinones, tannins and saponins were reported in *A. spinosus* extract by Ahamath and Sirajudeen (2014).

Phytochemical constituents present in the methanolic extract and other fractions *viz., n*-hexane, chloroform, ethyl acetate, *n*-butanol and aqueous extracts of *A. graecizans* subsp. *silvestris* was monitored by Ishtiaq et al. (2014). The analysis revealed that the amount of common bioactive components like terpenoids, alkaloids, phenolics, flavonoids and saponins were concentrated in medium polar and polar fractions (*i.e*., chloroform, ethyl acetate and *n*-butanol), while the quantity of sugars was good in remaining aqueous fraction. In this study the chloroform fraction revealed the highest amount of phenolic contents.

Rajasekaran et al. (2014) reported the presence of flavonoids, alkaloids, proteins, steroids and anthraquinones in the leaf extract of *A. spinosus*. Phytochemical analysis and estimation of extractive value was done at different extracts of *A. spinosus* by Khanal et al. (2015) and detected the presence of saponin, carbohydrate, tannin, protein, glycoside, flavonoid and phenol as phytoconstituents. The occurrence of phytoconstituents in different extracts of *A. spinosus* was as follows: hexane extract showed the presence of proteins, glycosides, flavonoids and phenols; chloroform extracts showed the presence of tannins, glycosides, flavonoids and phenol; ethanolic extracts showed the presence of saponins, proteins, flavonoids, phenols and cardiac glycosides; aqueous extract showed the presence of carbohydrates, proteins, flavonoids and phenols.

Pulipati and Narasu (2015) conducted a preliminary phytochemical screening of methanolic leaf extract of *A. tricolor* and revealed the presence of various phytoconstituents such as amino acids, carbohydrates, proteins, cardiac glycosides, steroids, alkaloids, flavonoids and tannins.

The phytochemical screening of ethanol and aqueous extracts of entire plant parts of *A. polygonoides* and *A. viridis* was done by Sharmila et al. (2017). The results indicated the presence of tannins, phenols, alkaloids, steroids, saponins, proteins and absence of flavonoids, terpenoids, cardiac glycosides and quinine in the ethanol extract of *A. polygonoides.* But all the components except tannin and saponin was absent in the aqueous extract. Tannin, saponin, phenols, proteins and steroids were found in the ethanol extract of *A. viridis,* whereas all the components except flavonoids, alkaloids, steroids, quinone and terpenoids were present in the aqueous fraction.

Estimation of phytochemical contents in the leaves of *A. hybridus* after different processing methods such as shredding, sun–drying, oven–drying, steaming and a combination of these were evaluated by Akubugwo et al. (2008). The phytochemicals examined were alkaloids, flavonoids, hydrocyanic acids, phenols, phytic acids and tannins. Sun–drying was found to be the most effective method for retaining the phytochemicals. For retention of alkaloids, flavonoids and saponins, oven–drying proved the second best method while steaming with sun – drying elicited the greatest reduction in the levels of toxic components *viz*., hydrocyanic acid, phenol, phytic acid and tannin. Hence processing methods can be employed while utilizing these leaves thereby giving the desired effect.

In a study by Mensah et al. (2008), twelve commonest green leafy vegetables from Edo State of Nigeria were selected to determine their nutritional and medicinal values. Fresh leaves were shredded and sun dried before milling into vegetable powder and then taken for qualitative and

quantitative phytochemical analysis. These vegetables were the major source of ascorbic acid and the mean values ranged from 100 to 421.6 mg/100 g with *A. cruentus* (408 mg/100 g) and *Celosia* (421 mg/100 g) species containing higher quantities. Among the studied plants, *A. cruentus* and *Talinum* recorded high mineral contents.

Phenolic content in different *Amaranthus* species have been extensively studied by several authors (Gerster, 1997; Amin et al., 2006; Pacifico et al., 2008). Isolation and structural elucidation of four new sesquiterpene glucosides from the methanolic extract of *A. retroflexus* revealed amarantholidosides IV–VII showing phytotoxic activity (Fiorentino et al., 2006). Several new polyhydroxylated terpenes and phenols in the methanolic leaf extract of redroot pigweed, *A. retroflexus* was isolated and characterized by Pacifico et al. (2008).

The total polyphenol content and antioxidant activity in the leaves of seven red amaranth (*A. tricolor*) cultivars were compared by Khandaker et al. (2008). The effect of the sunlight level on the accumulation of total polyphenol content and antioxidant activity was also investigated by growing plants under full sunlight and shaded conditions. The total phenolic content and antioxidant activity differed among the cultivars studied. Comparatively, red-fleshed cultivars had more total polyphenols and antioxidant activity than red green-fleshed cultivars. Total polyphenol and antioxidant activity were greater in leaves from plants grown under full sunlight without shading. The positive correlation between antioxidant activity and total polyphenols suggests that phenolic compounds are the major antioxidant components in red amaranth. The high amount of betacyanin in red amaranth, which gives its deep red hue, enhances antioxidant activity along with phenolic compounds. The results indicated that red amaranth containing high phenolics may act as a source of dietary antioxidants. The combination of cultivar variation and

responsiveness to specific growing conditions can create opportunities for the production and processing of vegetable red amaranth with improved antioxidant properties. Nsimba et al. (2008) mentioned a weak correlation between the phenolic concentration and antioxidant potency of various extracts and fractions from different *Amaranthu*s species studied and suggested that non-phenolic compounds may be the reason for the potential antioxidant activity. Pasko et al. (2009) reported the presence of phenolics and anthocyanins in *A. cruentus* seeds.

A. viridis have several active constituents like tannins, resins, reducing sugars and amino acids. The methanolic leaves extract was reported for the presence of rutin and quercetin (Kumar et al., 2009 a, b). It also possess spinosterol (24-ethyl-22,dehydrolathosterol) as major component along with 24-methyllathosterol, 24-ethyllathosterol, 24-methyl-22- dehydrolathosterol, 24-ethyl-cholesterol and 24-ethyl-22-dehydrocholesterol as minor components in sterol fraction. The roots of *A. viridis* possess a steroidal component namely, amasterol (24- methylene-20-hydroxycholesta- 5,7-dien-3β-ol) (Sowjanya et al., 2014).

Ozsoy et al. (2009) determined total phenolics in *A. lividus* leaf and flower extracts. The solvents used were water (by stirring), as well as methanol and ethyl acetate (by Soxhlet). Water and methanol extracts had more total phenolics (1.55 ± 0.098 and 1.51 ± 0.13 mg GAE/g DW, respectively) than the ethyl acetate extract.

Barba de la Rosa et al. (2009) reported the polyphenols in the seed flour such as rutin, isoquercitrin and nicotiflorin with relatively high antioxidant status in two commercial (Tulyehualco and Nutrisol) and two new (DGETA and Gabriela) varieties of *A. hypochondriacus.* Rutin was present at higher concentrations (10.1 mg/g flour) in Tulyehualco seed flour, while the highest amount of nicotiflorin (7.2 mg/g flour) was found in the Gabriela

variety. Tulyehualco and DGETA varieties had higher seed yield of 1475 and 1422 kg/ha. According to the authors this was the first study pertaining to the types of polyphenols present in amaranth seed flours. But rutin was already reported in amaranth leaves (Suryavanshi et al., 2007) rather than seed flours.

Characterization of polyphenols from *A. paniculatus* and *A. frumentaceus* using paper chromatography and their health implications was studied by Nambiar et al. (2010). Several

phenolic acids were identified in *A. frumentaceus* namely, vanillic acid, cis and trans ferulic acid, *p*-OH benzoic acid, *o*-coumaric acid, *p*-coumaric acid and melilotic acid. In addition syringic acid was also found in *A. paniculatus*.

Jimoh et al. (2010) conducted a quantitative determination for total phenolics, flavonoids, flavonols and proanthocyanidins present in the acetone, methanol and water extracts of the leaves of *A. asper* using standard analytical methods. The results showed the occurrence of abundant polyphenols mainly in the acetone extract. The higher amount of flavonoids was observed in water extract, where as proanthocyanidins and flavonols in methanol. Determination of anti-nutrients such as alkaloids, saponins and phytates were also analyzed and were observed to be 0.3, 7.0 and 9.25 mg/100 g (DW) respectively. Alvarez-Jubete et al. (2010 c) reported the presence of polyphenols in *A. caudatus* seeds. Repo-Carrasco-Valencia et al. (2010) observed flavonoids and betalains in *Amaranthus* seeds.

Methanol extracts of the dried leaves and seeds of *A. viridis* were screened for bioactive phytochemicals by Ahmed et al. (2013). Phytochemical investigation of this plant determines that tannins [6.07% (leaves), 5.96% (seed)], saponins (53%, 32%), alkaloids (13.14%, 11.42%), proteins (16.76%, 24.51%) and glycosides (63.2%, 32.3%) were rich in leaves except in the case of protein. The extracts also contained appreciable levels of total phenolic

contents [2.81 (leaves) and 3.61 (seed) GAE, g/100 g] and total flavonoid contents (18.4 and 5.42 QE, g/100 g). The existence of flavonoids, saponins, glycosides, terpenoids, amino acids, alkaloids, carbohydrates, phenolic compounds and proteins in the methanolic leaf extract of *A. viridis* was previously reported by Kumar et al. (2012). Sreelatha et al. (2012) observed the adequate content of total phenolics and flavonoids in *A. paniculatus* leaves. The ethanol extract of the plant was positive for alkaloids, phenolics, flavonoids and tannins. The amount of total phenolic and flavonoid content was found to be 25.23 mg/g and 11.60 mg/g respectively.

Pharmacological properties of different amaranth species have also been investigated. It was determined that *A. paniculatus* and *A. cruentus* are good sources of flavonoids, especially for rutin, which are mostly produced during the stage of blossoming (Martirosyan et al., 2003). Some other flavonols detected in *Amaranthus* leaves were isorhamnetin, kaempferol, myricetin and quercetin (Bhagwat et al., 2013). Detailed studies on the content of quercetin and rutin in different anatomical parts (leaves, stems, flowers and seeds) of 12 plant accessions belonging to 5 *Amaranthus* species, and their variations during plant vegetation, demonstrated that leafy parts of the plants contained many times higher amounts of flavonoids than the seeds. For instance, the content of rutin was as high as 30.65 g/kg DW in *A. retroflexus* leaves before harvest, whereas *A. tricolor* contained remarkably lower amount of rutin, from 0.459 (flowers) to 2.62 g/kg (leaves before harvest) (Kalinova & Dadakova, 2009). The extract of spiny amaranth (*A. spinosus*), a wild-growing weedy plant used in traditional African medicine, contained 305 mg/100 g of quercetin, hydroxycinnamates and kaempferol glycosides (Stintzing et al., 2004). Caffeic acid, ferulic acid, sinapic acid, *p*-coumaric acid, cinnamic acid (traces), together with *p*-hydroxybenzoic acid and vanilic acid were quantified in 7 varieties of *A. cruentus* from Poland, their total content was in the range of 287 to 385 mg/kg and the values were

significantly different for almost all tested accessions (Ogrodowska et al., 2012).

Amaranth extracts isolated sequentially by acetone and methanol/water from defatted plant leaves, flowers, stem and seeds yield rutin, nicotiflorin, isoquercitrin, 4- hydroxybenzoic acid and *p*-coumaric acid as major components (Kraujalis et al., 2013). In species specific studies, evaluation of bioactive substances and phenolic contents of *A. tricolor* and *A. hypochondriacus* leaves revealed high content of betacyanins and betaxanthins while isoquercetin and rutin were the most abundant flavonoids; salicylic, syringic, gallic, vanilic, ferulic, *p*-coumaric, ellagic and sinapic acids were the most common phenolic acids (Khanam & Oba, 2013). In addition to the known betalains, red-violet amaranthin, a novel betaxanthin methyl derivative of arginine betaxanthin and betalamic acid were detected in *A. tricolor* leaves (Biswas et al., 2013). Rutin and quercetin content was determined in individual plant parts of five *Amaranthus* species. Only *Amaranthus* leaves sampled at maturity stage contained quercetin or quercetin derivatives. The plants *A. hybridus* and *A. cruentus* were the best source of rutin (Kalinova & Dadakova, 2009). The amount of rutin and quercetin in methanolic leaf extract of *A. viridis* was found to be 58.52 and 9.12% w/v respectively (Kumar et al., 2009 b).

In an exclusive study, Jhade et al. (2011) reported alkaloids, glycosides, terpenes and sugars as the major phytochemicals in the roots of *A. spinosus*. A report from Castel et al. (2014) showed higher phenolic content in *A. mantegazzianus* seeds than other varieties of *Amaranthus* found in literature. López-Mejía et al. (2014) analyzed *A. hypochondriacus* seeds and they detected phenolics with antioxidant activities.

Quantitative estimation of total phenol, flavonoid, flavonols and carotenoid content present in different *Amaranthus* varieties was done by

Sravanthi and Rao (2015). The root, leaf, inflorescence and seed flavonoids of seven *Amaranthus* taxa from Tehran Province, Iran were compared by Noori et al. (2015). Aqueous and ethanolic extracts of collected plant materials were examined. The study included flavonoid detection, isolation and identification by 2-dimensional paper chromatography, thin layer chromatography, UV spectroscopy and comparison with available references. Results showed that all the examined taxa have flavonoid sulphate, flavon *C* & *C-/O* glycosides and aglycons in their roots and aerial parts with the exception of leaves that had no aglycons. Isorhamnetin, kaempferol, quercetin and rutin were found in the aerial parts of all the studied taxa. The roots of all the studied taxa had kaempferol, quercetin and rutin.

A study conducted by Cherian and Sheela (2016) estimated the alkaloid content present in different *Amaranthus* species namely *A. tricolor, A. viridis, A. dubius, A. caudatus and A. spinosus*. Maximum percentage of alkaloids was shown by *A. tricolor, A. viridis* and *A. caudatus* (8-8.8%) and the least by *A. spinosus* and *A. dubius* (5.8-6%). Karaseva et al. (2001) proposed *A. cruentus* as a new promising source for the preparation of natural phenolic compounds. Studies on the composition of the extracts have been reported and showed that ~40% of the total dry substance consists of phenolic compounds and makes up 3.5-4.5% of the total dry weight in amaranth. The phytochemical analysis using GC-MS analysis revealed the occurrence of five flavonoids like gallic acid, caffeic acid, rutin, ferulic acid and quercetin in *A. caudatus* leaves (Paranthaman et al., 2012). Isolation and structural elucidation of seven new triterpenoid saponins by GC-MS analysis from the seeds of *A. caudatus* was done by Rastrelli et al. (1995). The LC–MS/MS analysis of the amaranth grain sample revealed the free amino acid (FAA) profile with a total of 18 amino acids including 8 essential and rest non essential amino acids (Nimbalkar et al., 2012). The content of 18 FAA quantified by LC–MS/MS ranged between 0.61 and 10.70 μg/g. The highest

content was of Thr (10.70 µg/g) followed by Glu (9.20 µg/g), Try (7.79 µg/g) and Asp (7.28 µg/g). It is noticeable that both the acidic amino acids, Glu and Asp were among the dominant contributors. The essential amino acid, threonine is present in almost twice the amount than the other essential FAA quantified (Phe - 4.17, Met - 4.09, Val - 3.78, Lys - 3.33, Leu - 3.23, Ile - 3.20 µg/g). In a study by Ibrahim et al. (2015), fatty acids were identified and quantified from the stem and leaves of *A. hybridus* by using GC-MS system with split-less injection and the quantification of beta-carotene was done by using HPLC technique. The fatty acids obtained were linolenic acid, linoleic acid, palmitic acid, palmitoleic acid, stearic acid, lignoceric acid, behenic acid, arachidic acid and myristic acid.

The review by Venskutonis and Kraujalis (2013) revealed the fatty acid composition, tocopherols and tocotrienols, sterols, amino acids, phenolic constituents *etc*. present in different *Amaranthus* seeds and vegetables. The fatty acids observed were lauric, myristic, palmitic, palmitoleic, heptadacanoic, stearic, oleic, linoleic, linolenic, arachidic, eicosenoic, arachidonic and lignoceric acids. The tocopherols observed were *α*-tocopherol, *β*-tocotrienol, *γ*–tocotrienol, *δ*-tocotrienol *etc*. The sterols observed in *A. cruentus* from Austria were cholesterol, brassicasterol, campestanol, Δ^5,23-stigmastadienol, sistostanol, Δ^5,24-stigmastadienol, 24-methylen-cholesterol, campesterol, stigmasterol, Δ^7-campesterol, clerosterol, *β*-sitosterol, Δ^5-avenasterol, Δ^7-stigmastenol *etc*. (Leon-Camacho et al., 2001). Taraxerol (348.7 ppm), dammaradienol (189.0 ppm), *β*-amyrin (213.8 ppm), cycloartenol (401.8 ppm), 24-methylene-cycloartanol (446.7 ppm), citrostadienol (320.5 ppm) and 4 unidentified terpenic alcohols and methyl sterols were also found in *A. cruentus* (León-Camacho et al., 2001). Several other compounds were also reported and isolated from different *Amaranthus* species using various phytochemical techniques by various authors, which are consolidated in Table 2.

Table 2. Phytochemical compounds identified and isolated from different species of *Amaranthus*

Sl. No.	Species	Compounds	References
1	*A. albus*	Phenols; Catechins; Quercetin	(Muriuki et al., 2014)
2	*A. inamoenus*	Brassinosteroids; 24 ε-methyl-5α-cholest-7-en-3β-ol; 24 ε-ethyl-5α-cholest-7-en-3β-ol; 24-ethyl-5α-cholesta-7, 24 (28) Z-dien-3β-ol	(Takatsuto et al., 1999)
3	*A. palmeri*	2,6-dimethoxy- benzoquinone; Vanillin; Phytol; Chondrillasterol	(Bradow, 1985)
		Palmitic acid; Linoleic acid; Oleic acid; Chondrillasterol; Campesterol; Stigmasterol; Ergost-7-en-3β-ol, chondrillast-7-enol; 24-ethylidenecholest-7-en-3β-ol; β-amyrin; α-amyrin; Lupeol; Cycloartenol; 24-methylenecycloartenol	(Dailey et al., 1997)
4	*A. spinosus*	Spinoside	(Singh et al., 1993)
		Hentriacontane; α-spinasterol; Linoleic acid; Rutin; Betacarotene	(Barminas et al., 1998)
		7-*p*-coumaroyl apigenin 4-O-β-D-glucopyranoside; α-xylofuranosyl uracil; β-D-ribofuranosyl adenine; β-sitosterol glucoside	(Haq et al., 2004)
		Amaranthine; Isoamaranthine; Betanin; Isobetanin; Hydroxycinnamate; Quercetin; Kaempferol glycoside; Caffeoylquinic acid; Coumaroylquinic acid; Feruloylquinic acid; Quercetin diglycoside; Quercetin 3-*O*-rutinoside; Kaempferol diglycoside; Xylofuranosyl uracil; Betaxanthin; Betacyanin; Phenolic compounds	(Stintzing et al., 2004)
		Amaranthoside; Amaricin; Stigmasterol glycoside	(Haq et al., 2006)
		beta-sitosterol glycoside; Campesterol	(Odhava et al., 2007)

Sl. No.	Species	Compounds	References
		Amaranthine; Isoamaranthine	(Suryavanshi et al., 2007)
		Rutin; Quercetin	(Vijay et al., 2007; 2008)
		Alkaloids; Flavonoids; Glycosides; Phenolic acids; Steroids; Amino acids; Terpenoids; Lipids; Saponins; Betalains; *β*-sitosterol; Stigmasterol; Linoleic acid; Rutin; Catechuic tannins; Carotenoids	(Zeashan et al., 2008)
		α-spinasterols; Octacosanoate; Saponin; Oleanolic acid	(Manik et al., 2010)
		Hentriacontane; Octacosanoid; *α*-spinasterol; Saponin; Fatty acids	(Md et al., 2011)
		Terpenes; Alkaloids; Glycosides; Sugars	(Jhade et al., 2011)
		1,3,5-trihydroxy-7-methylanthracene-9,10-dione	(Ghosh et al., 2014)
		3,7,11,15-Tetramethyl-2-hexadecen-1-ol; n-Hexadecanoic acid; Phytol; Hexatriacontane; Linolenic acid, Methyl ester; Nonadecanoic acid, Ethyl ester; 2-[N-Methyl-N-cylohexylamino]ethanol; 3-(Ethyl-hydrazone)-butan-2-one; N-Formylglycine; 4-Hepten-2one, 5-ethyl-,3,3,4-trimethyl-	(Ahamath & Sirajudeen, 2014)
		Alkaloids; Tannins; Saponins; Glycosides; Proteins; Phenols; Flavonoids	(Amabye, 2015)
5	*A. hypochondriacus*	Saponin	(Kohda et al., 1991)
		Amaranthine; Isoamaranthine	(Cai et al., 1998; Scoles et al., 2000; Li et al., 2015 a)
		Glutelin	(Abugoch et al., 2003)
		Lunasin	(Herna´ndez-Ledesma et al., 2009)
		Rutin; Nicotiflorin; Isoquercitrin; 4-	(Barba de la Rosa

Sl. No.	Species	Compounds	References
		Hydroxybenzoic acid; Syringic acid; Vanillic acid	et al., 2009)
		Threonine; Glutamic acid; Tryptophan; Aspartic acid; Cystine; Phenyl alanine; Methionine; Valine; Lysine; Leucine; Isoleucine; Arginine; Tyrosine; Histidine; Proline; Serine; Glycine	(Nimbalkar et al., 2012)
		Nonanoic acid; α-Toluic acid; Myristic acid; n- Pentadecanoic acid; Palmitic acid; 11-*Cis* Octadecanoic acid; Stearic acid; Linoleic acid; Linolenic acid; 11-Eicosanoic acid; Docosanoic acid; Tetracosanoic acid; Carotenic acid; β-Sitosterol; Stigmasterol	(Srivastava et al., 2012)
		2H-1,2-oxazine, 6-(4-chlorophenyl) tetrahydro-2-methyl; Caffeine; 1-Naphthalenol, 4-methyl; Squalene; Phenol-4-(2-(dimethylamino)ethyl); 2-Propenoic, 3-(2,3-dimethoxyphenyl)-D acid; 2-Methoxy-4-vinylphenol; 24,25-Dihydroxyvitamin D	(López-Mejía et al., 2014)
		Phenols; Catechins; Quercetin	(Muriuki et al., 2014)
6	*A. cruentus*	Saponin	(Junkuszew et al., 1998)
		Amaranthine; Isoamaranthine	(Cai et al., 1998; Li et al., 2015 a)
		Linoleic acid; Oleic acid; Palmitic acid; Stearic acid; Linolenic acid; Triglycerides; 24-methylene cholesterol; Campesterol; Stigmasterol; Δ^7-campesterol; Clerosterol; β-sitosterol; Δ^5-avenasterol; Δ^7-stigmastenol; Δ^5-avenasterol; cholesterol; Bassicasterol; Campestanol; $\Delta^{5,23}$-stigmastadienol; Sistostanol; $\Delta^{5,24}$-stigmastadienol; Aliphatic alcohols; Hydrocarbons; β-amyrin; Cycloartenol; 24-methylene-cycloartanol	(León-Camacho et al., 2001)

Sl. No.	Species	Compounds	References
		Palmitic acid; Stearic acid; Oleic acid; Linoleic acid; Squalene	(Berganza et al., 2003)
		Anthocyanin; Polyphenols	(Pasko et al., 2009)
		Lauric acid; Myristic acid; Palmitic acid; Stearic acid; Oleic acid; Linoleic acid; Linolenic acid; Phytates; Tannins	(Mburu et al., 2011)
		Oleic acid; Linoleic acid, α-linolenic acid, α-tocopherol, β-tocopherol, γ-tocopherol	(Palombini et al., 2013)
		Phenols; Catechins; Quercetin	(Muriuki et al., 2014)
		Polyphenols	(Ghosh, 2016)
7	*A. caudatus*	Amaranthin; Isoamaranthin	(Cai et al., 1998; Li et al., 2015 a)
		α-tocopherol; β-tocopherol; γ-tocopherol; δ-tocopherol; Squalene; Ergost-7-en-3β-ol; Chondrillasterol; Chondrillastanol; 24-ethylidenecholest-7-en-3β-ol; Linoleic acid; Palmitic acid; Oleic acid; Stearic acid; Arachidic acid; Behenic acid; Lignoceric acid	(Bruni et al., 2001)
		Hexadecanoic acid methyl ester; Hexadecanoic acid; 9,12-Octadecadienoic acid methyl ester; 9,12-Octadecadienoic acid; Hexadecanoic acid, 2,3-dihydroxypropyl ester; 12-Octadecadien-1-ol; Squalene; β-tocopherol; γ-tocopherol; α-tocopherol; (3β, 5α)-ergost-7-en-3β – ol; (3β, 5α)-stigmasta-7,16-dien-3β – ol; (3β, 5α, 24S)-stigmasta-7-en-3β - ol	Conforti et al., 2005)
		Gallic acid; Caffeic acid; Quercetin; Rutin; Ferulic acid; Phytol; Pseudoephedrine (+)- ; 2,6-bis(1,1-dimethylethyl)-4- methyl-Phenol, methylcarbamate; Pyridine-3-carboxamide, 4-dimethylamino-N-(2,4-difluorophenyl)-	(Paranthaman et al., 2012)

Sl. No.	Species	Compounds	References
8	*A. tricolor*	Linoleic acid; Linolenic acid; Palmitic acid; Linolenic acid; Lignoceric acid; Arachidic acid; Spinasterol; 24-methylene cycloartenol	(Fernando & Bean, 1984)
		Amaranthine; Isoamaranthine	(Cai et al., 1998)
		Galactosyl diacylglycerols	(Jayaprakasam et al., 2004)
		Ascorbic acid; Thiamine; β-carotene; Oxalates; Tannins; Phytic acid	(Gupta et al., 2005)
		Amaranthine; Isoamaranthine; Methylated arginine betaxanthin; Betalamic acid	(Biswas et al., 2013)
9	*A. hybridus*	Amaranthine; Isoamaranthine	(Cai et al., 1998)
		Polyphenols; Myricetin; Quercetin; Kaempferol; β carotene; Xanthophyll	(Medoua & Oldewage-Theron, 2014)
		Phenols; Catechins; Quercetin	(Muriuki et al., 2014)
		Vitamin C; Carotenoids; Polyphenols; Tannins; Flavonoids; Oxalates; Phytates	(Patricia et al., 2014)
10	*A. lividus*	Amaranthine, Isoamaranthine	(Cai et al., 1998)
		β-sitosterol; Phytol; Eicosamethyl-cyclodecasiloxane; Tetradecane; Valeric acid; Cyclodecene; Nonadecane; Hexadecanoic acid, Methyl ester; Eicosane; Ergost-5-en-3-ol,(3.β.)-; Heptadecane; Cyclooctasiloxane hexadecamethyl-; Rolicyprine; Cyclononasiloxane, octadecamethyl-; 1,1,1,5,7,7,7-heptamethyl-3,3-bis(trimethylsiloxy) tetrasiloxane; 3,6-Dioxa-2,4,5,7-tetrasilaoctane, 2,2,4,4,5,5,7,7-octamethyl-; 5-Methyl-2-phenylindolizine; N-Methyl-1- adamantaneacetamide; 1,5-dioxaspiro[5.5]undecan-9-one, 3,3-dimethyl	(Nehal et al., 2016)
11	*A. paniculatus*	Amaranthine; Isoamaranthine	(Cai et al., 1998)
12	*A. viridis*	Amasterol	(Roy et al., 1982)

Sl. No.	Species	Compounds	References
		Rutin; *β*-carotene; Lutein; Quercetin	(Kumar et al., 2009 a, b)
		Squalene; Spinasterol; Trilinolein; Polyprenol; Phytol	(Ragasa et al., 2015)
		Polyphenols	(Ghosh, 2016)
13	*Amaranthus spp.*	Spinasterol; Stigmasterol; Δ^{-7} ergosterol; 24-methylene-cycloartenol; Linoleic acid; Oleic acid; Palmitic acid; Stearic acid; Myristic acid; Linolenic acid; Arachidic acid; Lignoceric acid	(Fernando & Bean, 1985)
		Spinasterol; 7-stigmastenol; Cholesterol; Campesterol; Stigmasterol; Sitosterol; 7,22-ergostadienol; 7,24(28)-ergostadienol; 7-ergostenol; 7,25-stigmastadienol; 7,24(28)-stigmastadienol; Stigmastanol; 24-methylenecycloartanol	(Xu et al., 1986)
		Lauric acid; Tridecanoic acid; Myristic acid; Myristic-oleic acid; Palmitic acid; Palmitic–oleic acid; Heptadecanoic acid; Stearic acid; Elaidic acid; Oleic acid; Linoleic acid; Linolenic acid; Arachidonic acid; Cis-11-eicosanoic acid; Cis-11.14-eicosadienic acid; Cis-11.14.17-eicosatrienic acid; Behenic acid	(Pisarikova et al., 2006 a)
		Triglycerides; Squalene; Phospholipids; Phytosterols; Tocopherols (vitamin E); Carotenoids	(Martirosyan et al., 2007)
		Isopropyl alcohol; 4-penten-2-ol; 2-methyl-3-buten-2-ol; 5-nonanol; 2-furanmethanol; Ethyl acetate; Methylbutyloxirane; 2,5-dihydrofuran; Methylfuran; 2-pentylfuran; 2-methylfuran; 2-ethylfuran; 2-*n*-butylfuran; 4-methylphenol; 2-ethylphenol; Pentanone; 2-heptanone; Furanylethanone; 2-methylheptenone;	(Ciganek et al., 2007)

Sl. No.	Species	Compounds	References
		1-methylheptenone; 2-methylpropanal; 2-methylbutanal; 3-methylbutanal; Acetic acid; Propanoic acid; Butanoic acid; Methylbutanoic acid; Methylhexanoic acid; Hexanoic acid; Heptanoic acid; Methylpyrazine; 2,5-dimethylpyrazine; 2,3-dimethylpyrazine; Dimethyldisulfide; Isopropylbenzene; *n*-propylbenzene; 1-ethyl-3-methylbenzene; 1-ethyl-4-methylbenzene; 1,3,5-trimethylbenzene; 1-ethyl-2-methylbenzene; 1,2,4-trimethylbenzene; *t*-butylbenzene; *sec*-butylbenzene; *iso*-butylbenzene; *p*-isopropyl-toluen; 1,2,3-trimethylbenzene; 1,3-diethylbenzene; *n*-butylbenzene; 1-methyl-3-*n*-propylbenzene; 1,4-diethylbenzene; 1,4-dimethyl-2-ethylbenzene; 1,3-dimethyl-4 ethylbenzene; 1,2-dimethyl-4-ethylbenzene; 2,3-dimethyl-1-ethylbenzene; 1,2,4,5-tetramethylbenzene; 1,2,3,5-tetramethylbenzene; 1,2,3,4-tetramethylbenzene; DiH-1H-indene; 2-methylnaphthalene, 1 methylnaphthalene, 2-methylpentane; Methylhexane; 2-methyl-1-propene; Methylcyclohexane; *n*-heptane; Dimethylcyclohexane; 4,5-dimethyl-1-hexene; Ethylmethylcyclohexane; *n*-undecane; *n*-dodecane; *n*-tridecane; *n*-tetradecane; *n*-pentadecane	
		Tetradecanoic acid, methyl ester; Pentadecanoic acid, methyl ester; Hexadecenoic acid, methyl ester; Heptadecanoic acid, methyl ester; 9,12-octadecadienoic acid (Z,Z)-, methyl ester, methyl linoleate; 9-Octadecenoic acid, methyl ester; Octadecanoic acid, methyl ester; Nonadecanoic acid, methyl ester; Eicosanoic acid, methyl ester; Docosanoic acid, methyl ester;	(Psodorov et al., 2015)

Sl. No.	Species	Compounds	References
		Tricosanoic acid, methyl ester; Tetracosanoic acid, methyl ester; Ergost-5-en-3-ol; Pentacosanoic acid, methyl ester; Squalene; Hexacosanoic acid, methyl ester; γ-sitosterol; Ethylcholestanol; 1,2,3,5-tetrakis-*O*-(trimethylsilyl)-pentose isomer; D-ribofuranose; 1,2,3,5-tetrakis-*O*-(trimethylsilyl)-; 1,2,3,5-tetrakis-*O*-(trimethylsilyl)-tetrose isomer; Glucofuranoside, methyl-tetrakis-O-(trimethylsilyl)-; Gluconic acid, 2,3,5,6-tetrakis-O-(trimethylsilyl)-, lactone	
14	*A. dubius*	Phenols; Catechins; Quercetin	(Muriuki et al., 2014)
15	*A. mangostanus*	Polyphenols; Anthocyanins; L-ascorbic acid	(Han & Xu, 2014)
16	*A. blitum*	2-Methoxy-4-vinylphenol; Bornyl chloride; Calamenene; Pentadecanal; (Z)-9,17-Octadecadienal; Methyl isopalmitate; Tritetracontane; Squalene; Ergosterol; 25,26-Dihydroelasterol	(Prajitha & Thoppil, 2018)

Consumers are increasingly avoiding foods containing synthetic colourants, which had compelled the food industries to replace them by natural pigments, such as carotenoids, betalains, anthocyanins and carminic acid. Betalains are water-soluble nitrogen-containing pigments, composed of two structural groups: the red-violet betacyanins and the yellow-orange betaxanthins (Azeredo, 2009). Betalains were recently identified as natural antioxidants. However, little is known about their bioavailability from dietary sources. Early studies on *Amaranthus* betacyanins in the 1960s and 1970s considered their botanical classification, application and biosynthetic pathways. The betacyanins in *A. tricolor* was identified as ‘amaranthin’ and ‘isoamaranthin’. Mabry and Dreiding (1968) designated the pigments

'amaranthine' rather than 'amaranthin' so as to avoid confusing it with 'amaranthin' (lectin). The word 'amaranthin' has also been used for a globulin protein from *Amaranthus* (Chen & Paredes-lopez, 1997).

Azeredo (2009) conducted a review regarding the properties, sources, applications and stability of betalains found naturally. Nature produces a variety of compounds adequate for food colouring, such as the water-soluble anthocyanins, betalains, carminic acid as well as the oil soluble carotenoids and chlorophylls. The health-benefit properties of natural pigments have been focussed by many works, especially those of carotenoids and anthocyanins, whose antioxidant properties have been extensively studied. Betalains, because of their relative scarceness in nature, have not been much explored as bioactive compounds, but some studies have indicated their potential as antioxidant pigments. These findings have helped to motivate utilisation of betalains as food colourants (Azeredo, 2009).

Amaranthus pigments have been used to colour beverages, bread and other foods in various locations *viz*., South Western United States, Mexico, Bolivia, Ecuador, Argentina *etc*. (Lehmann, 1990). Teutonico and Knorr (1985) reported that a red-colored extract of amaranth leaves had been used to colour foods throughout the world. Pigments from vegetable amaranth (*A. tricolor*) are approved for food use in China (Hygienic Standards for Food Additives in China, GB2760-89) (Jin, 1990). *Amaranthus* pigments are very similar to beet pigments (betanine) in structure and properties, even though it has not been used commonly as red beet pigments due to the lack of published data on food applications of *Amaranthus* pigments. Grain amaranth species with much higher biomass and more betacyanins over a longer growth period had greater commercial potential for the development of natural pigments than vegetable amaranth species (Cai et al., 1998).

The betalains are responsible for the red appearance of members belonging to the Amaranthaceae. Stem bark extracts of *A. spinosus* was characterized with respect to its phenolic profile including the betalains. The main betalains identified were amaranthine, isoamaranthine, betanin and isobetanin. The studied plant contained 24 mg betacyanins in 100 g of the ground plant material (Stintzing et al., 2004). The pigment patterns and colour qualities from the stem bark extracts of *A. spinosus* was comparable with other well known natural pigments.

The betacyanin pigments from 21 genotypes of 7 *Amaranthus* species were separated by gel filtration chromatography and HPLC in a study done by Cai et al. (1998). The study observed that the total betacyanins in the *Amaranthus* species ranged from 46.1 to 199 mg/100 g of fresh plant material and from 15.4 to 46.9 mg/g of dry extracts. A study done by Cai and Corke (1999) regarding the application of *Amaranthus* betacyanin pigments in model food systems revealed that the pigments have high potential for use as colorants in products such as jelly, ice cream and beverages. *Amaranthus* betacyanins could exhibit better colour characteristics than red radish anthocyanins at the same levels but were not as bright as a synthetic colourant. Betacyanins are generally not as stable as anthocyanins, especially acylated anthocyanins. At lower temperature (< 14°C), *Amaranthus* betacyanins had comparable colour stability to red radish and to the synthetic colorant in jelly, beverage and ice cream during 20 week storage. At room temperature (25°C), *Amaranthus* betacyanins were similar in colour stability to red radish and retained > 60% of colour during initial 4-week storage. At higher temperature (37°C), *Amaranthus* betacyanins were less stable than red radish. Ascorbic acid at 0.1% to 0.5% had a slight protective effect on *Amaranthus* betacyanin stability. Light is also found to be an absolute requirement for the synthesis of amaranthine in plants, but in *A. caudatus* the pigment accumulation take place even in the dark, increasing markedly under

the influence of white light (Rast et al., 1972). Scoles et al. (2000) had investigated the distribution of the betalain pigment amaranthine in the fresh leaf extract of *A. hypochondriacus* by spectroscopic techniques, for its possible application in the colouring of drinks and food at an industrial level. Knowledge of genotypic differences in pigment composition, content and stability is essential in the selection of *Amaranthus* varieties for the commercial production of food colorants.

Betalains exhibit broader pH stability than anthocyanins, thus they can be used in low-acid foods as a food colourant (Stintzing & Carle, 2007). Cai et al. (2003) showed that the amaranthine in *Amaranthus* possessed very strong antioxidant activities and could be used as a substitute source for the well-known betanins from red beets in the food colourants and natural antioxidants (Cai et al., 2003; Klimczak et al., 2002). Characterization of phenolics, betacyanins and antioxidant activities of the seed, leaf, sprout, flower and stalk extracts of three *Amaranthus* species *viz*., *A. hypochondriacus, A. caudatus and A. cruentus* by UHPLC and LC–ESI–MS was done by Li et al. (2015 a). The main betacyanins identified were amaranthine and isoamaranthine. The phenolic compounds identified were gallic acid, protocatechuic acid, chlorogenic acid, gentistic acid, 2,4-dihydroxybenzoic acid, ferulic acid, salicylic acid, rutin, ellagic acid, kaempferol-3-rutinoside and quercetin in the extracts of different parts of *Amaranthus*. The total phenolic content (TPC) ranged from 1.04 to 14.94 mg GAE/g DW, the total flavonoid content (TFC) ranged from 0.27 to 11.40 mg CAE/g DW, the total betalain content (TBC) ranged from 0.07 to 20.93 mg/100 g DW. There was a strong correlation between TPC, TBC, TFC and the antioxidant activity. Thus the result suggested that all parts of the *Amaranthus* plant can be a good source of antioxidants.

There are some evidences about the potential of beta-carotene in some taxa of *Amaranthus* (Marcone et al., 2002; Uzo & Okorie, 1983). The composition of carotenoids in five native Brazilian leafy vegetables including *A. viridis* was determined by Mercadante and Rodriguez-Amaya (1990). The range of total carotenoid contents of *A. viridis* was found to be 347-468 µg/g, with lutein and *β*-carotene predominating. Marcone et al. (2002), investigated beta-carotene, lycopene and lutein present in some amaranth species and are found to be good sources of both lutein as well as beta-carotene. The carotenoid content present in the green leaves of different *Amaranthus* species during the stage of blossoming was studied by Martirosyan et al. (2004). In a recent study, Nambiar and Sharma (2014) investigated the beta-carotene content in the leaves of red amaranth. The results obtained for moisture, total carotene and beta-carotene contents analyzed were as follows; 82.24%, 9886.326 µg/100 g FW and 2135.298 µg/100 g FW.

Amaranth grain consists of 6 to 9% of oil which is higher than most other cereals. Amaranth oil contains approximately 77% unsaturated fatty acids and is high in linoleic acid, which is necessary for human nutrition. The lipid fraction is unique due to the high squalene content. Fatty acid compositions of the seed oils from eighteen varieties of *Amaranthus* species have been determined after room temperature trans-esterification. Consistent with earlier studies, wide variations in the fatty acid composition are reported and appeared to be agronomically related. All varieties showed significant levels of squalene (2-5%) and a combined linoleic acid and oleic acid occurrence between 70-80%. This study represents the first report of fatty acid composition in grain *Amaranthus* cultivated in West Africa (Ayorinde et al., 1989). Novel fatty acids with strong α-glucosidase inhibitory activity namely (14E, 18E, 22E, 26E)-methyl nonacosa-14, 18, 22, 26 tetraenoate and b-sitosterol were purified from the methanol extract of whole plant of *A. spinosus* (Mondal et al., 2015). HPLC quantitative analysis revealed 0.15% of

the former compound and 0.06% of the latter compound in the plant extract. Accordingly this is the first report on the isolation and identification of (14E, 18E, 22E, 26E)-methyl nonacosa-14, 18, 22, 26 tetraenoate and b-sitosterol from *A. spinosus.*

A detailed and comprehensive study on *A. cruentus* oil and fatty profile was done by León-Camacho et al. (2001) and observed the presence of fatty acids, triglycerides, sterols, methyl sterols, terpenic and aliphatic alcohols, tocopherols and hydrocarbons by using HRGC and HPLC. The crude fat content of the amaranth seeds was 6.34% and amaranth oil contains considerable amounts of linoleic (38.2%) and oleic (33.3%) acids, about 20% palmitic acid and lower quantities of stearic (4%) and linolenic (1%) acids.

He and Cork, (2003) studied *Amaranthus* grain of 104 genotypes from 30 species cultivated in China for oil content, squalene concentration and fatty acid profile. The taxonomic relationship among the species in *Amaranthus* was carried out by multivariate statistical methods using the oil data. The study revealed that the oil and squalene content in *Amaranthus* grain ranges from 1.9 to 8.7% and trace to 7.3% respectively. The average contents of three major fatty acids observed in *Amaranthus* grain were palmitic acid (22.2%), oleic acid (29.1%) and linoleic acid (44.6%). The range of oil and squalene content in dried mature leaves of 45 *Amaranthus* genotypes were 1.08 to 2.18% and trace to 0.77% respectively, which is much lower than that of grains. The major fatty acids found in leaf extracts were linolenic acid (56.5 to 62.0%), linoleic acid (15.5 to 24.7%) and palmitic acid (13.5 to 15.5%) respectively. Observations were also made for the fatty acid compositions at different growth stages. The fatty acid content in leaf lipids was lower in mature leaves than in young leaves. The saturated/unsaturated ratio decreased when the leaf grows to maturity.

Milled *Amaranthus* seeds were subjected to HPLC and GC method for the analysis of triacylglycerols, squalene and fatty acid composition of oils (Kraujalis et al., 2013). The fatty acids detected in the seed oil were myristic, palmitic, palmitoleic, heptadecanoic, stearic, oleic, linoleic, linolelaidic, linolenic, arachidic, cis-11-eicosenoic and arachidonic acids.

Lipophilic fraction of *Amaranthus* contains valuable edible oil with high content of unsaturated fatty acids, squalene and a pool of minor compounds, such as tocopherols, phytosterols, waxes, terpenic alcohols and hydrocarbons. Health benefits of bioactive components present in unsaponifiable fraction of amaranth lipids, particularly those exhibited by squalene and tocopherols were reported in many studies (Martirosyan et al., 2007; Lippi et al., 2010). Kraujalis and Venskutonis (2013) extracted squalene and tocopherols from the lipophilic fraction of the black colour seeds of *Amaranthus* species by using supercritical carbon dioxide extraction method. The yields of tocopherols and squalene from amaranth seeds obtained was found to be 317.3 mg/kg and 0.289 g/100 g, respectively.

Amaranthaceae family is characterized by its diverse chemistry, including betalains, flavonoids, phenolic acids, essential oils, sesquiterpenes, diterpenes and triterpenes. Triterpene saponins are present abundantly in *Amaranthus* species. Saponins can be classified into two groups based on chemical structure of their aglycone skeleton (Vincken et al., 2007). The first group consists of the steroidal saponins, which are present mainly in monocotyledonous angiosperms (Sparg et al., 2004). The second group consists of the triterpenoid saponins, the more common form that occurs predominantly in the dicotyledonous angiosperms. Triterpenoid saponins are most interesting from the therapeutic point of view and are found in a wide variety of medicinal plants including *Amaranthus* species. In recent years, great interest has been shown to their characterization in plants and to the

investigation of their pharmacological and biological properties (Mroczek, 2015). Saponins were isolated from numerous Amaranthaceae plants and have been proved to be the major effective constituents in some species. They might be responsible for the pharmacological or nutraceutical activities of these plants. However, the saponin profiles of these plants have not been fully elucidated. Increasing attention is being paid to the characterization of saponins from Amaranthaceae plants for the screening of bioactive compounds and for quality control purpose. Several strategies were implemented for the isolation of Amaranthaceae saponins. In general, the extraction techniques employed in saponin extraction can be classified into two categories: the conventional and the green techniques (Cheok et al., 2014). The former one demand utilization of a large volume of solvent and are usually time consuming. The green techniques, utilizing minimal volumes of solvent and requiring shorter extraction time, were used seldom in case of Amaranthaceae saponins (Mroczek et al., 2012). A variety of chromatographic techniques namely LPLC, MPLC, HPLC *etc*. was employed for the isolation and separation of individual saponin. GC/MS analysis was used for the quantification of the sapogenins, i.e. oleanolic acid, hederagenin, phytolaccagenic acid and serjanic acid in *Chenopodium quinoa* seeds (Burnouf-Radosevich et al., 1985, Mastebroek et al., 2000).

Mroczek (2015) conducted a review on the distribution, structure and pharmacological properties of triterpene saponins present in Amaranthaceae plants. Different pattern of triterpene saponin occurrence was characterised in almost 30 species belonging to the family. The reported bioactivity of saponin mixtures or individual saponins *in vitro* and *in vivo* include cytotoxic, antiinflammatory, immunomodulatory, hepatoprotective, antidiabetic, hypolipidemic, antiosteoporosis, antiviral, antifungal and anthelmintic activities. Herbal and edible Amaranthaceae plants can be considered as

promising and highly available sources of biologically active triterpene saponins.

Chenopodiaceae and Amaranthaceae are two previously constituted different families and are still divided into two families in some taxonomic analyses, when occurrence of specific saponin structures and patterns could provide reliable evidence for the taxonomic integrity of the extended Amaranthaceae family. Although the data on saponin content in Amaranthaceae plants are still limited in terms of the number of analyzed plants as well as the number of elucidated compounds, these compounds could be presumed as promising biomarkers in chemotaxonomic analysis aiming to clarify the relationships of Amaranthaceae and Chenopodiaceae. However, treating saponin content as evidence in such analyses require more detailed analysis of saponin profile in a large number of species.

Oleszek et al. (1999) studied about the toxicity of four triterpene saponins present in the seeds of *A. cruentus* after determining their concentration by using HPLC. It was shown that the total concentration of saponins in seeds was 0.09-0.1% of dry matter. In germinating seeds an increase in concentration to 0.18% was observed after 4 days of germination, which remained stable for the next 3 days and later dropped to 0.09%. Highly purified extracts from the seeds were tested for their toxicity against hamsters. The maximum dose which cannot produce toxicity was found to be 1500 mg/kg since it does not show any toxicity symptoms and behavioural changes in hamsters. As per their study the approximate lethal dose value estimated for *Amaranthus* saponin was between 1500 and 1750 mg/kg of body weight. According to their conclusion, low contents of saponins in *A. cruentus* seeds and their relatively low toxicity guarantee that amaranth-derived products create no significant hazard for the consumers. Isolation and structural elucidation of seven triterpenoid saponins and ionol-derived glycosides from

the leaves of *A. caudatus* was done by Rastrelli et al. (1998). Among them three are new natural products.

In recent years extensive scientific evidence has been provided for the existence of biologically active peptides and proteins derived from foods that might have beneficial effects upon human health (Möller et al., 2008). Biologically active peptides are food-derived peptides that exert beyond their nutritional value as a physiological, hormone-like effect in humans. They are found in milk, egg, meat and fish of various kinds as well as in many plants. Bioactive peptides are inactive within the sequence of their parent protein and can be released by enzymatic hydrolysis either during gastrointestinal digestion or during food processing. They usually contain 2–20 amino acid residues per molecule, but in some cases may consist of more than 20 amino acids. Following digestion, bioactive peptides can either be absorbed through the intestine to enter the blood circulation intact and exert systemic effects, or produce local effects in the gastrointestinal tract. Depending on the sequence of amino acids, these peptides can exhibit diverse activities (Erdmann et al., 2008). Many of the known bioactive peptides are multifunctional and can exert more than one of the effects (Korhonen & Pihlanto, 2003; Meisel & FitzGerald, 2003; Meisel, 2004). Because of their health-enhancing potential and safety profiles they may be used as components in functional foods or nutraceuticals. Research is being conducted on the production of active peptides or biopharmaceutical proteins and peptides using transgenic plants (Giddings et al., 2000).

Silva-Sánchez et al. (2008) reported the occurrence of lunasin in *Amaranthus* seeds. Lunasin is a bioactive peptide derived mainly from soybean seed protein having cancer-preventive efficacy. Maldonado-Cervantes et al. (2010) reported a cancer-preventive peptide in *Amaranthus* that has activities similar to those of soybean lunasin. The amaranth lunasin-

like peptide, however, requires less time than the soybean lunasin to internalize into the nucleus of NIH-3T3 cells, and inhibits histone acetylation thereby inhibiting the transformation of NIH-3T3 cells to cancerous foci. A detailed review on bioactive peptides production and functionality by Korhonen and Pihlanto (2006) represented the enzymatic hydrolysis, microbial fermentation and fractionation methods for the production of bioactive peptides and their functionalities such as regulation of the gastrointestinal system, nervous system, cardiovascular system, immune system *etc*. The acidic-subunit of amaranthin, the main seed storage protein of *A. hypochondriacus*, was reported to be carrying four antihypertensive biopeptides (German-Baez et al., 2014). Udenigwe and Aluko (2012) reviewed the production, processing and potential health benefits of food-derived bioactive peptides, since there is a growing trend and interest in the use of food protein-derived peptides as intervention agents against chronic human diseases and for maintenance of general well-being. A 30-residue antimicrobial peptide Ar-AMP was isolated from the seeds of *A. retroflexus* essentially by a single step procedure using reversed-phase HPLC, and its *in vitro* biological activities were studied by Lipkin et al. (2005).

Amaranthus, especially the grain types are rich sources of bioactive peptides having various health benefits. The plant produces seeds that contains high quality protein (Barba de la Rosa et al., 1992), compounds with health-promoting potential such as rutin and nicotiflorin (Barba de la Rosa et al., 2009) and peptides with various bioactivities (Barrio & Anon, 2010, Silva-Sánchez et al., 2008; Tiengo et al., 2009, Tovar-Pérez et al., 2009; Barba de la Rosa et al., 2010). For these reasons, *Amaranthus* is a "natural biopharmaceutical" plant that could reduce hunger while increasing human health (Kitts & Weiler, 2003; Korhonen & Pihlanto, 2003). Fritz et al. (2011) reported the *in vivo* and *in vitro* antihypertensive activity of *A. mantegazzianus* seed protein hydrolysates. An *in silico* approach by Vecchi

and Añón (2009) revealed the interaction of angiotensin-I converting enzyme 1 (ACE) with two new tetrapeptides only found in *Amaranthus* 11S globulin, which can inhibit the activity of such enzyme *in vitro*. In addition, Tovar-Pérez et al. (2009) has recently shown the existence of ACE-inhibitory peptides in the albumin 1 and globulin fractions from *A. hypochondríacus.* The presence of dipeptidyl peptidase IV (DPP-IV) inhibitory peptides encrypted in *Amaranthus* protein having antidiabetic potential was studied by Velarde-Salcedo et al. (2012). The highest inhibition of DPP-IV, an enzyme known to deactivate incretins, (hormones involved in insulin secretion) by *A. hypochondriacus* peptides released after simulated gastrointestinal digestion when compared to other seeds such as soybean, black bean and wheat, reflects its anti-diabetic potential. This is the report of the identification of inhibitory DPP-IV peptides from amaranth hydrolysates and the prediction of their binding modes at the molecular level, leading to their possible use as functional food ingredients in the prevention of diabetes (Velarde-Salcedo et al., 2013). ACE-inhibitory peptides (ALEP, VIKP) and anti-hypertensive peptides (IKP, LEP) were also found in amaranth 11S globulin in an *in silico* study, which utilized 3D models and simulated protein docking technologies (Vecchi & Añón, 2009). Kabiri et al. (2010) reported an antiatherosclerotic effect of amaranth in hypercholesterolemic rabbits via reducing levels of LDL, triglycerides and oxidized low density lipoproteins (ox-LDL). Quiroga et al. (2015) gave an account of the potential antitumor activity of a biopeptide namely lectin from *A. mantegazzianus*. Immunomodulatory effect of a bioactive peptide (SSEDIKE) from *A. hypochondriacus* grains in an IgE-mediated food allergy mouse model was examined by Moronta et al. (2016 a, b). Identification and characterization of four more peptides (AWEEREQGSR, YLAGKPQQEH, IYIEQGNGITGM, TEVWDSNEQ) with antioxidant activity, obtained after gastrointestinal digestion of amaranth proteins was also made recently (Delgado et al., 2016).

Sabbione et al. (2016) studied about the antithrombotic peptides from *A. hypochondriacus* seed flour obtained by gastrointestinal digestion. Identification and characterization of antioxidant peptides obtained by gastrointestinal digestion of *A. mantegazzianus* proteins was done recently by Delgado et al. (2016).

Cavazos and Gonzalez de Mejia (2013) identified bioactive peptides from cereal storage proteins (wheat, oat, barley and rice) and studied their potential role in prevention of chronic diseases particularly diabetes, cardiovascular disease and cancer. This study supports the notion that cereal grains are a nutritious part of a healthy diet by preventing chronic diseases (Cavazos & Gonzalez de Mejia, 2013). Biologically active peptides are considered to promote diverse activities, including ACE inhibitory (Vermeirssen et al., 2004), antioxidant (Peña-Ramos et al., 2004), anticancer (Wang et al., 2008; Hernandez-Ledesma et al., 2009), antithrombotic (Fiat et al., 1989), hypocholesterolemic (Wergedahl et al., 2004), antidiabetic (Velarde-Salcedo et al., 2012; 2013), antiobesity (Nishi et al., 2001), hypotriglyceridemic (Kagawa et al., 1996), immunomodulatory (Playford et al., 2000), antinflammatory (Mine & Kovacs-Nolan, 2006), antihypertensive (Kanauchi et al., 2005; Germán-Báez et al., 2014), osteoprotective (Aoe et al., 2005), antilipidemic (Tsutsumi et al., 2000) and antiatherosclerotic activities (Kabiri et al., 2010) as well as by reducing the risk of cardiovascular diseases (Erdmann et al., 2008) by modulating and improving physiological functions. Hence bioactive peptides may provide new therapeutic applications for the prevention or treatment of chronic diseases. As components of functional foods or nutraceuticals with certain health claims, bioactive peptides are of commercial interest as well (Erdmann et al., 2008).

Extracts of *Amaranthus* have been used to treat several ailments since ancient times. However, *Amaranthus* species has seen a resurgence of interest

in recent decades. Literature summarization of *in vitro* and *in vivo* studies established that *Amaranthus* species has several protective and curative properties attributed mainly to strong antioxidant activity (Peter & Gandhi, 2017). A very recent comprehensive review by Peter and Gandhi (2017) critically analyzed the folklore claims of *Amaranthus* with scientific evidences, delineating its phytochemical based nutrapharmaceutical properties and emphasizes clinical utility of the plant in various chronic diseases, also defining gap areas for future clinical research. Data on 13 edible *Amaranthus* species was acquired and the results provided an in-depth analysis of biological effects of major bioactive ingredients present in crude extracts of specific bioparts. Tanmoy et al. (2014) and Kumar et al. (2014 a) described the morphological, phytochemical geographical, pharmacological and traditional uses of *A. spinosus.* The reported bioactivities of different *Amaranthus* species were represented in Table 3.

Table 3. Pharmacological properties reported in different *Amaranthus* species

Sl. No.	Species	Bioactivities Reported	References
1	*A. gangeticus*	Anticancer activity	(Sani et al., 2004)
		Protective effect against degenerative diseases	(Dutta & Singh, 2011)
		Antioxidant activity	(Dutta & Singh, 2011)
2	*A. tricolor*	Antitumor activity	(Jayaprakasam et al., 2004)
		Hepatoprotective activity	(Al-Dosari, 2010; Aneja et al., 2013)
		Antidiabetic and hypo-cholesterolemic activities	(Clemente & Desai, 2011)
		Gastro protective activity	(Devaraj & Krishna, 2011, 2013)
		Antiinflammatory and antinociceptive activities	(Bihani et al., 2013)
		Antinociceptive and antidiabetic activities	(Rahmatullah et al., 2013)
		antinociceptive activity	(Bihani et al., 2013)
		Antioxidant activity	(Clemente & Desai, 2011; Amornrit & Santiyanont, 2016)
		Neuroprotective effect	(Amornrit & Santiyanont, 2016)
3	*A. spinosus*	Vermifuge effects	(Olufemi et al., 2003)
		Haematological activity	(Olufemi et al., 2003)
		Antiinflammatory activity	(Olajide et al., 2004)
		Antimalarial activity	(Hilou et al., 2006; Pannu et al., 2013)
		Antipyretic and antioxidant activities	(Mishra et al., 2007; Kumar et al., 2010 b)
		Antidiabetic, anti-hyperlipidemic and spermatogenic effects	(Sangameswaran & Jayakar, 2008)
		Antidiarrheal and antiulcer activities	(Zeashan et al., 2009 a)

Sl. No.	Species	Bioactivities Reported	References
		Hepatoprotective, antioxidant, antinociceptive activities	(Zeashan et al., 2009 a, b)
		Anthelmintic activity	(Baral et al., 2010)
		Antipyretic activity	(Kumar et al., 2010 b)
		Antitumor activity	(Joshua et al., 2010)
		Antihyperglycemic and antihyperlipidemic activities	(Sangameswaran & Ramdas, 2010)
		Antidiabetic and anticholesterolemic activities	(Girija et al., 2011)
		Antifertility activity	(Jhade et al., 2011)
		Antibacterial and cytotoxic activities	(Bulbul et al., 2011)
		Antimicrobial activity	(Harsha, 2011; Sheeba et al., 2012)
		Antianaphylactic activity	(Patil et al., 2012)
		Gut modulatory and bronchodilator activities	(Chaudhary et al., 2012)
		Diuretic activity	(Amuthan et al., 2012)
		Anti peptic ulcer activity	(Mitra et al., 2013)
		Antidepressant activity	(Kumar et al., 2014 b)
		Genotoxic and antigenotoxic potential	(Prajitha & Thoppil, 2016)
		Cytotoxic and apoptotic activities	(Prajitha & Thoppil, 2017 b)
4	*A. blitum*	Antioxidant activity	(Ozsoy et al., 2009; Amornrit & Santiyanont, 2016)
		Anticancer activity	(Al-Mamun et al., 2016)
		Neuroprotective effect	(Amornrit & Santiyanont, 2016)
5	*A. retroflexus*	Antioxidant activity	(Pacifico et al., 2008)
6	*A. hypochondriacus*	Antihypertensive activity	(Silva-Sánchez et al., 2008)
		Hepatoprotective activity	(López et al., 2011)
		Antiatherosclerotic activity	(Montoya-Rodríguez et al., 2014)

Sl. No.	Species	Bioactivities Reported	References
		Antioxidant activity	(López-Mejía et al., 2014)
7	*A. paniculatus*	Hypoglycemic, hypolipidemic and antiinflammatory activities	(Nawale et al., 2017)
		Antitumor activity	(Sreelatha et al., 2012)
8	*A. viridis*	Antinociceptive and antipyretic activities	(Kumar et al., 2009 c)
		Anthelmintic activity	(Kumar et al., 2010 a)
		Heaptoprotective and antioxidant activities	(Kumar at al., 2011)
		Antidiabetic and anticholesterolemic activities	(Girija et al., 2011)
		Antiinflammatory activity	(Macharla et al., 2011)
		Antidiabetic, antihyperlipidemic and antioxidant activities	(Kumar et al., 2012)
		Antiphytopathogenic and antiproliferative activities	(Carminate et al., 2012)
		Cardioprotective a ctivity	(Saravanan et al., 2013)
		Antimicrobial activity	(Malik et al., 2016)
9	*Amaranthus* spp.	Antioxidant activity	(Gupta & Prakash, 2009)
10	*A. caudatus*	Antibacterial activity	(Maiyo et al., 2010)
		Antinociceptive activity	(Kumar et al., 2010 c)
		Antidiabetic and anticholesterolemic activities	(Girija et al., 2011)
		Antiatherosclerotic activity	(Kabiri et al., 2011)
		Antipyretic and antioxidant activities	(Kumar et al., 2011)
		Anticancer activity	(Adewale & Olorunju, 2013)
11	*A. cruentus*	Hepatoprotective activity	(Escudero et al., 2011)
		Anticancer activity	(Gandhi et al., 2011)

Sl. No.	Species	Bioactivities Reported	References
		Hypocholesterolemic activity	(Tiengo et al., 2011)
		Antioxidant activity	(Soares et al., 2015)
12	*A. hybridus*	Antibacterial activity	(Maiyo et al., 2010)
		Antinociceptive and antiinflammatory activities	(Singh & Sheoran, 2011)
		Antioxidant activity	(Nana et al., 2012)
		Anticancer activity	(Adewale & Olorunju, 2013)
13	*A. tristis*	Antiproliferative activity	(Baskar et al., 2012)
14	*A. mantegazzianus*	Antitumor activity	(Barrio & Anon, 2010)
15	*A. roxburghianus*	Treatment for ulcerative colitis	(Nirmal et al., 2013)

Most developing countries depend on starch-based foods as the main staple food for the supply of both energy and protein. This accounts in part for protein deficiency which prevails among the populace as recognized by Food and Agricultural Organization (Ladeji et al., 1995). In most of the tropical countries like Africa, the daily diet is dominated by starchy staple food. Vegetables are the cheapest and most readily available sources of important proteins, vitamins, minerals and essential amino acids (Okafor, 1981). Many of the local vegetable materials are under-exploited because of inadequate scientific knowledge of their nutritional potentials. Though several works reporting compositional evaluation and functional properties of various types of edible wild plants in use in the developing countries abound in literature, much is still to be done. Many workers (Lockeett et al., 2000; Akindahunsi & Salawu, 2005; Edeoga et al., 2006; Hassan et al., 2006; Ekop, 2007) have reported the composition and functional properties of various types of edible wild plants in use in the developing countries.

The species of *Amaranthus* are valuable sources of nutrients with high quality proteins, vitamins, minerals, dietary fibres and bioactive compounds

such as phenolics (Silva-Sánchez et al., 2008). In addition to being consumed as a staple food, both the grain and vegetable *Amaranthus* have also been commercially exploited for natural dyes and pharmaceuticals (National Academy of Sciences, 1984). The grains of amaranth have become one of the most favoured new foods in recent years in North America, largely because they are gluten-free, and contain a balanced essential amino acid profile. Apart from the macronutrients, phytochemicals in both the grain and vegetable portion of *Amaranthus* plants have also been shown to possess many beneficial health effects.

Amaranth is a non-grass plant producing significant amounts of edible "cereal" grain. Some authorities call it as the "grain of the 21st century". The main grain amaranth species *viz*., *A. caudatus, A. cruentus* and *A. hypochondriacus* have been cultivated in Mexico, Central America and the Andean highlands of Southern America for several thousand years (Sauer, 1976). The species have gained high popularity in the Indian subcontinent as well. These grain amaranths are highly rich in protein and the essential amino acid lysine (Tucker, 1986; Bressani, 1989). In recent years amaranths have received global attention, being a quality protein crop that can stand remarkably well against abiotic stress under marginal management practices. Its protein content is as high as 16%, which is somewhat higher than that found among commercial varieties of common cereals. This protein is unusual because its balance of amino acids is very close to the optimum required in human nutrition. In comparison to other cereals it has high lysine and tryptophan contents. Low gluten concentrations make products based on amaranth very valuable for the diet of persons afflicted with diabetes (NRC, 1984). Amaranth grain contains ~8% oil, of which oleic and linoleic fatty acids comprise ~70%, ~20% steraric and ~1% linolenic acids. The oil also contains high level of squalene (Oleszek et al., 1999). Lipids are characterized by high degree of unsaturation, which is desirable from a nutritional point of

view. Linoleic acid is the most abundant fatty acid followed by oleic acid and palmitic acid (Alvarez-Jubete et al., 2009). Another interesting component of amaranth oil is squalene, a terpenoid compound ubiquitous in the unsaponifiable fraction of cereal grains, used in medicines and cosmetic industry. Amaranth seed oil contains approximately 6% of squalene (Escudero et al., 2004), a considerably higher amount than usually found in oils from other cereal grains (Becker, 1994). Its importance as a food constituent resides in its ability to lower cholesterol levels by inhibiting its synthesis in the liver (Escudero et al., 2006). He and Corke (2003) reported the occurance of large amount of squalene in *A. hybridus.* Plant sterols (phytosterols) are another group of biologically active components present in pseudocereal lipids.

The high protein content, the well-balanced amino acid profile, high content of fat, fibre and minerals all make the grain amaranth, candidates for a renewed major role as world food source. Rat feeding trials with different varieties of *A. caudatus* and *A. cruentus* have shown that the protein quality of amaranth seeds is far superior to that of conventional grains and that the amount of utilizable protein is higher (Gamel et al., 2004). Morales et al. (1988) showed in a study with small children that a relatively small percentage of amaranth protein raised the utilization of maize protein to that of amaranth alone. In a rat study Pedersen et al. (1987) found that addition of amaranth grain to cereals such as wheat, maize and sorghum, improved protein quality significantly without affecting the digestibility. Grain species of *Amaranthus* are not only richer in protein content than other cereals but also their properties for human diet are closer to the nutritional perfection (Gupta & Gudu, 1991). Mendonça et al. (2009) reported the health benefit, specifically the cholesterol-lowering effects of amaranth protein.

A detailed review on the functional attributes of *Amaranthus* was done by Velez-Jimenez et al. (2014), who reported the amino acid composition of *A. cruentus* grains with alanine (5.3), arginine (14.7), aspartic acid (12.2), glycine (13.8), isoleucine (5.5), leucine (8.6), glutamic acid (25.1), lysine (8.3), cystine (1.9), methionine (3.4), phenylalanine (6.1), tyrosine (5.4), threonine (4.3), proline (6.9), valine (6.0), histidine (3.8) and serine (8.8 mg/g).

Both grain and leaves of amaranth are utilized for human as well as animal food (Tucker, 1986). The nutritional value of amaranth has been extensively studied by Martirosyan (2001; 2003). It has been shown that amaranth leaves are also an excellent source of protein, with its maximal accumulation in the blossoming phases (17.2–32.6% from dry weight for various samples) (Kadoshnikov et al., 2005). The investigation of Martirosyan et al. (2005 a) indicated that amaranth grain is a good source of modern diet, particularly to make special products for the people who are at a high risk to cardiovascular diseases. Furthermore, new formulas were created on the base of natural ingredients only with amaranth flour, containing a significant amount of magnesium and dietary fibre. This is extremely beneficial for people who have high blood pressure and are at a high risk of heart attack (Martirosyan et al., 2005 b).

Grain amaranth is a pseudo cereal with high protein content. The seed contain about 17-19% high quality protein (5% lysine and 4% sulfur-containing amino acids) and 63% easily digestible carbohydrates, as compared to more traditional crops that have an average of ~10% proteins. In *Amaranthus* 50% of the total seed proteins at maturity are globulin and albumin (Raina & Datta, 1992). Usage of amaranth as livestock feed indicated relatively high protein qualities (Kadoshnikov et al., 2001; Byron et al., 2001). Amaranth proteins are mostly storage proteins with legumin-like

features. They comprise globulin (11S-globulin and globulin-p) and glutelins, which present different solubility in aqueous solvents (Tovar-Pérez et al., 2009). Nimbalkar et al. (2012) used LC–MS/MS method for analysing the free amino acid composition of amaranth grain and revealed a higher concentration of essential amino acids especially threonine and tryptophan than non essential amino acids. Several methods have been adopted for determination of amino acids from *Amaranthus viz*., GC/MS (Calder et al., 1999; Wood et al., 2006), capillary electrophoresis–mass spectroscopy (CE–MS (Soga & Heiger, 2000; Soga et al., 2004; Poinsot et al., 2010), LC/MS (Piraud et al., 2005; Armstrong et al., 2007) and many other complicated methods. This is the first report of use of LC–MS/MS method for analysing the free amino acid composition of amaranth grain. Composition and structural characterization of *A. hypochondriacus* protein isolates by electrophoretic and calorimetric study was previously done by Martínez and Añón (1996). Rastogi and Shukla (2013) reported protein content of *Amaranthus* as 12-17 g/100 g flour and is found to be higher than that of most cereals.

The amaranth species as a group is used for a wide variety of purposes. Leaves and stems are interesting vegetable suitable for soups, salads and other dishes, used across Africa, Asia and the America. *A. dubius, A. cruentus* and *A. tricolor* are adapted for growth as leafy vegetables in areas with hot climate, especially in the hot, humid tropics (http[6]). The leaves are edible, being a good source of carotene, iron, calcium, ascorbic acid, vitamins and proteins. In Africa and the Caribbean, amaranth is commonly eaten as a pot herb, with individual leaves picked off the plants periodically. The leaves of both the grain and vegetable types may be eaten raw or cooked. *Amaranthus* grown principally for vegetable use have better tasting leaves than the grain types (O'Brien, 1983). A dark-seeded strain of *A. cruentus* is commonly cultivated as a vegetable in West Africa (Grubben, 1976).

Among the green leafy vegetables, *Amaranthus* species are a rich store house of vitamins, including carotene, vitamin B6, vitamin C, riboflavin, folate, as well as essential amino acids and dietary minerals like Ca, P, Fe, Mg, K, Cu, Zn and Mn (Musa et al., 2011).

Analysis of the mineral composition of *A. hybridus* leaves after different processing methods such as shredding, sun–drying, oven–drying, steaming and a combination of these were evaluated by Akubugwo et al. (2008). The minerals examined are Na, K, Ca, Mg, Fe, P, and Zn. Oven–drying was the most effective method for retaining the minerals. Steaming with sun-drying caused the greatest lose of these minerals. Steaming with oven-drying produced leaves with the lowest phosphorus content.

Processing of *Amaranthus* vegetables may influence the contents of microconstituents: for instance, steam cooking of *A. hybridus* resulted in 29.2% vitamin loss; however, the content of flavonoids increased by 25%, probably due to the release of some flavonoids during cooking (Adefegha & Oboh, 2011). Green leafy vegetables are also prone to vitamin losses during pre-processing handling conditions, exposure to light, refrigerated storage and other factors (Faboya, 1990).

Evaluation of nutrient contents of *Amaranthus* leaves prepared using different cooking methods revealed the highest retainment of nutrient contents in sample A having finely chopped leaves without heat treatment (Funke, 2011). The sample B is prepared by steaming before chopping the leaves. The third method involved chopping of leaves before blanching (sample C). These three samples were subjected to proximate analysis and micro-nutrient determinations. Result of proximate analysis showed that sample B method of preparation has highest percentage of crude fat per gram of sample (2.31 ± 0.45), protein (4.35 ± 0.15) and fibre (1.09 ± 0.06). Sample A has highest percentage of moisture (90.35 ± 0.27) and ash content (1.36 ± 0.28) while

sample C has highest percentage of carbohydrate per gram of sample (4.89 ± 1.21). Micronutrient determination results showed that sample A was highest in vitamin C (1.57 mg ± 0.06) and iron (535.84 ppm ± 123.42), followed by sample C with lesser vitamin C (1.21 ± 0.07) and iron (501.88 ± 215.19), while sample B had the least amount of vitamin C (0.79 ± 0.06) and iron (354.18 ± 121.84).

A comparative analysis of nutritional value, mineral content and bioactive compounds present in the leaves of *A. hybridus* after drying and cooking was done by Medoua and Oldewage-Theron (2014). Results showed that *A. hybridus* contained a significant amount of protein (3.6 ± 0.1 g/100 g FW) and minerals (level of which exceeds 1% of fresh weight). Total polyphenols (109.4 ± 7.5 mg GAE/100 g FW), vitamin C (36.6 ± 1.0 mg/100 g FW) and carotenoids represented by *β*-carotene (25.3 ± 1.3 mg/100 g FW) and xanthophylls (7.48 ± 0.31 mg/100 g FW) formed a significant part of bioactive contents of the leaves. Since the boiling can cause significant losses of compounds in the boiling water, it can be recommended to avoid the boiling step with discard of boiling water.

The influence of home cooking on bioactive components and antioxidant capacities of an edible amaranth *viz*., *A. mangostanus* was done by Han and Xu (2014). The amaranth was cooked by simmering, boiling, frying, blanching and steaming. The contents of total phenolics, anthocyanins, *L*-ascorbic acid, carotenoids, lutein, beta carotene and ferric reducing antioxidant power (FRAP) of edible amaranth were determined after the cooking by colorimetric assays. Home cooking proved to degrade anthocyanins but increased carotenoids. Steaming increased total phenol content (TPC) about 50% while simmering reduced 31.1% of TPC. Simmering, frying and blanching reduced *L*-ascorbic acid content by 18.6%, 17.2% and 14.0% respectively. Steaming increased *L*-ascorbic acid by 21.7%.

Both lutein and beta-carotene content was reduced by frying but increased by other methods. FRAP values of cooked vegetable were higher than the raw counterpart, which indicated that the cooking increased the antioxidant capacities of the edible amaranth.

Shukla et al. (2006) assessed and compared various mineral components in 30 strains of *A. tricolor*, which are being widely consumed as a leafy vegetable in many parts of the world. The study showed that vegetable *Amaranthus* is a rich source of a number of macro and micronutrients such as K, Ca, Mg, Zn, Fe, Mn *etc*. In addition to mineral composition, leaf size, foliage yield and variability studies were also performed. This study proposed that some of the strain may serve as promising material for selection of plant types with increasing yield potential as well as mineral composition for which they showed high mean performance.

Akubugwo et al. (2007) examined the nutritional and chemical value of *A. hybridus* using standard analytical methods in order to assess the nutritional potential of the plant. In addition to moisture, ash, protein, lipid, fibre and carbohydrate content they also disclosed the mineral composition, vitamin composition, chemical composition and amino acid profile of the plant. Their study revealed high nutritional potential of *A. hybridus* for the reason that the leaves contain appreciable amount of proteins, fat, fibre, carbohydrate, calorific value, mineral elements, vitamins, amino acids and generally low level of toxicants. Thus, it can be concluded that *A. hybridus* leaves can contribute significantly to the nutrient requirements of man and should be used as a source of nutrients to supplement other sources. The chemical compositions analyzed in their study include alkaloid, flavonoid, saponin, tannin, phenols, hydrocyanic acid and phytic acid. The study disclosed the occurrence of hydrocyanic acid (16.99 mg/100 g) and alkaloids (3.54 mg/100 g) in abundance and other components are in tolerable limit.

According to their study, the alkaloid content obtained was higher than the values reported for other leafy vegetables specifically *Aspilia africana*, *Bryophyllum pinnatum*, *Cleome rutidosperma* and *Emilia coccinea* consumed in Nigeria (Edeoga et al., 2005; Okwu & Josiah, 2006). As per their opinion, chemical analysis should not be the sole criterion for judging the nutritional value of this plant. It is necessary to consider other aspects such as the biological evaluation of the nutrient content of the plant in order to determine the bioavailability of the nutrients and also the effects of processing on the chemical and nutritive value of the plant.

A detailed phytochemical and proximate analysis of three *Amaranthus* species *viz*., *A. graecizans*, *A. lividus* and *A. viridis* was done by Ziada et al. (2008). The result showed that *A. lividus* contained a relatively high percentage of moisture content (9.51%), ash content (20.67%), water soluble ash (11.17%), total protein (214.8 mg/100 g DW) and total lipid (13.73%). The plant, *A. graecizans* contained a relatively high percentage of acid insoluble ash (2.45%) and total carbohydrates (196.5 mg/100 g DW). The highest value for total nitrogen content was recorded for *A. viridis* is 271.14 mg/100 g DW. Preliminary phytochemical screening revealed the occurrence of alkaloids, carbohydrates, flavonoids, sterols and tannins in all the parts of the species studied. Saponin is detected in the leaves of *A. lividus* as well as in the leaves and stem of *A. viridis*. Sulphates are found in all the organs of the species studied except in the leaves of *A. graecizans*. Chloride is found in all the parts of the species except in the leaves and roots of *A. graecizans.* The highest extractive value is obtained for the leaves of *A. lividus*, while the lowest is in the roots of *A. graecizans.* Elemental analysis revealed the occurrence of K, Fe, Cu and Cd at highest concentration in *A. graecizans* where as Na, Ca, Mg, Mn and Zn occur in *A. lividus.* The data obtained from amino acid investigation shown the occurrence of 15 amino acids in each of the species studied. They are aspartic acid, threonine, serine, glutamic acid,

proline, glycine, alanine, valine, leucine, isoleucine, phenyl alanine, tyrosine, histidine, lysine and arginine. An amino acid namely cysteine is found only in *A. graecizans.*

Proximal composition of two commercial (Tulyehualco and Nutrisol) and two new (DGETA and Gabriela) varieties of *A. hypochondriacus* was analyzed by Barba de la Rosa et al. (2009), where Gabriela had the highest protein content of 17.3%, but all varieties had an adequate balance of essential amino acids. The fat contents were similar among the varieties, however, at higher proportions (7–9%), than for cereals such as wheat (2.1%) and maize (4.5%). There were no differences in ash contents between varieties, but Gabriela had the highest fibre content of 2.5%.

The proximate analysis of the leaves of *A. asper* using standard analytical methods was done by Jimoh et al. (2010), which showed that the leaves contained moisture, ash, crude protein, crude lipid, crude fibre, energy and carbohydrate. Elemental analysis in mg/100 g (DW) indicated that the leaves contained sodium (0.0373), potassium (2.872), calcium (2.504), magnesium (0.569), iron (419), zinc (26), phosphorus (0.177), copper (18), manganese (91) and nitrogen (1.78 mg/100 g). Comparing the nutrient and chemical constituents with recommended dietary allowance (RDA) values, the results reveal that the leaves contain an appreciable amount of nutrients, minerals and phytochemicals and low levels of toxicants.

Calcium bioavailability of raw and extruded *A. caudatus* grains was assessed in a biological assay in rats. The results showed that amaranth can be a complementary source of dietary calcium, bioavailability of which is favourably modified by the extrusion process (Ferreira & Arêas, 2010).

Multi-elemental analysis and classification of the seeds of *A. hypochondriacus, A. cruentus* and *A. dubius* using inductively coupled plasma

optic atomic spectroscopy was done by Aguilar et al. (2011). The elements analyzed were Ag, Al, Ba, Ca, Co, Cr, Cu, Fe, K, La, Li, Mg, Mn, Mo, Na, Ni, P, S, Sr, V, Zn and Zr. The lowest mineral content was found in *A. hypochondriacus* and highest one was found in *A. dubius*.

Physicochemical analysis of the roots of *A. spinous* by Jhade et al. (2011) showed the following components: total ash (6.60% w/w), acid insoluble ash (2.09% w/w), water soluble ash (2.44% w/w), water soluble extractive value (5.03% w/w), ethyl alcohol soluble extractive value (6.60% w/w), moisture content (2.07%) and pH value (6.9).

Evaluation for physicochemical parameters and phytochemical constituents in the roots of *A. tricolor* was done by Aneja et al. (2011). They revealed the occurrence of total ash, acid insoluble ash, water soluble ash, ethanol soluble extractive and water soluble extractive as 12.8%, 6.89%, 5.0%, 7.6% and 20.0% w/w respectively. Phytochemical screening of the aqueous extract showed the presence of alkaloids, flavonoids, carbohydrates, glycosides, tannins, proteins, amino acids, fats, fixed oil, saponins, mucilages, steroids and phenolic compounds (Aneja et al., 2011, 2013).

A detailed review on the nutritional composition and phytochemical constituents of *A. viridis* was done by Sowjanya et al. (2014), and found that the leaves and seeds are highly nutritious. The nutritional components present in 100 g DW was as follows; water 81.8%, protein 34.2 g, fibre 6.6, fat 5.3, carbohydrates 44.1, ash 16.4, calcium 2243 mg, phosphorous 500 mg, iron 27 mg, sodium 336 mg, potassium 2910 mg, vitamin A 50 mg, riboflavin 2.43 mg, thiamine 0.07 mg, niacin 11.8 mg, vitamin C 790 mg and calories 283 kcal.

Proximate characterization of *A. hypochondriacus* seeds and leaves are analyzed by López-Mejía et al. (2014). Protein and fat content in seeds was

found to be 18.03% and 5.88% respectively, which are significantly higher than in leaves (15.49% and 1.52%). The ash and crude fibre contents are found to be higher in leaves than grains.

In a study of Bhat et al. (2015), the dried *A. hypochondriacus* grains were investigated for their nutraceutical properties. A thorough nutritional characterization of the dried amaranth grains demonstrated them to be a good source of natural antioxidants and minerals like phosphorus, calcium, magnesium and iron followed by other nutrients. Phytochemical analysis of the dried grains revealed the presence of phenolics, flavonoids, alkaloids and saponins. Fortified cookies were formulated using the amaranth grains flour for nutritional analysis. From the nutritional analysis, it was observed that the cookies can act as a good source of protein, carbohydrates and dietary fibre and hence a potential source of energy.

Several other studies were also reported regarding the proximate composition of various *Amaranthus* species which are consolidated in the Table 4.

Table 4. Components reported by proximate analysis of different *Amaranthus* species

Sl. No.	Species	Components	References
1	*A. albus*	Moisture, protein, fat, ash, crude fibre, carbohydrate; Ca, Zn, Fe	(Muriuki et al., 2014)
2	*A. hybridus*	Oil, ash, protein	(Dhellot et al., 2006)
		Moisture, ash, crude protein, crude lipid, crude fibre, carbohydrate; Na, K, Ca, Mg, Fe, Zn, P; *β*-carotene, thiamine, riboflavin, niacin, pyridoxine, ascorbic acid, α-tocopherol; isoleucine, leucine, lysine, methionine, cysteine, phenyl alanine, tyrosine, threonine, valine, alanine, arginine, aspartic acid, glutamic acid, glycine, histidine, proline, serine	(Akubugwo et al., 2007)
		Moisture, protein, energy, fat, fibre, ash, carbohydrate; Ca, P, Na, Zn, Mg, Mn, Cu, Fe	(Odhav et al., 2007)
		Na, K, Ca, Mg, Fe, P, Zn	(Akubugwo et al., 2008)
		Moisture, protein, fat, ash, crude fibre, carbohydrate; Ca, Zn, Fe	(Muriuki et al., 2014)
		Moisture, pH, ash, crude fibre, lipids, proteins, carbohydrate, calorific value, vitamin C, carotenoid; Ca, Mg, P, K, Fe	(Patricia et al., 2014)
		Ash, fat, carbohydrate, fibre, Vitamin C, protein	(Medoua & Oldewage-Theron, 2014)
		Moisture, ash, crude protein, vitamin C; Fe, Zn, Ca, K, Mg	(Kachiguma et al., 2015)
3	*A. spinosus*	Moisture, protein, energy, fat, fibre, ash, carbohydrate; Ca, P, Na, Zn, Mg, Mn, Cu, Fe	(Odhav et al., 2007)
		Total ash, acid insoluble ash, water-soluble ash and sulfated ash, Alcohol soluble extractive value,	(Mathur et al., 2010)

Sl. No.	Species	Components	References
		water-soluble extractive, moisture content	
		Total ash, acid insoluble ash, water soluble ash, water soluble extractive, ethyl alcohol soluble extractive, moisture content, pH	(Jhade et al., 2011; Baral et al., 2011)
		Protein, carbohydrate; Na, K, Ca, Fe	(Srivastava, 2011)
		Total ash, acid soluble ash, water soluble ash, alcohol soluble extractive, water soluble extractive, moisture content	(Amabye, 2015)
4	*A. cruentus*	Crude protein, fibre, ash, organic matter, nitrogen-free extractive,	(Pisarikova et al., 2006 a, b)
		Protein, vitamin A, vitamin C; Ca, Fe, Zn	(Yang & Keding, 2009)
		Moisture, protein, fat, ash, fibre, nitrogen-free extractive; ascorbic acid, pyridoxine, niacin, thiamine, riboflavin, tocopherol; Ca, Cu, Mn, Mg, Fe, Zn, K, Na, P; lysine, threonine, valine, cysteine, methionine, isoleucine, leucine, phenyl alanine, arginine, alanine, histidine, aspartic acid, glutamic acid	(Mburu et al., 2011)
		Ag, Al, Ba, Ca, Co, Cr, Cu, Fe, K, La, Li, Mg, Mn, Mo, Na, Ni, P, S, Sr, V, Zn, Zr	(Aguilar et al., 2011)
		Alanine, arginine, aspartic acid, glycine, isoleucine, leucine, glutamic acid, lysine, cysteine, methionine, phenyl alanine, tyrosine, threonine, proline, valine, histidine, serine; Fe, Cu, Mn, Na, Ca, Mg, K, P	(Palombini et al., 2013)
		Ca, Mg, K, P, Fe, Zn; protein, chlorophyll, carotenoid	(Kamga et al., 2013)
		Moisture, protein, fat, ash, crude fibre, carbohydrate; Ca, Zn, Fe	(Muriuki et al., 2014)
		Moisture, ash, crude protein, vitamin C; Fe, Zn, Ca, K, Mg	(Kachiguma et al., 2015)
		Protein, fat, starch, ash,	(Das, 2016 a)

Sl. No.	Species	Components	References
		carbohydrate; Ca, Fe, P	
5	*A. hypochondriacus*	Crude protein, fibre, ash, organic matter, nitrogen-free extractive	(Pisarikova et al., 2006 a, b)
		Isoleucine, lysine, leucine, threonine, valine, protein, fat, ash, fibre	(Barba de la Rosa et al., 2009)
		Ag, Al, Ba, Ca, Co, Cr, Cu, Fe, K, La, Li, Mg, Mn, Mo, Na, Ni, P, S, Sr, Zn, Zr	(Aguilar et al., 2011)
		Moisture, ash, fat, protein, crude fibre, carbohydrate	(López-Mejía et al., 2014)
		Moisture, protein, fat, ash, crude fibre, carbohydrate; Ca, Zn, Fe	(Muriuki et al., 2014)
		Crude protein, vitamin C, moisture, ash; Fe, Zn, Ca, K, Mg	(Kachiguma et al., 2015)
6	*A. lividus*	Moisture, ash, crude protein, vitamin C; Fe, Zn, Ca, K, Mg	(Kachiguma et al., 2015)
		Moisture, ash, protein, crude fat, carbohydrate, crude fibre, calorific value; K, Ca, Na, Mg, P, Fe, Mn, Zn, Cu	(Nehal et al., 2016)
7	*A. blitum*	Protein, vitamin A, vitamin C; Ca, Fe	(Yang & Keding, 2009)
		Protein, carbohydrate; Na, Ca, Fe	(Srivastava, 2011)
		Moisture, ash, crude fibre, protein, carbohydrate, lipid, calorific value	(Prajitha & Thoppil, 2018)
8	*A. dubius*	Moisture, protein, energy, fat, fibre, ash, carbohydrate; Ca, P, Na, Zn, Mg, Mn, Cu, Fe	(Odhav et al., 2007)
		Protein, vitamin A, vitamin C; Ca, Fe, Zn	(Yang & Keding, 2009)
		Ag, Al, Ba, Ca, Co, Cr, Cu, Fe, K, La, Li, Mg, Mn, Mo, Na, Ni, P, S, Sr, V, Zn, Zr	(Aguilar et al., 2011)
		Moisture, protein, fat, ash, crude fibre, carbohydrate; Ca, Zn, Fe	(Muriuki et al., 2014)
		Dry matter, crude protein, ash, crude fibre	(Molina et al., 2015)
9	*A. viridis*	Moisture, ash; N, P, Na, K, Ca, Mg, Fe, Cu, Zn, Mn	(Guerrero et al., 1998)
		Protein, vitamin A, vitamin C;	(Yang & Keding,

Sl. No.	Species	Components	References
		Ca, Fe	2009)
		Protein, carbohydrate; Na, K, Ca, Fe	(Srivastava, 2011)
10	*Amaranthus sp.*	Moisture content, dry matter content, total ash, crude protein, crude fibre, ascorbic acid, nitrate, total oxalate, soluble oxalate; Zn, Fe	(Onyango et al., 2008)
		Moisture, protein, fibre, fat, carbohydrate, energy, vitamin A, ascorbic acid, riboflavin, folate; Ca, Fe, Mg, Zn	(Uusiku et al., 2010)
		Protein, starch, lipid; Ca, Mg, K, P, Mn, Na, Se, Si, Sn, Zn, Rb	(Bolaños et al., 2016)
11	*A. caudatus*	Moisture, ash, protein, fibre, fat, starch, amylose; Li, V, Ni, Cu, Mn, Fe, Zn, Mg, Ca, P, K	(Nascimento et al., 2014)
12	*A. tricolor*	Dietary fibre, soluble dietary fibre	(Gupta et al., 2005)
		Moisture, protein, ash; Ca, P, K, Na, Mg, Fe, Zn, Cu, Cr	(Gupta et al., 2005)
		Protein, vitamin A, vitamin C; Ca, Fe, Zn	(Yang & Keding, 2009)
		Protein, carbohydrate; Na, K, Ca, Fe	(Srivastava, 2011)

MATERIALS AND METHODS

The present study is an attempt to characterize cytogenetical, micromorphological, molecular and phytochemical features of eight species of the genus *Amaranthus* collected from different locations in Kerala. Entire details pertaining to the materials and methods adopted during the study are mentioned in this section. The experiments were carried out at Cell and Molecular Biology Division, Department of Botany, University of Calicut; Central Sophisticated Instrumentation Facility (CSIF), University of Calicut; Rajiv Gandhi Centre for Biotechnology (RGCB), Thiruvananthapuram; Sophisticated Test and Instrumentation Centre (STIC), Cochin; Indian Institute of Technology (IIT), Bombay and the Cashew Export Promotion Council of India (CEPCI Laboratory and Research Institute), Kollam.

A. PLANT MATERIALS

Eight monoecious species of the genus *Amaranthus* (Amaranthaceae) reported to be present in Kerala was collected for conducting the present study, they are *Amaranthus blitum* L., *A. caudatus* L., *A. dubius* Mart. ex Thell., *A. hybridus* subsp. *hybridus* C. C. Towns., *A. hybridus* subsp. *cruentus* (L.) Thell., *A. spinosus* L., *A. tricolor* L. and *A. viridis* L. (Plate 1). These plants were collected from Malappuram, Kozhikode, Kannur, Palakkad, Ernakulam and Idukki districts of Kerala. The plants were authenticated by Dr A. K. Pradeep, Assistant Professor, Angiosperm Taxonomy Division, Department of Botany, University of Calicut and voucher specimens were deposited at the Herbarium (CALI) of Department of Botany, University of Calicut, Kerala, India. The collected plant specimens were cultivated under uniform conditions in the green house of Department of Botany, University of Calicut. The details of plant specimens were given below:

1. *Amaranthus blitum* L. (CALI 123758)

Collection locations: Karappara regions of Nelliyampathi, Palakkad district in Kerala

Synonyms: *A. oleraceus* L., *A. lividus* L., *A. blitum* var. *polygonoides* Moq., *A. mucronatus* Poir. *etc*.

Common name: Wild amaranth, purple amaranth, Guernsey pigweed *etc*. **(http[7]).**

Habit: Herb

Native region: Mediterranean region

Distribution: *A. blitum* is a wild and cultivated species used as a home garden leafy vegetable in Africa, South-Eastern Europe and in South India. The species is widespread in warmer parts of Europe, east to middle Asia, China, Japan and Africa.

A. blitum is a monoecious annual herb, small, usually erect, sometimes ascending. Stem simple or branched, glabrous; leaves green, simple, entire, arranged spirally without stipules, lamina angular ovate 4-8.5 x 2-5 cm, cuneate at base, notched at apex, pinnately veined; inflorescence an axillary many-flowered cluster, forming a false spike at apex of the plant with male and female flowers intermixed; bracts up to 1mm long; flowers unisexual, subsessile, with 3 tepals, male flowers commonest in the upper whorls with 2/3 stamens opposite tepals, female flowers with superior 1-celled ovary crowned by 3 stigmas, lobes 2 mm long, recurved; anthers oblong, bithecous, 1 mm long, yellowish. Fruit 1-seeded indehiscent subglobular to broadly ovoid-ellipsoid capsule, 2.3-3.5 mm long, gradually to abruptly narrowed towards the stigma region, glabrous, distinctly exceeding the perianth lobes;

pericarp coarsely wrinkled when dry; seeds orbicular 1.25 x 1.25 mm, glossy, brownish black (Anilkumar, 2014).

2. *Amaranthus caudatus* L. (CALI 123767)

Collection locations: Feroke Chungam (Kozhikode district)

Synonyms: *A. edulis* Speg., *A. mantegazzianus* Pass. *etc*.

Common name: Love-lies-bleeding, pendant amaranth, tassel flower, velvet flower, foxtail amaranth, quilete *etc*. (http[8]).

Vernacular name (Malayalam): Chaulai/Cheera

Habit: Annual herb

Native region: Andean regions of Ecuador, Peru and Bolivia

Distribution: Mostly distributed throughout the tropics, occasionally in temperate regions. It is a widely distributed staple food in South America and in India.

Monoecious erect annual herb; stem stout, sparingly branched upto 1.5 m, glabrous or glabrescent; leaves alternate, 3-15 x 2-7 cm, ovate-oblong or rhomboid-ovate, base cuneate to attenuate, apex obtuse to subacute at the mucronate tip; petiole 0.5-5 cm long, glabrous; inflorescence terminal and axillary spikes or paniculate up to 20 cm long, green; flowers unisexual, male and female flowers intermixed throughout the spikes; bract deltoid ovate, pale, membraneous; bracteoles 2, deltoid ovate, longer than tepals, pale-membraneous; perianth with 5 tepals, 2-3.5 mm long, oblong-elliptic, subequal, outwardly curved or erect, aristate. Stamens 5, filament 1 mm long, glabrous, yellow; stigma 3-lobed, erect or flexuose, rough, style indistinct; capsule ovoid-globose, 2 mm long, glabrous, circumscissile, longer than the tepals, slightly urceolate, the lid smooth or furrowed below, abruptly

narrowed to a short thick neck; ovary ovoid, 1 mm long, glabrous, ovule solitary; seeds 1-1.5 mm across, lenticular, compressed, black round, smooth shiny, between the hilum and radical a furrow extends almost to the middle of the seed (Anilkumar, 2014; http[9]).

3. *Amaranthus dubius* Mart. ex Thell. (CALI 123753)

Collection locations: Malappuram, Kozhikode, Kannur and Ernakulam districts

Synonyms: *A. tortuosus* Hornem., *A. tristis* Willd., *A. dubius* var. *flexuosus* Thell. *etc*.

Common name: Red spinach, Chinese spinach, spleen amaranth *etc*. (http[10]).

Vernacular name (Malayalam): Cheera

Habit: Annual herb

Native region: South America

Distribution: It is widespread throughout the humid lowland tropics. It originated from South America, where it is common in the Caribbean region and from Southern Mexico to Northern South America. The cultivated type may have been developed from the weedy ancestor in tropical Asia (Indonesia and India) and is found in several African and Central American countries, where immigrants have introduced it. In South India it is fairly common along roadsides and waste places along with *A. viridis* and/or *A. spinosus*.

Monoecious erect herb, up to 150 cm tall; stem slender to stout, branched, glabrous; leaves simple, arranged spirally without stipules, petiole up to 2-5 cm long, lamina ovate or rhomboid-ovate, 5-8 x 3-5 cm, cuneate at the base, blunt or retuse at the apex, mucronate, entire, glabrous or shortly pilose, sometimes the centre of the lamina blotched red. Inflorescence

spikelike or paniculate, axillary and terminal, the terminal one up to 25 cm long, consisting of glomerules more or less isolated at the base of inflorescence and agglomerated towards apex; bracts up to 2.5 mm long, awned, deltoid ovate in shape; flowers unisexual, sub-sessile with 5 tepals up to 1 mm long; male flowers usually near the apex of inflorescences, with 5 stamens, 2 mm long; female flowers with superior 1-celled ovary crowned by 3 stigmas. Fruit is a one seeded ovoid-urceolate capsule, 1.5 mm long with a short inflated beak below the stigmas, dehisce circularly, the lid strongly rugulose below the beak; seeds lenticular, compressed, 1 mm long, black shiny (http[11]).

4. *Amaranthus hybridus* subsp. *hybridus* C. C. Towns. (CALI 123756)

Collection locations: Calicut University (Malappuram district)

Synonyms: *A. chlorostachys* Willd., *A. paniculatus* L., *A. quitensis* Kunth *etc*.

Common name: Green amaranth, slim amaranth, smooth amaranth, smooth pigweed *etc*. (http[12]).

Habit: Annual herb

Native region: Eastern North America

Distribution: *A. hybridus* is a native riverbank pioneer of Eastern North America and parts of Mexico, Central America and Northern South America (Sauer, 1967). It is much more common in the eastern than the western half of USA. Its range has expanded to Africa, South-Central Asia and Australia, possibly because of its use as a green vegetable (http[13]).

Monoecious erect herb, stem branched up to 110 cm, angular, glabrous or thinly furnished with short or long hairs. Leaves rhomboid-lanceolate, 4-23 x 1-6.5 cm, thinly pilose on the lower margins and underside on the nerves,

margins entire, apex subacute, mucronate, base cuneate, petiole 1-9 cm long, glabrous. Inflorescence terminal and axillary spikes formed of cymose clusters. Flowers unisexual, male commonest in terminal whorls, green, red or pink in color; bracts deltoid or lanceolate, 6 x 1 mm, apex with reddish-tipped erect arista; bracteoles 2, 3 x 0.8 mm long, pale membraneous; tepals 5, subequal, 2 x 0.75 mm, glabrous; stamens 5, filaments 1 mm long, glabrous; anthers oblong, bithecous, 0.6 mm, yellow, glabrous; ovary ovoid, globose, 2 mm long, glabrous; ovules solitary, style short, indistinct, stigma 3, lobes slender, recurved, 1 mm long; capsule ovoid, globose, circumscissile. Seeds discoid, 1.25 x 1.25 mm, brownish black (Anilkumar, 2014).

5. *Amaranthus hybridus* subsp. *cruentus* (L.) Thell. (CALI 123764)

Collection locations: Aalinchuvadu, Vandoor, Vaniyambalam para (Malappuram district) and Aluva, Vypin (Ernakulam).

Synonyms: *A. cruentus* L., *A. nepalensis* Moq., *A. paniculatus* L. var. *cruentus* (L.) Moq., *A. sanguineus* L. *etc*.

Common name: Blood amaranth, red amaranth, Prince's feather, Mexican grain amaranth, caterpillar amaranth, purple amaranth *etc*. (http[14]).

Habit: Annual herb

Native region: Mexico

Distribution: Widespread in tropical and subtropical regions of the world, it is cultivated as ornamental and pseudocereal crop almost worldwide from tropical to warm-temperate regions.

Monoecious annuals, glabrous or slightly pubescent distally, especially when young. Stems erect, green or reddish purple, 0.4-2 m, branched distally, mostly in inflorescence. Leaves glabrous, blade rhombic-ovate or ovate to

broadly lanceolate, 6.5-10 x 2-4 cm, occasionally larger in robust plants, base cuneate to broadly cuneate, margins entire, plane, apex acute or subobtuse to slightly emarginated. Petiole 2-4 cm long; inflorescences in terminal spike and axillary clusters, erect, usually dark red, purple or deep beet-red, less commonly almost green or greenish red; flowers unisexual, male commonest in the upper whorls; bracts narrowly spathulate, 2-3 mm, equal or slightly longer than tepals, apex short-spinescent; bracteoles 2, ovate to lanceolate 2 x 0.75 mm; tepals 5, oblong to lanceolate, not clawed, equal or subequal, 1.25-1.75 x 0.5 mm, apex acute; stigmas 3; stamens 5, filament 0.25 mm long, glabrous; anthers oblong, bithecous, 1 mm long, yellowish. Utricles obovoid to elongate-obovoid, 2-2.5 mm, smooth or slightly rugose distally, dehisce circularly, circumscissile. Seeds are dark brown to dark reddish brown, broadly lenticular to elliptic-lenticular, 1.2-1.6 mm diameter, smooth or indistinctly punctuate (Anilkumar, 2014).

6. *Amaranthus spinosus* L. (CALI 123743)

Collection locations: Malappuram, Kozhikode, Kannur and Palakkad districts.

Synonyms: *A. spinosus* var. *basiscissus* Thell., *A. spinosus* var. *circumscissus* Thell., *A. spinosus* var. *pygmaeus* Hassk. *etc*.

Common name: Spiny amaranth, spiny pigweed, prickly amaranth, thorny amaranth *etc*. (http[15]).

Vernacular name (Malayalam): Mullencheera

Habit: Annual herb

Native region: Tropical America

Distribution: The native distribution range of *A. spinosus* is uncertain. It is probably a native of tropical America and was introduced into other warmer parts of the world. It extends into the temperate zone in Japan and the USA. It is a problematic weed principally around the Caribbean Sea, the west and south of Africa, around the Bay of Bengal and in east and South-East Asia from Japan to Indonesia (Holm et al., 1991).

Monoecious herb, glabrous or sparsely pubescent in the distal younger parts of stem and branches. Stem erect or sometimes ascending proximally, much-branched and bushy, each node with paired divergent spines (modified bracts) of 1.5-2 cm; petiole equaling or longer than blade; blade rhombic-ovate, ovate, or ovate-lanceolate, 3-10 x 1.5-6 cm, base broadly cuneate, margins entire, plane or slightly undulate, apex acute or subobtuse. Inflorescences simple or compound terminal staminate spikes and axillary subglobose mostly pistillate clusters, erect or with reflexed or nodding tips, usually green to silvery green upto 16 cm long. Lower clusters entirely female and upper male; flowers unisexual, green; bracts lanceolate to ovate-lanceolate, shorter than tepals, apex attenuate; tepals 5, obovate-lanceolate or spatulate-lanceolate, equal or subequal, 1.2-2 mm, apex mucronate; stamens 5, styles erect or spreading; stigmas 3; utricles ovoid to subglobose, 1.5-2.5 mm, membranaceous proximally, wrinkled and spongy or inflated distally, dehisce by a line around the centre. Seeds black, lenticular or subglobose, 0.7-1 mm diameter, smooth and shiny (http[16])

7. *Amaranthus tricolor* L. (CALI 123761)

Collection locations: Thalassery (Kannur district) and Malappuram district.

Synonyms: *A. gangeticus* L., *A. tristis* L., *A. melancholicus* L., *A. mangostanus* L. *etc*.

Common name: Edible amaranth (http[17]).

Vernacular name (Malayalam): Cheera

Habit: Annual herb

Native region: Tropical Asia

Distribution: *A. tricolor was* originated from tropical Asia. In South and South-East Asia it is one of the major leafy vegetable. Its domestication took place in prehistoric times and the wild ancestor is not known. *A. tricolor* occurs as a quite rare exotic vegetable in several African countries, apparently introduced by Indian immigrants and occasionally cultivated around the big cities, especially in East and Southern Africa. Its cultivation has been reported from Benin, Nigeria, Kenya and Tanzania, Southern Africa and throughout India (Aneja et al., 2011; http[18]).

Ascending or erect monoecious annual herb up to 75 cm tall, with stout stem, usually much branched; stem and branches angular, glabrous or in the upper part with crisped hairs. Leaves arranged spirally, simple, without stipules; petiole up to 8 cm long; lamina broadly ovate, rhomboid-ovate or broadly elliptical to lanceolate-oblong, varies in size, shortly cuneate to attenuate at base, emarginate to obtuse or acute at apex, glabrous or thinly pilose on lower surface of primary venation, green to reddish. Inflorescence an axillary, globose cluster up to 2.5 cm in diameter, the upper clusters sometimes forming a terminal spike, with male and female flowers intermixed; bracts broadly ovate, about as long as tepals, awned. Flowers unisexual, subsessile, with 3 tepals up to 5 x 1.2 mm, having a long awn; male flowers with 3 stamens, 0.5-0.75 mm long filaments, anthers oblong, bithecous, 0.75-1 mm long; female flowers with superior 1-celled ovary crowned by 3 stigmas, erect or recurved, 1-1.5 mm. Fruit an ovoid-urceolate capsule with a short beak below the stigmas, 2.5-3.5 mm, circumscissile,

membraneous, obscurely wrinkled. Seed 1–1.75 mm, shining black or brown, faintly reticulate (http[18]).

8. *Amaranthus viridis* L. (CALI 123771)

Collection locations: Calicut University, Moonniyur (Malappuram district), Kozhikode, Palakkad and Kannur district.

Synonyms: *A. gracilis* Desf. ex Poir., *Euxolus viridis* (L.) Moq., *A. acutilobus* Uline & W. L. Bray *etc*.

Common name: Slender amaranth, green amaranth, African spinach, rough pigweed, wild amaranth *etc*. (http[19])

Vernacular name (Malayalam): Kuppacheera

Habit: Herb

Native region: Southern United States and Mexico

Distribution: *A. viridis* is cosmopolitan in all warm regions of the world. It is one of the most common weeds in the tropics, subtropics and warm temperate regions.

Monoecious herb; stem erect or usually ascending, 6-80 (sometimes up to 100) cm tall, glabrous to pubescent, pubescent especially upwards. Leaves glabrous or pubescent on the veins of the lower surface; petioles long, 2-6 cm, occasionally longer than the blade; blade ovate to rhombic-oblong, 2-7 x 1.5-5.5 cm, base tapered to blunt, tip rounded, minutely mucronate, barely to clearly emarginate. Flowers green or pinkish tinged, unisexual, male and female intermixed in slender axillary to terminal paniculate spikes (2-12 cm long) or in dense axillary clusters in the lower part of the stem. Bracts deltoid to lanceolate-ovate, white membraneous 1 x 0.5 mm with a short awn from the green midrib; bracteoles 2, deltoid ovate-lanceolate ovate, 0.6 x 0.25 mm,

whitish, membraneous. Perianth-segments 3, oblong-oval, 1.2 x 0.5 mm, acute, concave, shortly mucronate; stamens 3, filament 1.2 mm long, hyaline, glabrous; anthers oblong, bithecous 0.25 mm long; stigmas 2-3 lobes, short, 0.25 mm long. Capsule nearly globose 1.25-1 mm, slightly exceeding the perianth, indehiscent or rupturing irregularly, very strong rugose throughout the surface. Seed 1-1.25 mm, round, slightly compressed, dark brown to black with a pale thick border (http[19]).

B. METHODOLOGY

For conducting cytogenetical, micromorphological, molecular and phytochemical studies of the eight *Amaranthus* species collected, standard protocols and techniques were adopted and followed, which are explained here upon.

1. MOLECULAR CHARACTERIZATION

Molecular characterization was performed for all the species of *Amaranthus* collected to resolve the phylogeny clearly and thereby to alleviate problems related to plant authentication. Species discriminations based solely on taxonomic and morphological descriptors are inefficient due to influences from the environment (Viljoen et al., 2015). So the species were first phylogenetically characterized using inter and intraspecies informative chloroplast barcoding genes *viz*., matK, rbcL and the nuclear gene *viz*., ITS region of rDNA.

ISOLATION AND PURIFICATION OF GENOMIC DNA

Total genomic DNA was extracted from silica dried specimens using the protocols of MACHEREY-NAGEL-09/2013, Rev.07, Nucleospin® Plant II kit. To isolate the genomic DNA, young leaves of the *Amaranthus* species

were used for better yield. The genomic DNA isolation involves the following steps:

Homogenization of the sample

Homogenize 20 mg of silica dried plant material to fine powder using a mortar and pestle. Then proceed the cell lysis using Buffer PL1 or alternatively Buffer PL2.

Cell lysis using Buffer PL1

i. Transfer the resulting powder to a new tube and add 400 µl Buffer PL1 (CTAB buffer). Vortex the mixture thoroughly.

ii. Add 10 µl RNAase A solution and mix the sample thoroughly.

iii. Incubate the suspension for 10 min at 65^0C.

iv. Vortex (Spinix) the mixture and centrifuge the crude lysate for 5 min at 11,000×g (Eppendorf 5804R) and transfer the supernatant to a new tube or pass the precleared supernatant through the Nucleospin® filter to remove solid particles completely.

Filtration/ Clarification of crude lysate

i. Place a Nucleospin® filter (violet ring) into a new collection tube (2 ml) and load the lysate onto the column.

ii. Centrifuge the column for 2 min at 11,000×g, then collect the clear flow-through and discard the Nucleospin® filter (If all liquid do not pass through the filter, repeat the centrifugation step).

Adjust DNA binding conditions

Add 450 µl Buffer PC (high salt concentration) and mix thoroughly by pipetting up and down (5 times) or by vortexing.

Binding the DNA

i. Place a Nucleospin® Plant II column (green filter having silica membrane) into a new collection tube (2 ml) and load a maximum of 700 µl of the sample.

ii. Centrifuge for 1 min at 11,000×g and discard the flow-through and retain the collecting tube.

iii. The green filter contains the silica membrane to which the DNA will bind at higher salt concentration (PC buffer).

Washing and drying of silica membrane

First wash

Add 400 µl Buffer PW1 to the Nucleospin® Plant II column, specifically on the filter. Centrifuge for 1 min at 11,000×g and discard flow-through and retain the collecting tube.

Second wash

Add 700 µl Buffer PW2 (diluted with methanol) to the Nucleospin® Plant II column. Centrifuge for 1 min at 11, 000×g and discard flow-through and retain the collecting tube.

Third wash

Add another 200 µl Buffer PW2 to the Nucleospin® Plant II column. Centrifuge for 2 min at 11, 000×g in order to remove wash buffer and dry the silica membrane completely.

Elution of DNA

Place the Nucleospin® Plant II column into a new 1.5 ml micro centrifuge tube. Add 60 µl Buffer PE (65^0C) onto the membrane. Incubate the

Nucleospin® Plant II column for 5 min at 65^{0}C. Centrifuge for 1 min at 11, 000×g to elute the genomic DNA. Discard the green filter and keep the flow through containing DNA. At low salt concentration of elution buffer (PE) the DNA will detach from the silica membrane.

DNA QUANTIFICATION AND QUALITY ANALYSIS

After isolation of DNA, quantification and analysis of quality are necessary to ascertain the approximate quantity of DNA obtained and the suitability of DNA sample for further analysis such as PCR amplification of target DNA. The methods used for the quantification and quality analysis were according to Hoisington et al. (1994) as mentioned below:

Agarose gel electrophoresis

The genomic DNA extracted was visualized on agarose gel (0.8%) for its quality and stored at -20^{0}C.

i. Prepare 0.8 % agarose gel (detailed procedure is given in the PCR amplification section).

ii. Add 1µl of 6X gel loading dye to 5 µl of each DNA sample before loading the wells of the gel. Addition of dye allows us to note the extent to which the samples might have migrated during electrophoresis, so that it can be halted at an appropriate stage.

iii. Load at least 1 or 2 wells with good quality λ DNA or any previously quantified DNA samples (50 ng and 100 ng) as molecular weight standards.

iv. Run the submarine electrophoretic gel at 75V till the dye has migrated one-third of the distance in the gel.

v. DNA can be visualised using UV transilluminator (Bio RAD, Gel Doc –1000) and quantified in comparison with the fluorescent yield of the standards.

Spectrophotometric determination

The isolated DNA was quantified using ELICO SL 218 double beam UV-VIS spectrophotometer. Analysis of UV absorption by the nucleotides provides a simple and accurate estimation of the concentration of nucleic acids in a sample. The ratio of OD_{260}/OD_{280} should be determined to assess the purity of the sample.

i. Take 1 ml TE (Tris-EDTA) buffer in a cuvette and calibrate the spectrophotometer at 260 nm as well as 280 nm.

ii. Add 10 µl of each DNA sample to 900 µl TE buffer and mix well.

iii. Use TE buffer as a blank in the other cuvette of the spectrophotometer.

iv. Note the OD_{260} and OD_{280} values on spectrophotometer.

v. Calculate the OD_{260}/OD_{280} ratio.

The ratio 1.8 - 2.0 denotes that the absorption in the UV range is due to nucleic acids. The ratio lower than 1.8, indicates the presence of proteins and/or other UV absorbers. The ratio higher than 2.0 indicates that the sample may be contaminated with chloroform or phenol. In either case (< 1.8 or > 2.0) it is advisable to re-precipitate the DNA. The amount of DNA can be quantified using the formula:

$$\textbf{DNA concentration } (\mu g/ml) = \frac{\textbf{A260} \times \textbf{dilution factor} \times \textbf{5 0}}{\textbf{1 000}}$$

Where, an OD of 1 corresponds to ~ 50 µg/ml of double stranded DNA, A_{260} is the OD of the sample at 260 nm and

$$\textbf{Dilution factor} = \frac{\textbf{Total volume of the sample}}{\textbf{Volume of DNA used}}$$

PCR AMPLIFICATION USING Phire II DNA POLYMERASE

The nuclear ribosomal DNA sequences of the internal transcribed spacers (ITS1, 5.8S rRNA gene, ITS2), and plastidial genes, rbcL (ribulose-1, 5-bisphosphate carboxylase/oxygenase large subunit) and matK (maturase K) were used in the study for the phylogenetic analysis. For PCR amplification Phire II DNA polymerase was used instead of Taq polymerase because Phire II has high processivity which reduces the time taken for PCR reaction. The reaction mixture consists of the following:

5× PCR buffer (with 25 mM $MgCl_2$)	2.0 µl
2 mM dNTPs	1.0 µl
Phire II DNA polymerase	0.1 µl
Primer [Forward] (10 µM)	0.2 µl
Primer [Reverse] (10 µM)	0.2 µl
Sample DNA (10 ng/µL)	1.0 µl
Distilled water	5.5 µl
Total volume	**10.0 µl**

Primers used

Different forward and reverse primers used for the amplification of the gene regions selected were mentioned below:

Primer sequence for rbcL gene

Primer [Forward] (rbcLa-F)	5′-ATGTCACCACAAACAGAGACTAAAGC- 3′	Levin et al., 2003
Primer [Reverse] (rbcL724R)	5′-TCGCATGTACCTGCAGTAGC- 3′	Fay et al., 1997

Primer sequence for matK gene

Primer [Forward] (matK-xF)	5′-TAATTTACGATCAATTCATTC- 3′	Ford et al., 2009
Primer [Reverse] (matK-MALPR)	5′-ACAAGAAAGTCGAAGTAT- 3′	Dunning and Savolainen (2010)

Primer sequence for ITS region of rDNA

Primer [Forward] (ITS-5F)	5′-GGAAGTAAAAGTCGTAACAAGG-3′	White et al., 1990
Primer [Reverse] (ITS-4R)	5′-TCCTCCGCTTATTGATATGC- 3′	White et al., 1990

Mix the PCR mix by gentle spin. To each PCR tubes add 9 µl PCR mix, 1 µl sample DNA (diluted as per the quality and quantity of DNA obtained). Gently mix the contents. PCR reaction was performed in GeneAmp PCR thermal cycler (PCR system 9700, Applied Biosystems)

Thermal profile of PCR for rbcL, matK and ITS region of rDNA genes were as follows:

PCR conditions for rbcL gene/ITS region of rDNA

Initial denaturation	-	98^0C	-	30 sec	
Denaturation	-	98^0C	-	5 sec	40 cycles
Annealing	-	55^0C	-	10 sec	
Extension	-	72^0C	-	15 sec	
Final elongation	-	72^0C	-	1min	
4^0C	-	∞			

PCR conditions for matK gene

Initial denaturation		98^0C	-	30 sec	
Denaturation	-	98^0C	-	5 sec	
Annealing	-	45^0C	-	10 sec	10 cycles
Extension	-	72^0C	-	15 sec	
Repeat the steps					
Denaturation	-	98^0C	-	5 sec	
Annealing	-	50^0C	-	10 sec	30 cycles
Extension	-	72^0C	-	15 sec	
Final elongation	-	72^0C	-	1 min	
4^0C	-	∞			

Agarose gel electrophoresis

Agarose gel electrophoresis is the method based on the ethidium bromide fluorescent staining to visualize the amplified DNA fragments. The amplified PCR products were subjected to electrophoresis. For this, 1% agarose solution was prepared and then heated in a microwave oven until the solution became clear. 7.5 µl ethidium bromide (EtBr) was added to the melted agarose, at this point to facilitate visualization of DNA after electrophoresis and swirled well. The melted agarose solution was poured carefully into the casting tray without forming any bubbles and a suitable comb was placed in the gel. The gel was allowed to solidify and the comb was pulled out carefully. The gel was transferred to the electrophoresis chamber containing 1X TBE (Tris-Borate-EDTA) electrophoresis buffer in such a way that the buffer just covered the surface of the gel. 1 µl of 6X gel loading dye

(Bromophenol blue) was added to 5 µl of the PCR amplified products before loading the wells of the gel. Thereafter the sample DNA was loaded into the well along with a commercially available DNA ladder (Genei PG100-500D1: 100bp) as size standard. Electrophoresis was performed at 75V power supply with 1X TBE buffer for 1-2 h, until the Bromophenol blue front had migrated to almost the bottom of the gel. After the electrophoresis, the gel was carefully transferred and placed on a UV transilluminator (Bio RAD, Gel Doc –1000) for visualising the resultant bands. The DNA samples showing perfect bands were selected for further analysis.

PCR CLEAN-UP

ExoSAP-IT Treatment

ExoSAP-IT reagent is designed for simple, quick PCR clean-up for downstream applications, such as DNA sequencing or Single Nucleotide Polymorphism (SNP) analysis. When PCR amplification is complete, any unconsumed dNTPs, primers and primer dimers remaining in the PCR product mixture will interfere with these methods. ExoSAP-IT removes these contaminants. ExoSAP-IT utilizes two hydrolytic enzymes, exonuclease I and Shrimp alkaline phosphatase, together in a specially formulated buffer, to remove unwanted dNTPs and primers from PCR products. Exonuclease I remove residual single-stranded primers and any extraneous single-stranded DNA produced in the PCR. Shrimp alkaline phosphatase removes the remaining dNTPs from the PCR mixture.

ExoSAP-IT	0.5 µl
PCR product (~ 100 ng)	5.0 µl

ExoSAP-IT is added directly to the PCR product and incubated at 37^0C for 15 min. After PCR treatment, ExoSAP-IT is inactivated simply by heating to 80^0C for 15 min.

SEQUENCING PCR

Sequencing PCR reaction

5x sequencing buffer	1.86 µl
Sequencing mix (Applied Biosystems)(ddNTPs)	0.28 µl
Primer (Forward/Reverse- 10 µM)	0.32 µl
Distilled water	6.54 µl
PCR product (~20 ng)	1.00 µl
Total volume	**10.00 µl**

For sequence PCR, forward and reverse reactions are carried out in separate tubes. The PCR product after ExoSAP-IT treatment was used for sequencing PCR reaction.

Sequencing PCR conditions

Initial denaturation	-	96^0C	-	2.00 min	
Denaturation	-	96^0C	-	9.00 sec	35 cycles
Annealing	-	50^0C	-	9.00 sec	
Extension	-	60^0C	-	3.00 min	
4^0C	-	∞			

POST SEQUENCING PCR CLEAN-UP

The composition of post sequencing PCR clean-up master mix is as follows:

Distilled water	9 µl
Sodium acetate (3M)	1 µl
EDTA (0.5 M)	0.1 µl
Ethanol (100%)	40 µl

i. Add 50 µl of master mix to each reaction.

ii. Contents are mixed by inverting the tube.

iii. Incubate at room temperature for 30 min.

iv. Spin at 14,000 rpm for 30 min.

v. Decant the supernatant and add 100 µl of 70% ethanol, vortex for 5 min.

vi. Spin at 14,000 rpm for 20 min.

vii. Decant the supernatant.

viii. Repeat wash with ethanol and air dry the pellet.

SEQUENCING

To the cleaned up air dried product add 10 µl HiDi (Highly deionized formamide) or equivalent to avoid complementary pairing thereby maintain DNA as single stranded. The HiDi added tubes were vortexed for 5 min and spun for few sec. and stored in refrigerator until sequencing was performed. The final product obtained was sequenced in a Genetic Analyzer 3500 (Applied Biosystems, Hitachi) using chain-termination method/Sanger method. The sequencing resulted in color-coded raw sequence data, which is analyzed using the software Sequence Scanner V 1.0. Raw data, quality showing data and sequence showing results were analysed to determine the quality of sequence data. Original sequences had been submitted to NCBI Genbank, with the aid of Sequin, a stand-alone software tool developed by the NCBI for submitting and updating entries to the GenBank sequence database. The details of GenBank submission were shown in the results section.

DETERMINATION OF SEQUENCE QUALITY

The generated sequences were edited manually using Geneious V 9. 0. 5 software (Biomatters Ltd., New Zealand), to correct the erroneously identified bases and were quality trimmed to remove indecipherable sequences at the 3’ and 5' ends. Standard practises and guidelines provided by Hyde et al. (2013) and Nilsson et al. (2012) were followed for establishing the basic authenticity and reliability of the newly generated sequences.

MULTIPLE SEQUENCE ALIGNMENT AND PHYLOGENETIC ANALYSIS

Sequence alignment, primer trimming and editing was done by using Geneious software. The ends of aligned sequences were trimmed to minimize the number of missing sites across taxa. The sequences were aligned pairwisely to compare base pair similarity between forward and reverse strands. The gene region having medium and low quality sequences were edited by comparing with the peak quality of the other strand and consensus identity. Positions containing gaps and missing data were completely eliminated. After editing, the consensus sequences were generated and were aligned together. Best-fitting nucleotide substitution models were tested using MEGA 6 software. The model with lowest Bayesian Information Criterion (BIC) score was selected. Based on this model selected, the pairwise distances between sequences were calculated in MEGA 6.

BLAST

The edited DNA sequences were then used separately for similarity searches using BLAST (Basic Local Alignment Search Tool), online tool in the NCBI GenBank DNA database. Sequences obtained after the BLAST search with an identity of ≥ 85% and zero e- value were considered for further phylogenetic analyses.

Based on the BLAST search results, available sequences of representatives of close out groups of *Amaranthus* were also included in the analyses, apart from the sequences of *Amaranthus* species selected. The out groups selected were the genus *Celosia*, *Pleuropetalum, Beta* and *Chenopodium* from the family Amaranthaceae. *Beta* and *Chenopodium* were in the family Chenopodiaceae, but the gene-based APG system I (1998) and II (2003) have included these plants in the family Amaranthaceae. The species, geographical location, accession number and length of the sequences retrieved from the GenBank (http[20]) as outgroups for the present phylogenetic analysis was mentioned in the Table 6.

Phylogenetic analysis was done by using Mega6 software. All the aligned sequences were converted into Mega format (.meg) and exported to MEGA 6 software. Various sequence details such as conserved sites, variable sites, parsimony-informative sites, singleton sites *etc*. were retrieved from MEGA 6. Phylogenetic trees were constructed by using maximum likelihood (ML) algorithm. Models with the lowest BIC source are considered to describe the substitution pattern the best. Substitution model obtained after BIC value analysis was used for constructing phylogenetic tree. Test of phylogeny is based on bootstrap method. 500 bootstrap replicates were performed under maximum likelihood criterion to estimate interior branch support. The genetic distance between species and overall mean distance was also calculated.

2. CYTOGENETICAL CHARACTERIZATION

Mitotic and meiotic squash experiments were conducted on the eight species of *Amaranthus* collected to characterize the chromosome complement and genetic variability. Cytogenetic studies were conducted by conventional staining (Aceto-orcein) method as well as fluorescence DAPI (4',6-diamidino-2-phenyl indole) staining.

MITOTIC CHROMOSOME STUDIES

Taxonomic characterization is very difficult in the species of *Amaranthus* due to the occurrence of many hybrids and broad geographical distribution, which is the reason for many synonyms. Cytogenetic analysis is usually performed to clarify systematic and evolutionary aspects, which offer added information to disputed taxonomic families. It helps for the karyological characterization of the species, increase the knowledge of genetic resources and explore evolutionary trends.

Aceto-orcein staining

Somatic chromosome studies have been conducted on eight species of the genus *Amaranthus* with the help of improved cytotechniques suggested by Sharma and Sharma (1990). Mitotic squash experiments were carried out on root tip meristem at mitotic metaphase stage. Mature seeds collected from the plants were allowed to germinate in petridishes lined with moist filter paper/cotton under laboratory conditions. The germinated roots were collected at the period showing peak mitotic frequency *i.e.* 09.00 AM to 10.00 AM. In seeds with a lesser germinating ability, young healthy twig of the plant was allowed to germinate in autoclaved sand. The germinated root tips thus collected was washed thoroughly in distilled water and subjected to pretreatment in cold saturated aqueous paradicholoro benzene (PDB).

The pretreatment solution was prepared after chilling of saturated aqueous PDB at 0-5ºC for 30 min. Root tips were immersed in the PDB and was kept under refrigeration at 12-15ºC for 1½ h. The pretreated root tips thus obtained were washed thoroughly in distilled water and fixed in modified Carnoy's fluid prepared with a composition of 1 acetic acid: 2 ethyl alcohol for 1 h. After thorough washing in distilled water for 2 or 3 times, fixed root tips were subjected to hydrolysis in 1N HCl for 1-10 min. Thorough washing

in distilled water was essential to remove the acid content from the root tips for proper staining. The staining was done with aceto-orcein for 3 h. Microslides were prepared after removing the excess stain by using 45% acetic acid. The slides prepared were screened for well spread mitotic metaphase chromosomes in Olympus CX21 Binocular Research Microscope (Olympus Corporation, Japan) under 100x oil immersion objective and photomicrographs were taken with the help of AmScope MU Series digital camera attached to the microscope.

DAPI staining

The root tips fixed for mitotic chromosome studies were used for the DAPI staining also. For the study, 0.1 mg of DAPI weighed and dissolved in 1 ml of phosphate buffered saline (PBS) and stored at 4ºC in the dark is saved as the stock (0.1 mg/ml). From the stock, working solution (0.01 mg/ml) was prepared by diluting the stock with PBS. For that 100 µl stock solution was pipetted and made up to 1 ml using PBS. The roots after fixation was washed thoroughly in distilled water and subjected to hydrolysis in 1N HCl for 1-10 min. The hydrolyzed root tips were washed again and equilibriated with PBS (pH – 7.4) for 15 min. Added 300 µl DAPI solution to the slide by covering the root tips. The slides were incubated for 15 min in the dark at RT. Washing of the slide was done to remove excess stain by using PBS and mounted in 50% glycerol. The slides prepared were screened under fluorescent microscope (Leica DM6 B, Leica Microsystems, Germany) and blue colour images were captured by using UV excitation filter. The captured images were further edited by using LAS X software provided by Leica Microsystems.

MEIOTIC CHROMOSOME STUDIES

Meiotic smear experiments were carried out by the analysis of pollen mother cells (PMC's). For this unopened flower buds were collected from the plant species and were fixed in modified Carnoy's fluid (1 acetic acid: 3 chloroform: 6 ethyl alcohol) for 1-3 days. During fixation the fixative was replaced frequently by fresh Carnoy's fluid to remove excess cell inclusions and pigments in order to clear the cytoplasm so as to enhance the visibility of chromosomes. After fixation the flower buds were washed thoroughly in distilled water and stored in 70% ethyl alcohol until further analysis. For meiotic smear preparation young anthers were dissected from the flower bud by using stainless steel needles under a stereo microscope (Labomed® CSM2, USA). The anthers dissected were transferred into a glass slide and smeared with a drop of aceto-orcein stain to obtain PMC's showing divisional stages. Keep the slide for 5-10 min and warmed gently in order to enhance stainability. Microslides were prepared and screened for different meiotic stages in Olympus CX21 Binocular Research Microscope (Olympus Corporation, Japan) under 100x oil immersion objective and photomicrographs were taken with the help of AmScope MU Series digital camera attached to the microscope.

KARYOMORPHOMETRIC STUDIES

The photomicrographs obtained with good quality and uniform focus was selected for further analysis such as karyogram and idiogram preparation using computer based programs such as AutoCAD, Adobe photoshop, Microsoft Paint and data based analyzing system such as Microsoft Excel. For the identification, each chromosome in the photograph was falsely numbered and loaded to AutoCAD program for karyomorphometric studies. Centromeric position of each chromosome was identified, the short arm and long arm length were measured and centromeric indices were calculated. On

the basis of chromosome length, arm ratio and centromeric indices, homologous chromosome pairs were identified and were classified according to Abraham and Prasad (1983) (Table 5). The paired chromosomes were subjected to karyogram and idiogram preparation with the help of Adobe Photoshop program.

Table 5: Chromosome nomenclature in relation to the position of centromere based on arm ratios and centromeric indices

Nomenclature	**Notation**	**R_1 s/l**	**R_2 l/s**	**I_1 100 s/c**	**I_2 100 l/c**
Median	M	1.00	1.00	50.00	50.00
Nearly median	nm	0.99 - 0.61	1.01 - 1.63	49.99 - 38.01	50.01 - 61.99
Nearly submedian	nsm(-)	0.60 - 0.34	1.64 - 2.99	38.00 - 25.01	62.00 - 74.99
Submedian	SM	0.33	3.00	25.00	75.00
Nearly submedian	nsm(+)	0.32 - 0.23	3.01 - 4.26	24.99 - 18.20	75.01 - 81.80
Nearly subterminal	nst(-)	0.22 - 0.15	4.27 - 6.99	18.19 - 12.51	81.81 - 87.49
Subterminal	ST	0.14	7.00	12.50	87.50
Nearly subterminal	nst(+)	0.13 - 0.07	7.01 - 14.38	12.49 - 5.01	87.51 - 94.99
Nearly terminal	nt	0.06 - 0.01	14.39 - 19.99	5.00 - 0.01	95.00 - 99.99
Terminal	T	0.00	∞	0.00	100.00

R_1 = arm ratio 1**, R_2 =** arm ratio 2**, I_1 =** centromeric index 1**, I_2 =** centromeric index 2**, s** = short arm length**, l** = long arm length**, c** = total length of chromosome

Karyotypic formula was determined based on the number of chromosome, position of centromere and presence or absence of secondary constriction. The karyograms and idiograms were drawn and arranged based on chromosome size in decreasing order. Various karyomorphometric and asymmetry indices calculated are mentioned below:

Disparity index (DI) of the chromosomes was calculated by the formula proposed by Mohanty et al. (1991)

$$DI = \frac{\text{Longest chromosome} - \text{Shortest chromosome}}{\text{Longest chromosome} + \text{Shortest chromosome}} \times 100$$

The variation coefficient (VC) among the chromosome complements was determined by Verma (1980) as follows,

$$VC = \frac{\text{Standard deviation}}{\text{Mean length of chromosomes}} \times 100$$

The total forma percentage (TF%) or mean centromeric index value was calculated (Huziwara, 1962) by the formula,

$$TF\% = \frac{\text{Total sum of short arm length}}{\text{Total sum of chromosome length}} \times 100$$

The karyotype asymmetry index (As K%) was calculated using the following formula developed by Arano (1963).

$$As\ K\% = \frac{\text{Total sum of long arm length}}{\text{Total sum of chromosome length}} \times 100$$

Greilhuber and Speta (1976) developed two indices, the index of karyotype symmetry and the index of chromosomal size resemblance, to evaluate karyotype asymmetry. These two indices were later called by Venora et al. (2002) as the Syi index and the Rec index, respectively (Paszko, 2006). The formulae are as follows:

$$Syi = \frac{\text{Mean length of short arms}}{\text{Mean length of long arms}} \times 100$$

$$Rec = \frac{\sum_{i=1}^{n} \frac{CLi}{LC}}{n} \times 100$$

here CLi is the length of each chromosome, LC is the longest chromosome and n is the number of chromosomes analyzed.

Zarco (1986) proposed two numerical parameters to estimate karyotype asymmetry, which are intrachromosomal asymmetry index (A_1) and interchromosomal asymmetry index (A_2). Karyotype asymmetry for the relations between the chromosome arms can be estimated using A_1 whereas karyotype asymmetry due to relations between sizes of different chromosomes can be estimated using A_2. A_1 and A_2 were calculated by the following equations:

$$A1 = 1 - \left(\frac{\sum_{i=1}^{n} \frac{bi}{Bi}}{n} \right)$$

where bi is the average length for short arms in every homologous chromosome pair or group, Bi is the average length for long arms in every homologous chromosome pair or group and n is the number of homologous chromosome pairs or groups.

$$A_2 = \frac{\text{Standard deviation of chromosome length}}{\text{Mean of chromosome length}}$$

The degree of asymmetry of karyotypes (A) was developed by Watanabe et al. (1999) using the formula,

$$A = \frac{\sum_{i=1}^{n} \frac{pi - qi}{pi + qi}}{n}$$

where p and q are the lengths of the long arm and short arm of the chromosome respectively and n is the haploid chromosome number of the species.

Asymmetry index (AI) was calculated using the formula proposed by Paszko (2006),

$$AI = \frac{CV_{CL} \times CV_{CI}}{100}$$

where CV_{CL} is the coefficient of variation of chromosome length and CV_{CI} is the coefficient of variation of centromeric index.

Statistical analysis

The numerical data represented were calculated based on well spread metaphase plates in triplicate and were analyzed statistically by using SPSS version 20 (SPSS Inc., Chicago, IL, USA). The karyomorphometric data obtained were subjected to one-way ANOVA followed by DMRT to confirm the variability of the data and validity of results. Each value represented the mean ± standard error (SE) of three independent experiments.

3. MICROMORPHOLOGICAL CHARACTERIZATION

Micromorphological studies are important in *Amaranthus* species, since it demonstrate taxonomic ambiguity at the basic morphological level. The micromorphology of pollen grain, seed coat and seed capsule was done for all the species of *Amaranthus* collected to reveal the variation among them, using scanning electron microscopy (SEM) with the following specifications: Make/model: JEOL Model JSM - 6390LV; Resolution: 3 nm (Acc V 30 KV, WD 8 mm, SEI), 8 nm (Acc V 3.0 KV, WD 6 mm, SEI), 15 nm (Acc V 1.0 KV, WD 6 mm, SEI); Magnification: 5 × to 300, 000 × (Both in High and Low Vacuum Modes); Image Modes: SEI and BEI; Probe Current: 1 pA to 1mA; High vacuum resolution: 3 nm; Low vacuum resolution: 4 nm; Specimen Stage: 4 nm, Eucentric, +90º; Specimen holder: 10 mm dia.; Maximum loadable specimen size: 150 mm dia. Scanning Electron Microscopy is a method for high resolution surface imaging using an electron beam having greater magnification and much larger depth of field. Different elements and surface topographies emit different quantity of

electrons, due to which the contrast in a SEM micrograph is representative of the surface topography and distribution of elemental composition on the surface.

MICROMORPHOLOGICAL STUDIES OF POLLEN GRAINS

Comparative studies on the pollen grain morphology of eight *Amaranthus* species were conducted by using SEM analysis in order to assess the taxonomic value of pollen traits. The pollen morphological studies were initially done by the conventional light microscopic method such as acetolysis and then by SEM. Both qualitative and quantitative parameters were analyzed and observed for similarities and variations among the species studied.

Light Microscopy - Acetolysis

Acetolysis is a technique for recovering pollen by dissolving organic debris, proteins, lipids and carbohydrates from the surface of the pollen grains (Erdtman, 1960). This makes the pollen grains easier to stain, so that they can be photographed and identified. For the study, anthers from fresh flower buds at pre-anthesis stage were collected, crushed with a glass rod and the mixture is filtered through cheesecloth. The filtrate thus obtained was subjected to centrifugation (1000 rpm, 3 min), discarded the supernatant and the sediment was retained. To the sediment, 2-3 ml of 70% ethyl alcohol was added, centrifuged (1000 rpm, 10 min) and collected the sediment. Since the acetolysis mixture reacts violently with water, a wash with glacial acetic acid is necessary to replace water with acetic acid, before adding acetolysis solution. For that 2-3 ml of glacial acetic acid was added to the sediment collected, kept for 10 min and mixed thoroughly and centrifuged (1000 rpm, 3 min) and collected the sediment again. Freshly prepared acetolysis mixture consisting of glacial acetic acid and concentrated H_2SO_4 in the ratio of 9:1 was poured in the centrifuge tube containing washed pollen. Conc. H_2SO_4

was carefully added to acetic acid drop by drop to avoid excess heating. The centrifuge tube containing pollen in suspension in the acetolysis mixture is placed in a water bath at boiling point (100°C) for 2-3 minutes and the contents were stirred with a glass rod intermittently. The pollen after completion of acetolysis process turns light to golden brown in colour. The solution was allowed to cool for few minutes. Thereafter the acetolyzed pollen suspension was centrifuged and discarded the supernatant. The pollen sediment in the tube was immersed in glacial acetic acid for few minutes and centrifuged at 2000 rpm for 5 min, and then the supernatant was discarded. The finally obtained sediment was washed with distilled water for 2-3 times by centrifugation. The washed pollen material was then used for mounting on the glass slides for microscopic examination. It can also be stored in this condition in vials containing 50% glycerine. For staining put a drop of glycerine, one drop of stain (Safranin O) and sample of pollen on the microscopic slide, mixed well and kept for few min and covered with cover slip (http[21]). The slides prepared were screened under Olympus CX21 Binocular Research Microscope (Olympus Corporation, Japan) at 40x and 100x magnification, photographed with AmScope MU Series digital camera attached to the microscope and observed for various palynological characteristics.

Scanning Electron Microscopy (SEM)

Pollen morphological studies were carried out with the help of SEM to determine similarities and variations in pollen micromorphology between the selected species of *Amaranthus*. For the analysis, mature flower buds were collected and fixed in 70% ethyl alcohol. The fixed samples were then transferred in to 100 % ethyl alcohol to enable complete dehydration. Pollen grains were collected from the flower bud by dissecting the anthers, air dried and mounted on aluminum stubs with double-sided sticky tape and sputter-

coated with gold in IB-2 ion coater. The coated materials were examined with different magnifications in a SEM and photographed by a Jeol JSM-840 at an accelerating voltage of 30 KV. For each species, three specimens were examined. Palynological characteristics, such as equatorial diameter (E), polar axis length (P), P/E, pollen shape, exine ornamentation and pore dimensions were calculated for each sample. The shape of pollen grain was defined on the basis of P/E ratios according to Erdtman (1943).

MICROMORPHOLOGICAL STUDIES OF SEED CAPSULE

A comparative analysis of seed capsule micromorphology was done for all species of *Amaranthus* collected by SEM analysis, and observed for variations in size, seed capsule ornamentation, nature of dehiscence *etc*. from 5 seed capsules for each species. For the analysis, mature seed capsules were dissected from the flower and stored in 100 % ethyl alcohol. After drying, the samples were mounted on aluminum stubs with double-sided sticky tape and sputter-coated with gold in IB-2 ion coater. The coated materials were examined with different magnifications in a SEM and photographed by a Jeol JSM-840 at an accelerating voltage of 30 KV.

MICROMORPHOLOGICAL STUDIES OF SEED COAT

Seed coat micromorphologies of eight *Amaranthus* species was done by SEM analysis, and observed for variations in exomorphic parameters including shape, length, width, colour, mass and surface texture from 5 seeds for each species. For the analysis, dry seeds were collected from the mature inflorescence of the *Amaranthus* species. The collected seeds were scanned under a stereomicroscope (Labomed® CSM2, USA) and mature good quality seeds were selected for further studies. Mature dry seeds (without fixation) were mounted on aluminum stubs with double-sided sticky tape and sputter-coated with gold in IB-2 ion coater. The coated materials were examined with

different magnifications in a SEM and photographed by a Jeol JSM-840 at an accelerating voltage of 30 KV.

Statistical Analysis

All the parameters analyzed in the micromorphological studies were subjected to statistical analysis using SPSS version 20 (SPSS Inc., Chicago, IL, USA). Data obtained were subjected to one-way ANOVA followed by DMRT to confirm the variability of the data and validity of results. All results were expressed as mean ± SE.

4. PHYTOCHEMICAL CHARACTERIZATION

Qualitative and quantitative phytochemical analysis was done by using standard protocols. Quantitative estimation of phenol, flavonoid, terpenoid, alkaloid, tannin, carotenoid and saponin was done. GC/MS and HR-LC/MS analysis was carried out for the identification of specific phytocomponents present in the extract.

PREPARATION OF METHANOLIC EXTRACT

Eight species of *Amaranthus* collected from wild were cultivated under uniform conditions in the net house. After sufficient growth, the aerial parts were collected and subjected to further phytochemical analysis. The collected plant twigs were washed thoroughly to remove debris and shade dried. The dried samples were powdered and subjected to methanol extraction using Soxhlet apparatus. 10 g of the plant material was extracted for 6 h in 100 ml of 100% methanol. The extract so obtained was cooled, filtered and concentrated to dryness in a vacuum evaporator. GC-MS and HR-LCMS analyses were performed using this methanol extract. For analysis, the extract was dissolved in 10 ml HPLC Grade methanol (Merck) and filtered through 0.45 micron membrane filter to ensure minimum contamination.

QUALITATIVE PHYTOCHEMICAL SCREENING

All the collected species were subjected to preliminary phytochemical analysis as per the standard methods. Chemical tests were carried out on the aqueous, methanol, chloroform extract and on the powdered sample using standard procedures to identify the presence of active chemical constituents in the plant extract by the following procedures:

1. Test for carbohydrate

Molisch's test:

Few drops of Molisch's reagent were added to 2 ml of the various extracts. This was followed by addition of 2 ml of conc. H_2SO_4 down the side of the test tube. The mixture was then allowed to stand for two to three minutes. Formation of a red or dull violet colour at the interphase of the two layers indicates a positive test.

Benedict's test:

Test solution was mixed with few drops of Benedict's reagent (alkaline solution containing cupric citrate complex) boiled in water bath and observed for the formation of reddish brown precipitate, which reveal the presence of carbohydrate.

2. Test for proteins

Millon's test:

Crude extract was mixed with 2 ml of Millon's reagent. A white precipitate which turned red upon heating, confirms the presence of proteins.

3. Test for phenols (Kumar et al., 2007)

Ferric chloride test:

A fraction of the extracts was treated with 5% aqueous ferric chloride and observed. Formation of deep blue or black colour indicates a positive result for phenols.

Spot test:

Extract was spotted on to the filter paper, few drops of phosphomolybdic acid was added to the spot and was exposed to ammonia vapour. Blue colouration of the spot indicates the presence of phenol.

4. Test for flavonoids (Trease & Evans, 2002)

Alkaline reagent test:

2 ml of the extract was treated with few drops of 20% sodium hydroxide solution. Formation of intense yellow colour, which becomes colourless on addition of dilute hydrochloric acid, indicates the presence of flavonoids.

Lead acetate solution test:

Test solution was treated with few drops of lead acetate (10%) solution. Formation of yellow precipitate confirms the presence of flavonoids.

5. Test for alkaloids (Siddiqui & Ali, 1997)

Mayer's test:

A fraction of methanolic extract was mixed with 2 ml of 1% HCl and heated gently. Mayers reagent (Evans & Evans, 2002) was then added to the mixture. Turbidity of the resulting precipitate was taken as evidence for the presence of alkaloids.

Wagner's test:

A fraction of methanolic extract was treated with 3-5 drops of Wagner's reagent (1.27 g of iodine and 2 g of potassium iodide in 100 ml of water) and observed for the formation of reddish brown precipitate (or colouration).

6. Test for terpenoids (Edeoga et al., 2005)

Salkowski's test:

2 ml of chloroform was added to 5 ml of the extract and mixed well. 3 ml of conc. H_2SO_4 was carefully added to the solution to form a layer. The presence of terpenoids can be confirmed by the formation of reddish brown colouration at the interface of the solutions.

10 mg of the extract was dissolved in 1 ml of chloroform; 1 ml of acetic anhydride was added following the addition of 2 ml of conc. H_2SO_4. Formation of reddish violet colour indicates the presence of terpenoids.

7. Test for saponins (Sofowara, 1993)

Foam test:

1 ml of aqueous extract was taken in a test tube and diluted with 20 ml of distilled water. It was shaken by hand for 15 min. A foam layer was obtained on the top of the test tube, which is stable for 15 min. This foam layer indicated the presence of saponins.

8. Test for steroids (Sofowara, 1993)

Liebermann-Burchard test:

1 ml of methanol extract was treated with one drop of chloroform, acetic anhydride and conc. H_2SO_4. Formation of dark pink or red colour confirms the presence of steroids.

9. Test for carotenoids (Ajayi et al., 2011)

1 g of sample was extracted with 10 ml of chloroform in a test tube with vigorous shaking. The resulting mixture was filtered and 85% sulphuric acid was added. A blue colour at the interface showed the presence of carotenoids.

10. Test for tannins (Evans & Evans, 2002)

Braymer's test:

2 ml of extract was treated with 10% alcoholic ferric chloride solution and observed for formation of blue or greenish colour solution, which indicated the presence of tannins in the sample.

11. Test for phlobatannins (Edeoga et al., 2005)

Precipitate test:

Deposition of a red precipitate when 2 ml of extract was boiled with 1ml of 1% aqueous hydrochloric acid was taken as evidence for the presence of phlobatannins.

12. Test for cardiac glycosides (Siddiqui & Ali, 1997)

Keller Killiani's test:

5 ml of each extract was treated with 2 ml of glacial acetic acid in a test tube and a drop of ferric chloride solution was added to it. This was carefully underlayed with 1 ml of concentrated sulphuric acid. A brown ring at the interface indicated the presence of deoxysugar characteristic of cardiac glycosides. A violet ring may appear below the ring while in the acetic acid layer, a greenish ring may form gradually throughout the thin layer.

The extract was hydrolysed with HCl for few hours on a water bath. To the hydrolysate, 1ml of pyridine and few drops of sodium nitroprusside solutions were added and then it was made alkaline with sodium hydroxide solution. Appearance of pink to red colour shows the presence of cardiac glycosides.

13. Test for quinones and anthraquinones (Kumar et al., 2007)

A small amount of extract was treated with concentrated HCl and observed for the formation of yellow precipitate (or colouration), which indicates the presence of quinones.

Borntrager's test:

50 mg of the extract was stirred with 1 ml of 10% ferric chloride solution and 1 ml of conc. HCl and heated. The extract was cooled and filtered. The filtrate obtained was shaken well with equal amount of diethyl ether. The ether extract thus obtained was further extorted with strong ammonia. Development of pink or deep red colouration in the aqueous layer indicates the presence of anthraquinones.

QUANTITATIVE ESTIMATION OF MAJOR PHYTOCOMPONENTS

Quantification of major classes of compounds such as phenol, flavonoid, terpenoid, alkaloid, tannin, carotenoid and saponin was done as per the standard methods described below:

Estimation of total phenolic content

The total phenolic content of the plant extract was determined using Folin-Ciocalteu reagent based technique described by Oueslati et al. (2012). Gallic acid was used as the standard for estimation. An aliquot of sample/standard solution of gallic acid was mixed with 0.5 ml of distilled water and 0.125 ml of Folin-Ciocalteu reagent (1 N) and shaken well. After 6 min of incubation, 1.25 ml of 7% Na_2CO_3 was added and made to a final volume of 3 ml with distilled water and mixed well. The reaction mixture thus obtained was incubated in dark at ambient temperature for 90 min. The absorbance of the standard and sample were measured against blank (distilled water) at 760 nm in a spectrophotometer (Elico SL 218, India). Calibration curve of gallic acid was plotted and the phenolic content was expressed in mg of gallic acid equivalents per gram of dry weight (mg GAE/g DW) by using regression equation. Samples were analysed in triplicates.

Estimation of total flavonoid content

The total flavonoid content of the plant extracts was determined using the aluminium chloride colorimetric method proposed by Dewanto et al. (2002) with slight modifications. Quercetin was used as the standard for estimation. An aliquot of diluted sample/standard solution of quercetin was mixed with 75 µl of $NaNO_2$ solution (7%) for 6 min. The solution thus obtained was added with 0.15 ml of $AlCl_3$ (10%). After 5 min, 0.5 ml of 1 M NaOH solution was also added and the final volume was adjusted to 2.5 ml. The absorbance of the mixture, after thorough mixing, was determined at 510

nm against blank *i.e.*, methanol. Calibration curve of quercetin was plotted and the total flavonoid content expressed as mg quercetin equivalent per gram of dry weight (mg QE/g DW) was determined using regression equation. Samples were analysed in triplicates.

Estimation of total terpenoid content

The total terpenoid content of the plant extracts of *Amaranthus* species was determined based on an assay described by Ghorai et al. (2012) with some modifications. Linalool was used as the standard for estimation. An aliquot of the reaction mixture obtained after Salkowski test employed for the qualitative analysis of terpenoids in the extract was transferred to colorimetric cuvette. The absorbance was measured at 538 nm against blank *i.e.*, 95% (v/v) methanol. For the standard curve, 200 µl of linalool solution in methanol was added with 1.5 ml chloroform and serial dilutions [dilution level-100 mg/200 µl to 1 mg/200 µl linalool Conc.] were prepared in which total volume of 200 µl was made up by the addition of 95% (v/v) methanol. Calibration curve of linalool was plotted and the total terpenoid content expressed as milligrams of linalool equivalent per grams of dry weight (mg linalool/g DW) was determined using the regression equation. Samples were analysed in triplicates.

Estimation of total alkaloid content

The total alkaloid content present in the extract of different *Amaranthus* species was determined based on an assay described by Harborne (1973). 5 g of the plant sample was weighed into a 250 ml beaker and 200 ml of 10% acetic acid in ethanol was added, covered and allowed to stand for 4 h. This was filtered and the extract was concentrated on a water bath to one-quarter of the original volume. Concentrated ammonium hydroxide was added drop wise to the extract until the precipitation was complete. The whole

solution was allowed to settle and the precipitate was collected and washed with dilute ammonium hydroxide and then filtered. The residue is the alkaloid, which was dried and weighed. Samples were analysed in triplicates.

Estimation of total tannin content

Tannin content in different *Amaranthus* species was quantified according to Bainbridge et al. (1996). For this, 1 ml of the methanolic extract was mixed with 5 ml of vanillin reagent and the mixture was allowed to incubate at ambient temperature for 30 min. After that, the absorbance was read at 500 nm by using a UV-Visible spectrophotometer (Elico SL 218, India). Tannin content of samples was estimated using the calibration curve of tannic acid as standard and the total tannin content expressed as milligrams of tannic acid equivalent per grams of dry weight (mg tannic acid/g DW) was determined using the regression equation. Samples were analysed in triplicates.

Estimation of total carotenoid content

Total carotenoid content was determined according to Song and Xu (2013) with slight modifications. 0.2 g of raw amaranth (Vegetative stage) was extracted with 5 ml of acetone in a water bath at 50°C for 15 min. The mixture was centrifuged at 4200 rpm for 3 min. The supernatant was collected in 25 ml volumetric flask. The residue was extracted two more times with acetone under the same procedures. All the supernatants were collected in the 25 ml volumetric flask and brought to scale with acetone. 1 ml of extract was diluted with 9 ml acetone. Absorbance of extracted fraction was then read at 475 nm by using a spectrophotometer (Elico SL 218, India) against the acetone blank. The total carotenoid content was subsequently estimated using a calibration curve of β-carotene as standard. Calibration curve of β-carotene was plotted and the total carotenoid content expressed as milligrams of

carotenoid equivalent per gram of dry weight (mg *β*-carotene/g DW) was determined using regression equation. Samples were analyzed in triplicates.

Estimation of total saponin content

The total saponin content present in different *Amaranthus* species was quantified according to the method described by Obadoni and Ochuka (2002). 20 g of dried powdered sample was put into a conical flask and 100 ml of 20% aqueous ethanol was added. The samples were heated over a hot water bath for 4 h with continuous stirring at about 55°C. The mixture was filtered and the residue was re-extracted with another 200 ml of 20% ethanol. The combined extracts were reduced to 40 ml over water bath at about 90°C. The concentrate was transferred into a 250 ml separating funnel and 20 ml of diethyl ether was added and shaken vigorously. The aqueous layer was recovered while the ether layer was discarded. The purification process was repeated. 60 ml of n-butanol was added to the aqueous layer. The combined n-butanol extracts were washed twice with 10 ml of 5% aqueous sodium chloride. The remaining solution was heated in a water bath. After evaporation the samples were dried in the oven to a constant weight and the saponin content was calculated as percentage. Samples were analysed in triplicates.

PHYTOCHEMICAL ANALYSIS FOR THE IDENTIFICATION OF VOLATILE AND NON-VOLATILE COMPONENTS

Analytical techniques were employed for the detection of specific bioactive components present in the extract of different *Amaranthus* species. For the identification of bioactive volatile components gas chromatography coupled with mass spectrometry techniques (GC/MS) was employed. High resolution liquid chromatography coupled with mass spectrometry techniques was done for the characterization of non-volatile components present. HR-

LC/MS is considered as one of the most powerful tools because of the fast separation, high sensitivity and excellent resolution (Bertrand et al., 2013), that reveals the metabolites that are not volatile and have not been derivatized. Hence both techniques are done to fulfil a complete phytochemical profile of the *Amaranthus* species studied.

Gas chromatography-mass spectrometry (GC/MS) analysis

The identification and quantification of the volatile fraction present in the different species was done by GC/MS analysis of plant methanol extract. Varian model CP-3800 GC interfaced with Varian Saturn 2200 Ion Trap Spectrometer (ITMS) operating at 70eV and 250^{0}C equipped with a CP-1177 Split/Splitless capillary injector and Combi PAL autosampler was employed for GC/MS analyses. A cross linked factor for capillary column, VF 5ms with 30 m × 0.25 mm ID and 0.25 µm film thickness was utilized. Carrier gas used was Helium at a flow rate of 1 ml/min. Injection volume was 1 µl. The split ratio was 1:20. The temperature programme for the chromatographic analysis was set at 60^{0}C for 1 min (initial) and then heated up at a rate of 3^{0}C/min to 280^{0}C. Run time was 60 min. Quantification was carried out using percentage peak area calculations and identification of individual components was done using the NIST-MS search. The relative concentration of each compound in the methanolic extract was quantified based on the peak area integrated by the analysis programme.

High-resolution liquid chromatography mass spectrometry (HR-LC/MS)

The identification of the non-volatile fraction present in the extract of different *Amaranthus* species was done by HR-LC/MS analysis of plant methanol extract. Experiments were performed using an Agilent 1290 Infinity UHPLC System, 1260 infinity Nano HPLC with Chipcube, 6550 iFunnel Q-TOFs (Palo Alto, CA, USA). The chromatographic separation was achieved

on a reverse-phase Agilent Zorbax-C18 110 Å analytical column (250 × 4.6 mm ID., 5 μm) operated at 40^0C (Thermo Electron, Auchtermuchty, UK). Elution was achieved with a gradient mobile phase consisting of methanol (A) and 0.5 μM ammonium formate (B) (9:91) in water (C) at a flow rate of 1 ml/min. The gradient system used was as follows: Mobile phase C was held constant throughout the run and a linear gradient of B was maintained for 30 min. Mode of injection was ALS (Automatic liquid sampling). Electrospray ionization (ESI) was used as the interface and was operated in positive selected ion monitoring (SIM) mode. The analytes were monitored by tandem-mass spectrometry with positive electrospray ionization. The injection volume was 20 μl. The probe temperature was set at 500^0C and needle voltage was set at 20 V. The cone voltage was set at 50 V for all selected ion monitoring (SIM) scans.

PROXIMATE COMPOSITION ANALYSIS

Eight species of *Amaranthus* collected from wild were cultivated under uniform conditions in the net house. After sufficient growth, the aerial parts were collected and subjected to proximate composition analysis using standard protocols. The nutritional constituents determined were moisture, ash, dietary fibre carbohydrate, crude protein and crude fat content. In addition, the calorific value was also determined. The proximate analysis of the samples for moisture, ash and dietary fibre contents were determined as described by Association of Official Analytical Chemists (AOAC, 1995). Quantitative estimation of protein was done by a standard method described by Lowry et al. (1951). The crude lipid content in different plant species was estimated by the method proposed by Bligh and Dyer (1959). Determination of total carbohydrate content was done by Anthrone method proposed by Hedge and Hofreiter (1962).

Determination of moisture content

The total moisture content present in all the species were determined by oven dry method (AOAC, 1995). This method is used to determine the percentage of water in a sample by drying the sample to a constant weight. The water content is expressed as the percentage, by weight, of the dry sample. 1 g of fresh leaf sample was weighed and recorded as 'fresh weight of sample'. The fresh samples were dried to a constant weight in a hot air oven at 105 ºC. Allowed the sample to cool and weighed it again, recorded as the 'dry weight of sample'. The percentage moisture content of the sample was calculated using the following equation. Samples were analysed in triplicate.

$$\textbf{Moisture content}\ \% = \frac{(\textbf{Fresh weight of the sample taken} - \textbf{Dry weight of the sample taken})}{\textbf{Fresh weight of the sample taken}} \times \mathbf{1\ 00}$$

Determination of ash content

Total ash content was determined by dry mineralization method (AOAC, 1995). For this study plant samples were initially shade dried and powered. 1 g of this dried powdered sample was weighed in a pre-conditioned crucible. The samples were first charred by flame to eliminate smoking before being incinerated in the muffle furnace (HF Electronic Furnace) at 550ºC for 5 h until the ash turned white. The residues were then cooled in desiccators and the weights were taken to determine the concentration of the ash present. Samples were analysed in triplicate.

$$\textbf{Crude ash}\ \% = \frac{\textbf{Weight of residue}}{\textbf{Weight of sample}} \times \mathbf{1\ 00}$$

Determination of dietary fibre content

The occurrence of nutritionally significant dietary fibre in different *Amaranthus* species was estimated by the method of AOAC (1995).

Sample preparation: Approximately 1 g of sample was weighed and transferred into a 250 ml conical flask. 100 ml of boiled sulphuric acid (1.25%) was then added and boiling continued for 30 min under reflux. Filtration was done under slight vacuum with Pyrex glass filter (crucible type) and the residue was washed to completely remove the acid with boiling water. 100 ml of boiling sodium hydroxide (1.25%) was then added to the washed residue and boiling was continued under reflux for another 30 min. Filtration was done using the same glass filter previously used and the residue was rinsed with boiling water followed by hydrochloric acid (1%) and again washed with boiling water to rinse the acid from the residue. The residue was then washed twice with alcohol and thrice with ether.

Drying and incineration: The residue was dried with glass filter at 100ºC, cooled to room temperature and weighed to get constant weight. This was incinerated at 450-550ºC for about 1 h in the muffle furnace (HF Electronic Furnace) and transferred to a desiccator, cooled to room temperature then weighed to get constant weight. For all the samples the determination was made in triplicate.

$$\textbf{Fibre } \% = \frac{(\textbf{Weight before incineration} - \textbf{ weight after incineration})}{\textbf{Weight of sample}} \times \mathbf{1\ 00}$$

Determination of total carbohydrate content

The quantification of carbohydrate present in different *Amaranthus* species was done by Anthrone method proposed by Hedge and Hofreiter (1962). Carbohydrate is first hydrolysed into simple sugars using dilute HCl. In hot acidic medium, glucose is dehydrated to hydroxymethyl furfural. This compound together with anthrone forms a green coloured product with an absorption maximum at 630 nm. About 100 mg of the sample was weighed and added in a boiling tube and hydrolysed by keeping it in a boiling water bath for 3 hrs with 5 ml of 2.5 N HCl and cooled to room temperature. The

mixture obtained was neutralized with solid sodium carbonate until the effervescence ceases. The volume was made up to 100 ml and centrifuged. The supernatant was collected and pipetted out 0.5 and 1 ml sample aliquots for further analysis. The standard glucose stock solution was prepared by dissolving 100 mg glucose in 100 ml distilled water. 10 ml of this stock solution was diluted to 100 ml using distilled water in order to prepare the working standard. From the working solution a series of standard concentrations *viz*., 0.02, 0.04, 0.06, 0.08 and 0.1 mg/ml were prepared. Pipette out 0.2, 0.4, 0.6, 0.8 and 1 ml of the working standard into a series of test tubes. Made up the volume to 1 ml in all the test tubes including the sample tubes by adding distilled water. About 4 ml of anthrone reagent (200 mg anthrone in 100 ml of ice cold 95% H_2SO_4) was added to all the test tubes. A tube with 1 ml distilled water and 4 ml anthrone reagent serves as blank. Heated the tubes in a boiling water bath for 8 min. Cooled rapidly and read the absorbance at 630 nm. Drawn a standard graph by plotting concentration of the standard on X-axis versus absorbance on the Y-axis. From the graph the amount of carbohydrate present in the sample tube was calculated.

Amount of carbohydrate present in 100 mg of the sample was calculated by the following formula:

$$\textbf{Amount of carbohydrate in 100 mg sample} = \frac{\textbf{Concentration of glucose}}{\textbf{volume of test sample}} \times \textbf{100}$$

Determination of total protein content

Quantitative estimation of protein content in different *Amaranthus* species was done by the method described by Lowry et al. (1951). For extracting protein from the sample 10 mg of plant powder was ground with 1 ml of phosphate buffer. The sample was centrifuged and collected the supernatant for protein estimation. Bovine Serum Albumin (BSA) was used as the working standard (500 µg/ml) for the estimation. Pipette out 2, 4, 6, 8

and 10 ml of working standard into a series of test tubes. Pipette out 0.1 ml of sample into other test tube. Make up the volume to 10 ml in all test tubes using distilled water. A tube with 10 ml distilled water alone serves as a blank. About 5 ml of alkaline copper sulphate reagent was added to all test tubes and incubated at room temperature for 10 minutes. Thereafter 0.5 ml of Folin Ciocalteu reagent was added to each tube and mixed well. The tubes were incubated in dark for 30 minutes. The absorbance at 660 nm was recorded against blank using a spectrophotometer. The absorbance was plotted against protein concentration to get a standard calibration curve. The absorbance of plant sample was noted and determined its unknown concentration using regression equation. Samples were analysed in triplicate.

Determination of crude lipid content

The determination of crude lipid content in different *Amaranthus* species was done by Bligh and Dyer's method with slight modifications (Bligh & Dyer, 1959). For the study, 1 g fresh weight of *Amaranthus* leaves was macerated using a mortar and pestle on ice. To the macerated tissue 1 ml of chloroform: methanol (1:2 v/v) was added and homogenized well. The homogenate obtained was transferred to a glass tube with a stopper. Added another 2 ml of chloroform: methanol (1:2 v/v) to the mortar for wash and transferred the wash into the same glass tube. The homogenate was subjected to centrifugation at 3000 rpm for 5 min. using a laboratory bench-top centrifuge (REMI, R-8C). The supernatant obtained after centrifugation was transferred into another glass tube with a stopper. Added 3 ml of chloroform: methanol (1:2 v/v) and 0.8 ml of 1% KCl to the pellet left after centrifugation and vortexed well. The mixture thus obtained was centrifuged at 3000 rpm for 5 minutes. Transferred the supernatant obtained to the same tube collected before. 2 ml of chloroform and 1.2 ml of 1% KCl was added to the collected supernatant and vortexed well. The obtained mixture was centrifuged at 3000

rpm for 5 minutes. The lower layer (lipid extract) was transferred to another pre-weighed glass tube with a Pasteur pipette and was dried under vacuum in a desiccator. The tube with lipid was weighed and calculated the weight of total lipids per gram of sample taken.

Determination of calorific value

Energy was calculated (kcal/100 g fresh weight) using the Atwater system as described by the World Health Organization (WHO, 1985). The calorific value for each plant sample was estimated by multiplying the percentage crude protein, crude lipid and carbohydrate by the recommended factors (2.44, 8.37 and 3.57 respectively) used in vegetable analysis (Asibbey-Berko & Tavie, 1999; FAO, 2002).

Statistical analysis

All the parameters analyzed in the proximate composition studies were subjected to statistical analysis using SPSS version 20 (SPSS Inc., Chicago, IL, USA). Data obtained were subjected to one-way ANOVA followed by DMRT to confirm the variability of the data and validity of results. All results were expressed as mean ± SE.

ELEMENTAL ANALYSIS (ICP-AES)

The mineral elements comprising Na, K, Ca, Mg, Fe, Zn, P, Mn were determined according to the method of Shahidi et al. (1999) and Nahapetian and Bassiri (1975) with slight modifications. 2 g of each plant powder sample was weighed and subjected to dry ashing in a well-cleaned crucible at 550ºC in a muffle furnace (HF Electronic Furnace). From the ash obtained, 0.5 g was weighed and dissolved in 5 ml HNO_3 and heated gently on a hot plate until brown fumes disappeared. To the remaining material in each crucible, 5 ml of de-ionized water was added and heated until a colourless solution was

obtained. The mineral solution in each crucible was transferred into a 100 ml volumetric flask by filtration through Whatman No. 42 filter paper and the volume was made to the mark with de-ionized water. This solution was used for elemental analysis by Inductively Coupled Plasma Atomic Emission Spectrometer (ICP-AES), make/model: Thermo Electron IRIS INTREPID II XSP DUO, flexible axial and radial view instrument with high concentration capabilities. The wavelength at which emission occurs identifies the element, while the intensity of the emitted radiation quantifies its concentration. Spectral range: 165 to >1000 nm, Resolution: 0.005 nm at 200 nm, Detection limit: ppb level, CID Detector, Atomization-Excitation Source: Inductively Coupled Plasma (ICP) device. Concentration of each element in the sample was calculated on percentage. Along with Ca/P and Na/K ratios were also calculated. Concentrations of mineral elements in foods are often at the trace level and so it is important to use very pure reagents when preparing samples for analysis. Similarly, one should ensure that glassware's are very clean and dry, so that it contains no contaminating elements. It is also important to ensure that there are no interfering substances in the sample whose presence would lead to erroneous results. An interfering substance could be something that absorbs at the same wavelength as the mineral being analyzed, or something that binds to the mineral and prevents it from being efficiently atomized (http[22]).

RESULTS

The present study analyzed the cytogenetical, micromorphological, molecular as well as phytochemical characterization with special emphasis on the nutritional potential of eight species of the genus *Amaranthus* L. (Plate 1), an economically important plant species, which led to the disclosure of the following cytogenetical, micromorphological, molecular and phytochemical features. The present study was designed in such a way that the taxonomic identity of the collected experimental plant materials has been confirmed by molecular analysis at first. After the proper identification of the plant materials, further studies have been focussed on the cytogenetical, micromorphological and phytochemical aspects.

1. MOLECULAR CHARACTERIZATION

Molecular characterization of the eight species of *Amaranthus* collected from the wild has been conducted for the precise conformation of their species identity. Isolation of genomic DNA from the young leaf tissues of all the species yielded good quality DNA of high molecular weight. PCR amplification of the genomic DNA obtained using two coding gene regions of chloroplast DNA *viz*., rbcL and matK and a non-coding spacer region *viz*., ITS region of rDNA gene were successful with distinct bands for all the species studied. The PCR product of the samples was successfully sequenced with a base pair length of 697, 893 and 701 for rbcL, matK and ITS region of rDNA genes respectively (Plates 2, 3 and 4). The resulted sequences showed high recoverability, sequence quality with few or no ambiguous base pairs. Ambiguous base pairs and deletions are found more in ITS region of rDNA sequences. BLAST searches were done with all the rbcL, matK and ITS sequences obtained from the collected taxa to find the close matches that were used to confirm the molecular identity of the species of interest. The

sequences obtained for the three gene regions of the test plant materials were almost similar in the order of their nucleotide sequences with BLAST search results.

The well resolved gene sequences were deposited in GenBank of NCBI and those details are as follows: The rbcL gene sequences of *A. blitum* (GenBank Accession No. KX090208); *A. caudatus* (GenBank Accession No. KX090209); *A. dubius* (GenBank Accession No. KX090210); *A. hybridus* subsp. *hybridus* (GenBank Accession No. KX090211); *A. hybridus* subsp. *cruentus* (GenBank Accession No. KX090212); *A. spinosus* (GenBank Accession No. KX090213); *A. tricolor* (GenBank Accession No. KX090214) and *A. viridis* (GenBank Accession No. KX090215) (Appendix 1).

The matK gene sequences of *A. blitum* (GenBank Accession No. KX090200); *A. caudatus* (GenBank Accession No. KX090201); *A. dubius* (GenBank Accession No. KX090202); *A. hybridus* subsp. *hybridus* (GenBank Accession No. KX090203); *A. hybridus* subsp. *cruentus* (GenBank Accession No. KX090204); *A. spinosus* (GenBank Accession No. KX090205); *A. tricolor* (GenBank Accession No. KX090206) and *A. viridis* (GenBank Accession No. KX090207) (Appendix 2).

The ITS region of rDNA gene sequences of *A. blitum* (GenBank Accession No. KX090192); *A. caudatus* (GenBank Accession No. KX090193); *A. dubius* (GenBank Accession No. KX090194); *A. hybridus* subsp. *hybridus* (GenBank Accession No. KX090195); *A. hybridus* subsp. *cruentus* (GenBank Accession No. KX090196); *A. spinosus* (GenBank Accession No. KX090197); *A. tricolor* (GenBank Accession No. KX090198) and *A. viridis* (GenBank Accession No. KX090199) (Appendix 3).

The percentage of nucleotide composition in the rbcL, matK and ITS region of rDNA sequences of the species studied was also noted and

documented in the Table 6. Highest G+C content was observed in ITS region of rDNA gene sequences of the studied taxa and was ranges from 58.2% (AB) to 59.7% (AS). Apart from ITS, more G+C content was found in rbcL sequences that ranges from 42.1% (AS) to 42.4% (AT), followed by matK sequences [33.0% (AT) to 33.7% (AV)].

The rbcL, matK and ITS region of rDNA sequence data analysis of the eight *Amaranthus* species using MEGA6 software revealed the information of the conserved sites (C), variable sites (V), parsimony informative sites (Pi) and singleton sites (S) as rbcL (C - 691, V – 6, Pi – 4, S – 2), matK (C - 878, V – 15, Pi – 8, S – 7) and ITS region of rDNA (C - 667, V – 35, Pi – 14, S – 21). From the result it is clear that more variable sites occur in the ITS region of rDNA gene sequences of the species studied. The gene regions of rbcL and matK were found to be highly conserved with few variable regions. Among the 3 gene regions studied the Pi sites were more in ITS region of rDNA gene sequences. The rbcL, matK and ITS sequences obtained from the taxa studied and their relative species were compared with the help of GENEIOUS software. Multiple sequence alignment of the three gene regions amplified from eight *Amaranthus* species showed similarity and variations by colour bands and consensus identity is confirmed. The maximum/complete consensus identity was represented by the high level of green colour in the scale. Multiple sequence alignment of the gene sequences obtained was represented in Plates 2-4.

Barcoding analysis was done by using the sequences generated from rbcL, matK and ITS gene regions of the samples together with related sequences and out-groups downloaded from GenBank. The out-groups selected were genera close to *Amaranthus* (*Celosia*, *Pleuropetalum, Beta* and *Chenopodium*). The details of the sequences retrieved from GenBank for the current phylogenetic analysis are represented in Table 7. The level of divergence between and within species was calculated by pairwise analysis of the sequences. Pairwise distance matrices were shown in Tables 8-10. Tree-

based method was used to assess species specific clusters. Maximum Likelihood (ML) method yielded well resolved phylograms with comparable topologies. Best fit model nucleotide substitution was found out after Bayesian Inference (BI) analysis of the sequences and it was based on the lowest BIC value scored, which were 1328.966 (rbcL), 3990.911 (matK) and 2262.450 (ITS) (Table 11-13). The Best fit model nucleotide substitution obtained after Bayesian Inference analysis for each gene region was Kimura 2-Parameter (K2) for rbcL, Tamura 3-Parameter (T92+G) for matK and Kimura 2-Parameter (K2+G) for ITS region of rDNA and were used for the phylogenetic tree construction having a node support of 500 bootstrap replicates.

Maximum Likelihood dendrogram of rbcL, matK and ITS gene region of selected taxa of *Amaranthus* and other related species based on K2, T92+G and K2+G model of substitution revealed 2 major clades represented by different colours (Plates 5-7). The dendrogram of rbcL and ITS gene region displayed the occurrence of the selected taxa of *Amaranthus*, other related species and the out-group *Chenopodium* in the same clade indicating their close matches. The mis match of *Beta*, *Celosia* and *Pleuropetalum* was clear by their position in separate clade. But in matK tree the *Beta* and *Chenopodium* (previously in Chenopodiaceae) was positioned in a distinct clade with a strong bootstrap support of 99% and all other species of the genus *Amaranthus* and their related genera *Pleuropetalum* and *Celosia* (Amaranthaceae) was placed in the same clade. The phylograms generated from the grain amaranth species in the present study *viz*., *A. caudatus* and *A. hybridus* subsp. *cruentu*s showed close position with the phylograms of *A. hypochondriacus and A. cruentus* obtained from NCBI GenBank. This shows the affinities of all these grain amaranths as revealed by their placement in a single subclade.

The present phylogenetic study successfully resolved species identity of the collected *Amaranthus* species, since all the collected species and their

close allies forms a monophyletic group/clade with a strong bootstrap support of >90%.

Table 6. Percentage of nucleotide composition in the rbcL, matK and ITS region of rDNA sequences of the species studied

Plant species	Sequence length (bp)	% of base pairs					Molecular weight of dsDNA (kDa)
		A	T	G	C	G+C	
rbcL gene							
AB	697	28.0	29.7	23.1	19.2	42.3	430.624
AC	”	28.2	29.6	22.8	19.4	42.2	430.623
AD	”	28.3	29.5	22.8	19.4	42.2	430.623
AH	”	28.1	29.7	22.8	19.4	42.2	430.623
AR	”	28.3	29.5	22.8	19.4	42.2	430.623
AS	”	28.3	29.6	22.7	19.4	42.1	430.623
AT	”	27.8	29.8	22.8	19.6	42.4	430.624
AV	”	28.0	29.8	22.8	19.4	42.2	430.623
matK gene							
AB	893	29.5	37.3	16.1	17.1	33.3	551.638
AC	”	29.6	37.2	16.2	17.0	33.3	551.638
AD	”	29.6	37.2	16.2	17.0	33.3	551.638
AH	”	29.5	37.3	16.0	17.2	33.3	551.638
AR	”	29.6	37.2	16.2	17.0	33.3	551.638
AS	”	29.5	37.2	16.2	17.1	33.4	551.639
AT	”	29.5	37.5	15.9	17.1	33.0	551.636
AV	”	29.0	37.3	16.1	17.6	33.7	551.642
ITS region of rDNA gene							
AB	701	20.5	21.3	30.8	27.4	58.2	433.204
AC	”	20.8	20.4	30.5	28.3	58.8	433.827
AD	”	20.8	20.4	30.5	28.3	58.8	433.827
AH	”	20.8	20.4	30.5	28.3	58.8	433.827
AR	”	20.8	20.4	30.5	28.3	58.8	433.827
AS	”	20.4	19.9	31.1	28.6	59.7	433.832
AT	”	20.4	20.8	31.1	27.7	58.8	433.208
AV	”	20.5	20.6	31.1	27.8	58.9	435.063

AB: *A. blitum*; AC: *A. caudatus*, AD: *A. dubius*, AH: *A. hybridus* subsp. *hybridus*, AR: *A. hybridus* subsp. *cruentus*, AS: *A. spinosus*, AT: *A. tricolor*, AV: *A. viridis*

Table 7. Details of the sequences retrieved from NCBI GenBank for the present phylogenetic analysis

Species	Accession Number	Length (bp)	Geographical Location
rbcL sequences			
Amaranthus asplundii	FR775301	1326	USA
Amaranthus australis	KJ773260	1323	USA
Amaranthus blitoides	HM849762	888	USA
Amaranthus blitum	HM849763	1363	USA
Amaranthus deflexus	HM849764	913	USA
Amaranthus greggii	AY270055	1343	Germany
Amaranthus hybridus	HM849765	772	USA
Amaranthus retroflexus	KM360629	1408	UK
Amaranthus spinosus	HM849766	745	USA
Amaranthus viridis	KJ773261	1265	USA
Beta vulgaris	KM360669	1408	UK
Celosia argentea	AY270072	1343	Germany
Chenopodium ficifolium	KM360714	1408	UK
Pleuropetalum sprucei	AY270117	1343	Germany
matK sequences			
Amaranthus albus	JF953137	713	China
Amaranthus australis	KJ772534	651	USA
Amaranthus blitoides	HM850678	813	USA
Amaranthus blitum	HM850679	813	USA
Amaranthus deflexus	HM850680	813	USA
Amaranthus hybridus	HM850681	813	USA
Amaranthus palmeri	KM403117	691	USA
Amaranthus paniculatus	AF204866	1128	Germany
Amaranthus retroflexus	HQ593163	782	USA
Amaranthus spinosus	HM850682	813	USA
Amaranthus tricolor	JF953164	713	China
Beta vulgaris subsp. *maritima*	HM850762	813	USA
Celosia cristata	GQ434271	808	China
Chenopodium ficifolium	KF986168	837	China
Pleuropetalum sprucei	AF542596	1018	Germany
ITS region of rDNA sequences			
Amaranthus arenicola	KP318853	632	Korea
Amaranthus blitoides	KF385442	681	China

Amaranthus blitum	KF493811	686	China
Amaranthus capensis	KF493810	681	China
Amaranthus caudatus	AF210907	630	China
Amaranthus cruentus	AF210912	630	China
Amaranthus graecizans	KF493809	656	China
Amaranthus hypochondriacus	KF385440	681	China
Amaranthus lividus	AF210916	629	China
Amaranthus palmeri	KF493822	688	China
Amaranthus paniculatus	AF210911	630	China
Amaranthus polygonoides	KF493813	675	China
Amaranthus powellii	KF493812	681	China
Amaranthus quitensis	KM438059	622	USA
Amaranthus retroflexus	KF493829	729	China
Amaranthus roxburghianus	AF210914	629	China
Amaranthus spinosus	KF493823	721	China
Amaranthus standleyanus	KF493831	680	China
Amaranthus taishanensis	AF210920	632	China
Amaranthus tenuifolius	KF385441	685	China
Amaranthus tricolor	KF385439	680	China
Amaranthus tuberculatus	KF493840	689	China
Beta vulgaris subsp. *orientalis*	DQ223065	241	Germany
Celosia cristata	KP318862	610	Korea
Chenopodium album	KC577848	678	India
Pleuropetalum darwinii	AY255506	632	USA

Table 8. Pairwise distance analyses matrice of rbcL gene sequence of selected taxa of *Amaranthus*

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
1	AB208																						
2	AC209	0.004																					
3	AD210	0.004	0.000																				
4	AH211	0.004	0.000	0.000																			
5	AR212	0.004	0.000	0.000	0.000																		
6	AS213	0.004	0.000	0.000	0.000	0.000																	
7	AT214	0.004	0.009	0.009	0.009	0.009	0.009																
8	AV215	0.000	0.004	0.004	0.004	0.004	0.004	0.004															
9	*Amaranthus asplundii*	0.004	0.000	0.000	0.000	0.000	0.000	0.009	0.004														
10	*Amaranthus australis*	0.000	0.004	0.004	0.004	0.004	0.004	0.004	0.000	0.004													
11	*Amaranthus blitoides*	0.000	0.004	0.004	0.004	0.004	0.004	0.004	0.000	0.004	0.000												
12	*Amaranthus blitum*	0.004	0.000	0.000	0.000	0.000	0.000	0.009	0.004	0.000	0.004	0.004											
13	*Amaranthus deflexus*	0.009	0.004	0.004	0.004	0.004	0.004	0.014	0.009	0.004	0.009	0.009	0.004										
14	*Amaranthus greggii*	0.000	0.004	0.004	0.004	0.004	0.004	0.004	0.000	0.004	0.000	0.000	0.004	0.009									
15	*Amaranthus hybridus*	0.004	0.000	0.000	0.000	0.000	0.000	0.009	0.004	0.000	0.004	0.004	0.000	0.004	0.004								
16	*Amaranthus retroflexus*	0.004	0.000	0.000	0.000	0.000	0.000	0.009	0.004	0.000	0.004	0.004	0.000	0.004	0.004	0.000							
17	*Amaranthus spinosus*	0.004	0.000	0.000	0.000	0.000	0.000	0.009	0.004	0.000	0.004	0.004	0.000	0.004	0.004	0.000	0.000						
18	*Amaranthus viridis*	0.000	0.004	0.004	0.004	0.004	0.004	0.004	0.000	0.004	0.000	0.000	0.004	0.009	0.000	0.004	0.004	0.004					
19	*Beta vulgaris*	0.056	0.061	0.061	0.061	0.061	0.061	0.056	0.056	0.061	0.056	0.056	0.061	0.066	0.056	0.061	0.061	0.061	0.056				
20	*Celosia argentea*	0.061	0.066	0.066	0.066	0.066	0.066	0.061	0.061	0.066	0.061	0.061	0.066	0.071	0.061	0.066	0.066	0.066	0.061	0.056			
21	*Chenopodium ficifolium*	0.042	0.037	0.037	0.037	0.037	0.037	0.046	0.042	0.037	0.042	0.042	0.037	0.042	0.042	0.037	0.037	0.037	0.042	0.061	0.056		
22	*Pleuropetalum sprucei*	0.051	0.056	0.056	0.056	0.056	0.056	0.051	0.051	0.056	0.051	0.051	0.056	0.061	0.051	0.056	0.056	0.056	0.051	0.046	0.037	0.056	

Table 9. Pairwise distance analyses matrice of matK gene sequence of selected taxa of *Amaranthus*

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
1	AB200																							
2	AC201	0.010																						
3	AD202	0.010	0.000																					
4	AH203	0.005	0.005	0.005																				
5	AR204	0.010	0.000	0.000	0.005																			
6	AS205	0.010	0.000	0.000	0.005	0.000																		
7	AT206	0.008	0.018	0.018	0.013	0.018	0.018																	
8	AV207	0.003	0.010	0.010	0.005	0.010	0.010	0.008																
9	*Amaranthus albus*	0.005	0.012	0.012	0.007	0.012	0.012	0.010	0.005															
10	*Amaranthus australis*	0.005	0.012	0.012	0.007	0.012	0.012	0.010	0.005	0.003														
11	*Amaranthus blitoides*	0.002	0.010	0.010	0.005	0.010	0.010	0.010	0.003	0.007	0.007													
12	*Amaranthus blitum*	0.005	0.005	0.005	0.000	0.005	0.005	0.013	0.005	0.007	0.007	0.005												
13	*Amaranthus deflexus*	0.000	0.010	0.010	0.005	0.010	0.010	0.008	0.003	0.005	0.005	0.002	0.005											
14	*Amaranthus hybridus*	0.007	0.007	0.007	0.002	0.007	0.007	0.015	0.007	0.008	0.008	0.007	0.002	0.007										
15	*Amaranthus palmeri*	0.003	0.010	0.010	0.005	0.010	0.010	0.008	0.003	0.002	0.002	0.005	0.005	0.003	0.007									
16	*Amaranthus paniculatus*	0.005	0.005	0.005	0.000	0.005	0.005	0.013	0.005	0.007	0.007	0.005	0.000	0.005	0.002	0.005								
17	*Amaranthus retroflexus*	0.010	0.007	0.007	0.005	0.007	0.007	0.015	0.007	0.008	0.008	0.010	0.005	0.010	0.007	0.007	0.005							
18	*Amaranthus spinosus*	0.010	0.000	0.000	0.005	0.000	0.000	0.018	0.010	0.012	0.012	0.010	0.005	0.010	0.007	0.010	0.005	0.007						
19	*Amaranthus tricolor*	0.005	0.005	0.005	0.000	0.005	0.005	0.013	0.005	0.007	0.007	0.005	0.000	0.005	0.002	0.005	0.000	0.005	0.005					
20	*Beta vulgaris* subsp. *maritima*	0.109	0.117	0.117	0.111	0.117	0.117	0.120	0.113	0.109	0.109	0.111	0.111	0.109	0.113	0.109	0.111	0.117	0.117	0.111				
21	*Celosia cristata*	0.116	0.118	0.118	0.111	0.118	0.118	0.125	0.116	0.116	0.113	0.116	0.111	0.116	0.113	0.116	0.111	0.115	0.118	0.111	0.133			
22	*Chenopodium ficifolium*	0.132	0.134	0.134	0.132	0.134	0.134	0.139	0.132	0.128	0.127	0.134	0.132	0.132	0.134	0.127	0.132	0.129	0.134	0.132	0.089	0.158		
23	*Pleuropetalum sprucei*	0.116	0.125	0.125	0.118	0.125	0.125	0.121	0.118	0.116	0.119	0.118	0.118	0.116	0.116	0.117	0.118	0.123	0.125	0.118	0.107	0.132	0.130	

Table 10. Pairwise distance analyses matrice of ITS region of rDNA gene sequence of selected taxa of *Amaranthus*

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34
1	AB192																																		
2	AC193	0.066																																	
3	AD194	0.066	0.000																																
4	AH195	0.066	0.000	0.000																															
5	AR196	0.066	0.000	0.000	0.000																														
6	AS197	0.073	0.031	0.031	0.031	0.031																													
7	AT198	0.018	0.045	0.045	0.045	0.045	0.051																												
8	AV199	0.025	0.038	0.038	0.038	0.038	0.044	0.006																											
9	*Amaranthus arenicola*	0.044	0.024	0.024	0.024	0.024	0.031	0.031	0.025																										
10	*Amaranthus blitoides*	0.074	0.059	0.059	0.059	0.059	0.044	0.052	0.045	0.045																									
11	*Amaranthus blitum*	0.000	0.066	0.066	0.066	0.066	0.073	0.018	0.025	0.044	0.074																								
12	*Amaranthus capensis*	0.074	0.059	0.059	0.059	0.059	0.044	0.052	0.045	0.045	0.000	0.074																							
13	*Amaranthus caudatus*	0.066	0.000	0.000	0.000	0.000	0.031	0.045	0.038	0.024	0.059	0.066	0.059																						
14	*Amaranthus cruentus*	0.066	0.000	0.000	0.000	0.000	0.031	0.045	0.038	0.024	0.059	0.066	0.059	0.000																					
15	*Amaranthus graecizans*	0.024	0.051	0.051	0.051	0.051	0.058	0.006	0.012	0.038	0.059	0.024	0.059	0.051	0.051																				
16	*Amaranthus hypochondriacus*	0.066	0.000	0.000	0.000	0.000	0.031	0.045	0.038	0.024	0.059	0.066	0.059	0.000	0.000	0.051																			
17	*Amaranthus lividus*	0.000	0.066	0.066	0.066	0.066	0.073	0.018	0.025	0.044	0.074	0.000	0.074	0.066	0.066	0.024	0.066																		
18	*Amaranthus palmeri*	0.073	0.031	0.031	0.031	0.031	0.012	0.051	0.044	0.031	0.044	0.073	0.044	0.031	0.031	0.058	0.031	0.073																	
19	*Amaranthus paniculatus*	0.066	0.000	0.000	0.000	0.000	0.031	0.045	0.038	0.024	0.059	0.066	0.059	0.000	0.000	0.051	0.000	0.066	0.031																
20	*Amaranthus polygonoides*	0.066	0.000	0.000	0.000	0.000	0.031	0.045	0.038	0.024	0.059	0.066	0.059	0.000	0.000	0.051	0.000	0.066	0.031	0.000															
21	*Amaranthus powellii*	0.059	0.006	0.006	0.006	0.006	0.024	0.038	0.031	0.018	0.052	0.059	0.052	0.006	0.006	0.045	0.006	0.059	0.024	0.006	0.006														
22	*Amaranthus quitensis*	0.066	0.000	0.000	0.000	0.000	0.031	0.045	0.038	0.024	0.059	0.066	0.059	0.000	0.000	0.051	0.000	0.066	0.031	0.000	0.000	0.006													
23	*Amaranthus retroflexus*	0.066	0.000	0.000	0.000	0.000	0.031	0.045	0.038	0.024	0.059	0.066	0.059	0.000	0.000	0.051	0.000	0.066	0.031	0.000	0.000	0.006	0.000												
24	*Amaranthus roxburghianus*	0.024	0.051	0.051	0.051	0.051	0.058	0.006	0.012	0.038	0.059	0.024	0.059	0.051	0.051	0.000	0.051	0.024	0.058	0.051	0.051	0.045	0.051	0.051											
25	*Amaranthus spinosus*	0.073	0.031	0.031	0.031	0.031	0.000	0.051	0.044	0.031	0.044	0.073	0.044	0.031	0.031	0.058	0.031	0.073	0.012	0.031	0.031	0.024	0.031	0.031	0.058										
26	*Amaranthus standleyanus*	0.038	0.051	0.051	0.051	0.051	0.058	0.018	0.012	0.038	0.045	0.038	0.045	0.051	0.051	0.024	0.051	0.038	0.058	0.051	0.051	0.045	0.051	0.051	0.024	0.058									
27	*Amaranthus tashanensis*	0.058	0.058	0.058	0.058	0.058	0.051	0.038	0.031	0.044	0.052	0.058	0.052	0.058	0.058	0.044	0.058	0.058	0.051	0.058	0.058	0.052	0.058	0.058	0.044	0.051	0.038								
28	*Amaranthus tenuifolius*	0.018	0.045	0.045	0.045	0.045	0.051	0.000	0.006	0.031	0.052	0.018	0.052	0.045	0.045	0.006	0.045	0.018	0.051	0.045	0.045	0.038	0.045	0.045	0.006	0.051	0.018	0.038							
29	*Amaranthus tricolor*	0.018	0.045	0.045	0.045	0.045	0.051	0.000	0.006	0.031	0.052	0.018	0.052	0.045	0.045	0.006	0.045	0.018	0.051	0.045	0.045	0.038	0.045	0.045	0.006	0.051	0.018	0.038	0.000						
30	*Amaranthus tuberculatus*	0.044	0.024	0.024	0.024	0.024	0.031	0.031	0.025	0.000	0.045	0.044	0.045	0.024	0.024	0.038	0.024	0.044	0.031	0.024	0.024	0.018	0.024	0.024	0.038	0.031	0.038	0.044	0.031	0.031					
31	*Beta vulgaris* subsp. *orientalis*	0.343	0.329	0.329	0.329	0.329	0.327	0.317	0.331	0.283	0.345	0.343	0.345	0.329	0.329	0.329	0.329	0.343	0.327	0.329	0.329	0.317	0.329	0.329	0.329	0.327	0.357	0.357	0.317	0.317	0.293				
32	*Celosia cristata*	0.382	0.367	0.367	0.367	0.367	0.341	0.370	0.386	0.353	0.386	0.382	0.395	0.367	0.367	0.367	0.367	0.382	0.331	0.367	0.367	0.355	0.367	0.367	0.367	0.341	0.414	0.414	0.370	0.370	0.353	0.427			
33	*Chenopodium album*	0.284	0.311	0.311	0.311	0.311	0.308	0.274	0.287	0.284	0.314	0.284	0.314	0.311	0.311	0.284	0.311	0.284	0.308	0.311	0.311	0.300	0.311	0.311	0.284	0.308	0.297	0.325	0.274	0.274	0.284	0.416	0.552		
34	*Pleuropetalum darwinii*	0.326	0.293	0.293	0.293	0.293	0.270	0.308	0.322	0.281	0.351	0.320	0.361	0.293	0.293	0.320	0.293	0.320	0.291	0.293	0.293	0.282	0.293	0.293	0.320	0.270	0.348	0.348	0.308	0.308	0.281	0.309	0.312	0.459	

AB – *A. blitum*; AC – *A. caudatus*; AD – *A. dubius*; AH – *A. hybridus* subsp. *hybridus*; AR – *A. hybridus* subsp. *cruentus*; AS – *A. spinosus*; AT – *A. tricolor*; AV – *A. viridis*

Table 11. Maximum Likelihood fits of different nucleotide substitution models of rbcL gene sequence analyzed

Model	Parameters	BIC	AICc	*lnL*	(+*I*)	(+*G*)	*R*	*f*(A)	*f*(T)	*f*(C)	*f*(G)	*r*(AT)	*r*(AC)	*r*(AG)	*r*(TA)	*r*(TC)	*r*(TG)	*r*(CA)	*r*(CT)	*r*(CG)	*r*(GA)	*r*(GT)	*r*(GC)
K2	42	1328.966	1056.592	-485.926	n/a	n/a	2.81	0.250	0.250	0.250	0.250	0.033	0.033	0.184	0.033	0.184	0.033	0.033	0.184	0.033	0.184	0.033	0.033
K2+G	43	1333.140	1054.299	-483.762	n/a	0.31	2.97	0.250	0.250	0.250	0.250	0.031	0.031	0.187	0.031	0.187	0.031	0.031	0.187	0.031	0.187	0.031	0.031
T92	43	1333.265	1054.425	-483.825	n/a	n/a	2.80	0.290	0.290	0.210	0.210	0.037	0.027	0.156	0.037	0.156	0.027	0.037	0.215	0.027	0.215	0.037	0.027
K2+I	43	1336.417	1057.576	-485.401	0.17	n/a	2.82	0.250	0.250	0.250	0.250	0.033	0.033	0.185	0.033	0.185	0.033	0.033	0.185	0.033	0.185	0.033	0.033
T92+G	44	1337.499	1052.191	-481.690	n/a	0.31	2.97	0.290	0.290	0.210	0.210	0.036	0.026	0.158	0.036	0.158	0.026	0.036	0.218	0.026	0.218	0.036	0.026
T92+I	44	1338.462	1053.154	-482.172	0.51	n/a	2.89	0.290	0.290	0.210	0.210	0.037	0.027	0.157	0.037	0.157	0.027	0.037	0.217	0.027	0.217	0.037	0.027
JC	41	1340.517	1074.611	-495.953	n/a	n/a	0.50	0.250	0.250	0.250	0.250	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083
K2+G+I	44	1341.553	1056.246	-483.717	0.36	0.72	2.98	0.250	0.250	0.250	0.250	0.031	0.031	0.187	0.031	0.187	0.031	0.031	0.187	0.031	0.187	0.031	0.031
JC+G	42	1345.262	1072.888	-494.074	n/a	0.37	0.50	0.250	0.250	0.250	0.250	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083
T92+G+I	45	1346.014	1054.241	-481.696	0.35	0.71	2.99	0.290	0.290	0.210	0.210	0.036	0.026	0.158	0.036	0.158	0.026	0.036	0.219	0.026	0.219	0.036	0.026
JC+I	42	1347.233	1074.859	-495.060	0.30	n/a	0.50	0.250	0.250	0.250	0.250	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083
HKY	45	1350.775	1059.002	-484.077	n/a	n/a	2.80	0.294	0.286	0.193	0.228	0.037	0.025	0.169	0.038	0.143	0.029	0.038	0.212	0.029	0.218	0.037	0.025
JC+G+I	43	1353.717	1074.876	-494.051	0.28	0.70	0.50	0.250	0.250	0.250	0.250	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083
HKY+G	46	1354.839	1056.601	-481.858	n/a	0.31	2.99	0.294	0.286	0.193	0.228	0.035	0.024	0.172	0.036	0.145	0.028	0.036	0.215	0.028	0.222	0.035	0.024
HKY+I	46	1355.260	1057.022	-482.068	0.66	n/a	2.98	0.294	0.286	0.193	0.228	0.035	0.024	0.171	0.036	0.145	0.028	0.036	0.215	0.028	0.221	0.035	0.024
TN93	46	1357.551	1059.313	-483.214	n/a	n/a	2.80	0.294	0.286	0.193	0.228	0.037	0.025	0.124	0.038	0.185	0.029	0.038	0.275	0.029	0.160	0.037	0.025
TN93+G	47	1361.486	1056.785	-480.930	n/a	0.29	3.02	0.294	0.286	0.193	0.228	0.034	0.023	0.119	0.035	0.196	0.027	0.035	0.291	0.027	0.153	0.034	0.023
TN93+I	47	1362.680	1057.978	-481.527	0.49	n/a	2.90	0.294	0.286	0.193	0.228	0.036	0.024	0.121	0.037	0.191	0.028	0.037	0.283	0.028	0.156	0.036	0.024
HKY+G+I	47	1363.269	1058.567	-481.821	0.36	0.71	3.01	0.294	0.286	0.193	0.228	0.035	0.024	0.172	0.036	0.145	0.028	0.036	0.216	0.028	0.222	0.035	0.024
TN93+G+I	48	1369.978	1058.813	-480.925	0.38	0.72	3.05	0.294	0.286	0.193	0.228	0.034	0.023	0.118	0.035	0.197	0.027	0.035	0.292	0.027	0.153	0.034	0.023
GTR	49	1377.376	1059.749	-480.372	n/a	n/a	1.92	0.294	0.286	0.193	0.228	0.035	0.041	0.126	0.036	0.154	0.069	0.062	0.228	0.000	0.162	0.087	0.000
GTR+I	50	1383.205	1059.116	-479.035	0.44	n/a	1.95	0.294	0.286	0.193	0.228	0.035	0.041	0.125	0.036	0.155	0.067	0.063	0.230	0.000	0.162	0.085	0.000
GTR+G	50	1383.354	1059.265	-479.110	n/a	0.37	1.86	0.294	0.286	0.193	0.228	0.027	0.041	0.125	0.028	0.149	0.065	0.062	0.220	0.021	0.162	0.081	0.018
GTR+G+I	51	1391.627	1061.078	-478.995	0.29	0.71	1.87	0.294	0.286	0.193	0.228	0.027	0.041	0.126	0.028	0.149	0.065	0.062	0.221	0.021	0.162	0.081	0.018

NOTE.-- Models with the lowest BIC scores (Bayesian Information Criterion) are considered to describe the substitution pattern the best. For each model, AICc value (Akaike Information Criterion, corrected), Maximum Likelihood value (*lnL*), and the number of parameters (including branch lengths) are also presented [1]. Non-uniformity of evolutionary rates among sites may be modeled by using a discrete Gamma distribution (+*G*) with 5 rate categories and by assuming that a certain fraction of sites are evolutionarily invariable (+*I*). Whenever applicable, estimates of gamma shape parameter and/or the estimated fraction of invariant sites are shown. Assumed or estimated values of transition/transversion bias (*R*) are shown for each model, as well. They are followed by nucleotide frequencies (*f*) and rates of base substitutions (*r*) for each nucleotide pair. Relative values of instantaneous *r* should be considered when evaluating them. For simplicity, sum of *r* values is made equal to 1 for each model. For estimating ML values, a tree topology was automatically computed. The analysis involved 22 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 224 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 [2].

Abbreviations: GTR: General Time Reversible; HKY: Hasegawa-Kishino-Yano; TN93: Tamura-Nei; T92: Tamura 3-parameter; K2: Kimura 2-parameter; JC: Jukes-Cantor.

Table 12. Maximum Likelihood fits of different nucleotide substitution models of matK gene sequence analyzed

Model	Parameters	BIC	AICc	*lnL*	(+*I*)	(+*G*)	*R*	*f*(A)	*f*(T)	*f*(C)	*f*(G)	*r*(AT)	*r*(AC)	*r*(AG)	*r*(TA)	*r*(TC)	*r*(TG)	*r*(CA)	*r*(CT)	*r*(CG)	*r*(GA)	*r*(GT)	*r*(GC)
T92+G	46	3990.911	3643.515	-1775.605	n/a	0.44	1.13	0.328	0.328	0.172	0.172	0.073	0.038	0.095	0.073	0.095	0.038	0.073	0.183	0.038	0.183	0.073	0.038
T92+G+I	47	4000.048	3645.107	-1775.394	0.32	1.03	1.13	0.328	0.328	0.172	0.172	0.073	0.038	0.095	0.073	0.095	0.038	0.073	0.183	0.038	0.183	0.073	0.038
HKY+G	48	4003.074	3640.588	-1772.127	n/a	0.45	1.11	0.289	0.367	0.169	0.174	0.082	0.038	0.096	0.065	0.093	0.039	0.065	0.203	0.039	0.160	0.082	0.038
GTR+G	52	4008.351	3615.686	-1755.648	n/a	0.52	1.17	0.289	0.367	0.169	0.174	0.024	0.075	0.078	0.019	0.105	0.051	0.129	0.228	0.028	0.129	0.107	0.027
HKY+I	48	4010.884	3648.397	-1776.032	0.41	n/a	1.10	0.289	0.367	0.169	0.174	0.083	0.038	0.096	0.065	0.093	0.039	0.065	0.202	0.039	0.159	0.083	0.038
TN93+G	49	4011.123	3641.092	-1771.372	n/a	0.46	1.12	0.289	0.367	0.169	0.174	0.082	0.038	0.081	0.065	0.106	0.039	0.065	0.230	0.039	0.134	0.082	0.038
HKY+G+I	49	4012.226	3642.195	-1771.924	0.31	1.04	1.12	0.289	0.367	0.169	0.174	0.082	0.038	0.096	0.065	0.094	0.039	0.065	0.203	0.039	0.160	0.082	0.038
TN93+I	49	4013.208	3643.177	-1772.415	0.56	n/a	1.11	0.289	0.367	0.169	0.174	0.083	0.038	0.080	0.065	0.106	0.039	0.065	0.230	0.039	0.133	0.083	0.038
T92+I	46	4014.832	3667.436	-1787.565	0.17	n/a	1.10	0.328	0.328	0.172	0.172	0.074	0.039	0.094	0.074	0.094	0.039	0.074	0.181	0.039	0.181	0.074	0.039
T92	45	4016.334	3676.484	-1793.095	n/a	n/a	1.10	0.328	0.328	0.172	0.172	0.074	0.039	0.094	0.074	0.094	0.039	0.074	0.180	0.039	0.180	0.074	0.039
GTR+G+I	53	4017.664	3617.457	-1755.526	0.27	1.04	1.17	0.289	0.367	0.169	0.174	0.024	0.075	0.078	0.019	0.105	0.051	0.129	0.228	0.028	0.129	0.107	0.027
TN93+G+I	50	4020.286	3642.710	-1771.174	0.32	1.11	1.12	0.289	0.367	0.169	0.174	0.082	0.038	0.081	0.065	0.106	0.039	0.065	0.231	0.039	0.134	0.082	0.038
GTR+I	52	4025.697	3633.033	-1764.321	0.55	n/a	0.81	0.289	0.367	0.169	0.174	0.066	0.074	0.081	0.052	0.078	0.051	0.127	0.170	0.028	0.135	0.108	0.028
HKY	47	4027.523	3672.581	-1789.131	n/a	n/a	1.10	0.289	0.367	0.169	0.174	0.083	0.038	0.095	0.065	0.093	0.039	0.065	0.201	0.039	0.159	0.083	0.038
TN93	48	4034.821	3672.335	-1788.001	n/a	n/a	1.10	0.289	0.367	0.169	0.174	0.083	0.038	0.079	0.066	0.106	0.039	0.066	0.231	0.039	0.131	0.083	0.038
GTR	51	4045.263	3660.143	-1778.883	n/a	n/a	0.78	0.289	0.367	0.169	0.174	0.065	0.077	0.078	0.052	0.078	0.050	0.131	0.169	0.033	0.129	0.106	0.032
K2+G	45	4050.206	3710.356	-1810.031	n/a	0.36	1.17	0.250	0.250	0.250	0.250	0.058	0.058	0.135	0.058	0.135	0.058	0.058	0.135	0.058	0.135	0.058	0.058
K2+I	45	4051.321	3711.471	-1810.589	0.61	n/a	1.16	0.250	0.250	0.250	0.250	0.058	0.058	0.134	0.058	0.134	0.058	0.058	0.134	0.058	0.134	0.058	0.058
K2+G+I	46	4059.362	3711.966	-1809.830	0.35	0.89	1.17	0.250	0.250	0.250	0.250	0.058	0.058	0.135	0.058	0.135	0.058	0.058	0.135	0.058	0.135	0.058	0.058
JC+G	44	4067.609	3735.305	-1823.512	n/a	0.37	0.50	0.250	0.250	0.250	0.250	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083
JC+G+I	45	4076.793	3736.943	-1823.325	0.34	0.88	0.50	0.250	0.250	0.250	0.250	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083
K2	44	4081.962	3749.658	-1830.689	n/a	n/a	1.10	0.250	0.250	0.250	0.250	0.060	0.060	0.131	0.060	0.131	0.060	0.060	0.131	0.060	0.131	0.060	0.060
JC+I	44	4096.339	3764.034	-1837.877	0.16	n/a	0.50	0.250	0.250	0.250	0.250	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083
JC	43	4097.701	3772.943	-1843.338	n/a	n/a	0.50	0.250	0.250	0.250	0.250	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083

NOTE.-- Models with the lowest BIC scores (Bayesian Information Criterion) are considered to describe the substitution pattern the best. For each model, AICc value (Akaike Information Criterion, corrected), Maximum Likelihood value (*lnL*), and the number of parameters (including branch lengths) are also presented [1]. Non-uniformity of evolutionary rates among sites may be modeled by using a discrete Gamma distribution (+G) with 5 rate categories and by assuming that a certain fraction of sites are evolutionarily invariable (+*I*). Whenever applicable, estimates of gamma shape parameter and/or the estimated fraction of invariant sites are shown. Assumed or estimated values of transition/transversion bias (*R*) are shown for each model, as well. They are followed by nucleotide frequencies (*f*) and rates of base substitutions (*r*) for each nucleotide pair. Relative values of instantaneous *r* should be considered when evaluating them. For simplicity, sum of *r* values is made equal to 1 for each model. For estimating ML values, a tree topology was automatically computed. The analysis involved 23 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 616 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 [2].

Abbreviations: GTR: General Time Reversible; HKY: Hasegawa-Kishino-Yano; TN93: Tamura-Nei; T92: Tamura 3-parameter; K2: Kimura 2-parameter; JC: Jukes-Cantor.

Table 13. Maximum Likelihood fits of different nucleotide substitution models of ITS region of rDNA sequence analyzed

Model	Parameters	BIC	AICc	*lnL*	(+*I*)	(+*G*)	*R*	*f*(A)	*f*(T)	*f*(C)	*f*(G)	*r*(AT)	*r*(AC)	*r*(AG)	*r*(TA)	*r*(TC)	*r*(TG)	*r*(CA)	*r*(CT)	*r*(CG)	*r*(GA)	*r*(GT)	*r*(GC)
K2+G	67	2262.450	1818.085	-841.240	n/a	0.92	1.43	0.250	0.250	0.250	0.250	0.051	0.051	0.147	0.051	0.147	0.051	0.051	0.147	0.051	0.147	0.051	0.051
K2+I	67	2264.064	1819.699	-842.047	0.35	n/a	1.41	0.250	0.250	0.250	0.250	0.052	0.052	0.146	0.052	0.146	0.052	0.052	0.146	0.052	0.146	0.052	0.052
K2+G+I	68	2270.905	1819.933	-841.140	0.18	1.75	1.43	0.250	0.250	0.250	0.250	0.051	0.051	0.147	0.051	0.147	0.051	0.051	0.147	0.051	0.147	0.051	0.051
K2	66	2273.849	1836.094	-851.268	n/a	n/a	1.31	0.250	0.250	0.250	0.250	0.054	0.054	0.142	0.054	0.142	0.054	0.054	0.142	0.054	0.142	0.054	0.054
T92+G	68	2279.265	1828.292	-845.320	n/a	0.98	1.42	0.206	0.206	0.294	0.294	0.042	0.060	0.175	0.042	0.175	0.060	0.042	0.122	0.060	0.122	0.042	0.060
T92+I	68	2280.807	1829.834	-846.091	0.34	n/a	1.39	0.206	0.206	0.294	0.294	0.042	0.060	0.173	0.042	0.173	0.060	0.042	0.121	0.060	0.121	0.042	0.060
JC+G	66	2281.839	1844.083	-855.263	n/a	0.98	0.50	0.250	0.250	0.250	0.250	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083
JC+I	66	2283.722	1845.966	-856.205	0.34	n/a	0.50	0.250	0.250	0.250	0.250	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083
T92+G+I	69	2287.342	1829.762	-845.030	0.12	1.45	1.41	0.206	0.206	0.294	0.294	0.042	0.060	0.174	0.042	0.174	0.060	0.042	0.122	0.060	0.122	0.042	0.060
T92	67	2288.479	1844.115	-854.255	n/a	n/a	1.31	0.206	0.206	0.294	0.294	0.044	0.062	0.169	0.044	0.169	0.062	0.044	0.118	0.062	0.118	0.044	0.062
JC+G+I	67	2290.361	1845.996	-855.196	0.15	1.61	0.50	0.250	0.250	0.250	0.250	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083
JC	65	2293.561	1862.415	-865.452	n/a	n/a	0.50	0.250	0.250	0.250	0.250	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083
HKY+G	70	2296.600	1832.413	-845.331	n/a	0.97	1.44	0.203	0.209	0.262	0.326	0.042	0.053	0.195	0.041	0.156	0.066	0.041	0.125	0.066	0.121	0.042	0.053
HKY+I	70	2298.553	1834.366	-846.307	0.34	n/a	1.42	0.203	0.209	0.262	0.326	0.042	0.053	0.194	0.041	0.155	0.066	0.041	0.124	0.066	0.121	0.042	0.053
TN93+G	71	2302.563	1831.771	-843.984	n/a	1.05	1.42	0.203	0.209	0.262	0.326	0.042	0.053	0.145	0.041	0.201	0.066	0.041	0.160	0.066	0.090	0.042	0.053
TN93+I	71	2304.720	1833.928	-845.063	0.32	n/a	1.38	0.203	0.209	0.262	0.326	0.043	0.054	0.152	0.042	0.191	0.067	0.042	0.153	0.067	0.094	0.043	0.054
HKY+G+I	71	2305.184	1834.391	-845.295	0.14	1.53	1.44	0.203	0.209	0.262	0.326	0.042	0.053	0.195	0.041	0.156	0.066	0.041	0.125	0.066	0.121	0.042	0.053
HKY	69	2308.154	1850.574	-855.436	n/a	n/a	1.31	0.203	0.209	0.262	0.326	0.044	0.056	0.188	0.043	0.150	0.069	0.043	0.120	0.069	0.117	0.044	0.056
TN93	70	2309.595	1845.408	-851.828	n/a	n/a	1.32	0.203	0.209	0.262	0.326	0.044	0.055	0.136	0.043	0.199	0.069	0.043	0.159	0.069	0.085	0.044	0.055
TN93+G+I	72	2310.723	1833.325	-843.736	0.02	1.12	1.42	0.203	0.209	0.262	0.326	0.042	0.053	0.145	0.041	0.200	0.066	0.041	0.160	0.066	0.091	0.042	0.053
GTR+G	74	2316.973	1826.367	-838.205	n/a	1.08	1.10	0.203	0.209	0.262	0.326	0.096	0.063	0.148	0.094	0.155	0.052	0.049	0.124	0.052	0.092	0.033	0.041
GTR+I	74	2321.451	1830.846	-840.444	0.31	n/a	1.08	0.203	0.209	0.262	0.326	0.094	0.063	0.155	0.092	0.147	0.051	0.049	0.117	0.057	0.096	0.033	0.046
GTR+G+I	75	2325.515	1828.306	-838.148	0.03	1.17	1.10	0.203	0.209	0.262	0.326	0.096	0.063	0.148	0.094	0.155	0.052	0.049	0.124	0.052	0.092	0.033	0.041
GTR	73	2326.660	1842.658	-847.377	n/a	n/a	1.00	0.203	0.209	0.262	0.326	0.095	0.059	0.135	0.092	0.156	0.061	0.046	0.124	0.060	0.084	0.039	0.048

NOTE.-- Models with the lowest BIC scores (Bayesian Information Criterion) are considered to describe the substitution pattern the best. For each model, AICc value (Akaike Information Criterion, corrected), Maximum Likelihood value (*lnL*), and the number of parameters (including branch lengths) are also presented [1]. Non-uniformity of evolutionary rates among sites may be modeled by using a discrete Gamma distribution (+*G*) with 5 rate categories and by assuming that a certain fraction of sites are evolutionarily invariable (+*I*). Whenever applicable, estimates of gamma shape parameter and/or the estimated fraction of invariant sites are shown. Assumed or estimated values of transition/transversion bias (*R*) are shown for each model, as well. They are followed by nucleotide frequencies (*f*) and rates of base substitutions (*r*) for each nucleotide pair. Relative values of instantaneous *r* should be considered when evaluating them. For simplicity, sum of *r* values is made equal to 1 for each model. For estimating ML values, a tree topology was automatically computed. The analysis involved 34 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 169 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 [2].

Abbreviations: GTR: General Time Reversible; HKY: Hasegawa-Kishino-Yano; TN93: Tamura-Nei; T92: Tamura 3-parameter; K2: Kimura 2-parameter; JC: Jukes-Cantor.

2. CYTOGENETICAL CHARACTERIZATION

Cytogenetical studies were conducted by analysing both mitotic and meiotic chromosomes of the selected *Amaranthus* species (Plates 8-26; Fig. 2; Tables 14-22). Mitotic squash preparation for the characterization of somatic chromosomes was done by using both aceto-orcein and DAPI staining technique. A comparative cytogenetical analysis of eight *Amaranthus* species helped to reveal the chromosome number, ploidy and karyomorphology of each species and led to the disclosure of the karyological identity. Recent reports on the cytogenetic studies to reveal mitotic and meiotic chromosome architecture as well as behaviour and comparison of different *Amaranthus* species were less. Apart from a cytogenetic study detailed karyomorphometric data of *Amaranthus* species was not available so far. The present comparative analysis helped to establish both unique and common cytogenetic features and evolutionary status of the species studied.

MITOTIC CHROMOSOME STUDIES

Detailed karyomorphometric data and the somatic chromosome number of eight *Amaranthus* species studied were represented in Tables 14-21 and were summarized in Table 22. The karyotype, karyogram and idiogram of somatic chromosomes of selected species using aceto-orcein staining were represented in the Plates 8-23. DAPI images of the karyotype of selected taxa were represented in Plate 24. The karyotype asymmetry was found out based on various asymmetry indices such as disparity index (DI), variation coefficient (VC), TF value (%), karyotype asymmetry index (As K%), Syi index, Rec index, intrachromosomal asymmetry index (A_1), interchromosomal asymmetry index (A_2), degree of asymmetry of karyotype (A) and asymmetry index (AI) of mitotic metaphase chromosomes in all the species studied.

The karyomorphological data of the somatic chromosomes of *A. blitum* was detailed in Table 14. The mitotic metaphase chromosome number was found to be 2n = 34 which consist of 17 pairs of chromosomes (Plates 8, 9). The length of chromosomes ranged from 2.10 µm to 1.11 µm. Total chromosome length and average chromosome length observed was 54.08 µm and 1.59 µm respectively. Secondary constriction was observed in the first two pairs of chromosomes. Karyotype has a predominance of nearly median chromosomes. 14 pairs of chromosomes were of nearly median, 2 pairs of nearly submedian and 1 pair have median type of primary constriction. The two largest pairs of chromosomes was nearly submedian type. The karyogram and idiogram of the chromosome complement were prepared based on chromosome size and centromeric index and arranged based on size in decreasing order (Plate 9 a-d).

The karyomorphological data of the somatic chromosomes of *A. caudatus* was detailed in Table 15. The mitotic metaphase chromosome number was found to be 2n = 32 which consist of 16 pairs of chromosomes (Plates 10, 11). The length of chromosomes ranged from 1.76 µm to 0.86 µm. Total chromosome length and average chromosome length observed was 38.86 µm and 1.21 µm respectively. Secondary constriction was observed in the first two pairs of chromosomes. Primary constriction of all chromosomes was of nearly median type. The karyogram and idiogram of the chromosome complement were prepared based on chromosome size and centromeric index and arranged in decreasing order (Plate 11 a-d).

The karyomorphological data of the somatic chromosomes of *A. dubius* is shown in Table 16. The mitotic metaphase chromosome number was found to be 2n = 64 which consist of 32 pairs of chromosomes (Plates 12, 13). The length of chromosomes ranged from 2.24 µm to 1.03 µm. Total chromosome length and average chromosome length observed was 94.12 µm

and 1.47 µm respectively. Secondary constriction was observed in the first four pairs of chromosomes. Karyotype has a predominance of nearly median chromosomes. 28 pairs of chromosomes were of nearly median and remaining was found to be with nearly submedian type of primary constriction. The four large pairs of chromosomes was nearly submedian type. The karyogram and idiogram of the chromosome complement were prepared based on chromosome size and centromeric index and arranged based on size in decreasing order (Plate 13 a-d).

The karyomorphological data of the somatic chromosomes of *A. hybridus* subsp. *hybridus* was detailed in Table 17. The mitotic metaphase chromosome number was found to be 2n = 32 which consist of 16 pairs of chromosomes (Plates 14, 15). The length of chromosomes ranged from 1.71 µm to 0.93 µm. Total chromosome length and average chromosome length observed was 39.66 µm and 1.23 µm respectively. Secondary constriction was observed in the first two pairs of chromosomes. Karyotype has a predominance of nearly median chromosomes. 13 pairs of chromosomes were of nearly median and the remaining 3 pairs were found to be with nearly submedian type of primary constriction. The two large pairs of chromosomes was nearly submedian type. The karyogram and idiogram of the chromosome complement were prepared based on chromosome size and centromeric index and arranged based on size in decreasing order (Plate 15 a-d).

The karyomorphological data of the somatic chromosomes of *A. hybridus* subsp. *cruentus* was detailed in Table 18. The mitotic metaphase chromosome number was found to be 2n = 34 which consist of 17 pairs of chromosomes (Plates 16, 17). The length of chromosomes ranged from 1.93 µm to 0.77 µm. Total chromosome length and average chromosome length observed was 44.64 µm and 1.31 µm respectively. Secondary constriction was observed in the first two pairs of chromosomes. Karyotype has a

predominance of nearly median chromosomes. 15 pairs of chromosomes were of nearly median and the remaining 2 pairs were found to be with nearly submedian type of primary constriction. The karyogram and idiogram of the chromosome complement were prepared based on chromosome size and centromeric index and arranged based on size in decreasing order (Plate 17 a-d).

The karyomorphological data of the somatic chromosomes of *A. spinosus* was detailed in Table 19. The mitotic metaphase chromosome number was found to be 2n = 34 which consist of 17 pairs of chromosomes (Plates 18, 19). The length of chromosomes ranged from 2.43 µm to 1.34 µm. Total chromosome length and average chromosome length observed was 57.68 µm and 1.69 µm respectively. Secondary constriction was observed in the first two pairs of chromosomes. Karyotype has a predominance of nearly median chromosomes, with 10 pairs of chromosomes having nearly median, 3 pairs with nearly submedian, 1 pair with nearly subterminal and the remaining 3 pairs were found to be with terminal type of primary constriction. The karyogram and idiogram of the chromosome complement were prepared based on chromosome size and centromeric index and arranged in decreasing size range (Plate 19 a-d).

The karyomorphological data of the somatic chromosomes of *A. tricolor* was detailed in Table 20. The mitotic metaphase chromosome number was found to be 2n = 34 which consist of 17 pairs of chromosomes (Plates 20, 21). The length of chromosomes ranged from 2.34 µm to 0.99 µm. Total chromosome length and average chromosome length observed was 47.32 µm and 1.39 µm respectively. Secondary constriction was observed in the first two pairs of chromosomes. Karyotype has a predominance of nearly submedian chromosomes. Out of the seventeen pairs of chromosomes, 10 pairs of chromosomes were of nearly submedian, 4 pairs with nearly median and the remaining 3 pairs were found to be with terminal type of primary

constriction. The karyogram and idiogram of the chromosome complement were prepared based on chromosome size and centromeric index and arranged in decreasing size range (Plate 21 a-d).

The karyomorphological data of the somatic chromosomes of *A. viridis* was detailed in Table 21. The mitotic metaphase chromosome number was found to be 2n = 32 which consist of 16 pairs of chromosomes (Plates 22, 23). The length of chromosomes ranged from 2.22 µm to 1.12 µm. Total chromosome length and average chromosome length observed was 47.84 µm and 1.49 µm respectively. Secondary constriction was observed in the first two pairs of chromosomes. Karyotype has a predominance of nearly submedian chromosomes. The chromosome complement shows 9 pairs of chromosomes having nearly submedian, 6 pairs with nearly median and the remaining 1 pair was found to be with terminal type of primary constriction. The karyogram and idiogram of the chromosome complement were prepared based on chromosome size and centromeric index and arranged in decreasing size range (Plate 23 a-d).

Karyotype analysis revealed that all the species possessed chromosome with small size of which reasonably higher size was shown by *A. spinosus* (2.43 µm) and lower size by *A. hybridus* subsp. *hybridus* (1.71 µm). Three species of *Amaranthus viz*., *A. caudatus*, *A. hybridus* subsp. *hybridus* and *A. viridis* have somatic chromosome number of 2n = 32. All other species except *A. dubius* (2n = 64) have diploid chromosome number of 2n = 34. The number of secondary constriction was in equity for all the species except *A. dubius* which possessed eight chromosomes with secondary constriction. Karyotype of all the species possessed predominantly nearly median primary constriction, whereas *A. tricolor* and *A. viridis* reveals predominance of nearly submedian primary constriction. One pair of chromosome in *A. blitum* was metacentric and the karyotype of *A. spinosus*, *A. tricolor* and *A. viridis* showed the presence of telocentric chromosomes. Chromosome with nearly subterminal primary constriction was found only in *A. spinosus* karyotype.

Karyomorphometric data of all the species was summarized and tabulated in Table 22 and the scatter plot representing the intrachromosomal and interchromosomal asymmetry of chromosomes was given in the Fig. 2. Phylogenetic scheme showing the probable evolution of chromosome numbers in the *Amaranthus* species investigated was represented in Plate 26. Karyotype, karyogram, idiogram, karyotype formula and various morphometric and asymmetric features of the karyotype provided a cytological identity to each *Amaranthus* species studied.

MEIOTIC CHROMOSOME STUDIES

Meiotic smear experiments were carried out by the analysis of pollen mother cells from young anthers of collected *Amaranthus* species. Meiotic studies conducted in eight *Amaranthus* species revealed normal meiotic cells with nearly perfect bivalent formation (Plate 25). The occurrence of univalent chromosomes was also found in some species. Cytological studies of meiotic chromosomes of *A. blitum* (Plate 25 a) and *A. spinosus* (Plate 25 f) showed n = 17 chromosomes with 17 bivalents without any univalents at diakinesis. Meiotic cells of *A. viridis* (Plate 25 h) showed normal metaphase of n = 16 with 16 bivalents. Meiotic cells of *A. caudatus* (Plate 25 b) showed n = 16 with 14 bivalents and 4 univalents. Haploid chromosome number of n = 32 with 28 bivalents and 8 univalents was observed in the pollen mother cells of *A. dubius* (Plate 25 c). Meiotic cells of *A. hybridus* subsp. *cruentus* (Plate 25 e) and *A. tricolor* (Plate 25 g) showed the occurrence of n = 17 chromosomes with 14 bivalents and 6 univalents. Gametic chromosome number of n = 16 with 15 bivalents and 2 univalents was observed in the meiotic cells of *A. hybridus* subsp. *hybridus* (Plate 25 d). The haploid chromosome complements observed in the meiotic cells revealed that the same chromosome complement exist both in the mitotic and meiotic cells, which was further confirmed by the fluorescent imaging studies of somatic chromosomes effected by DAPI staining in all the taxa analyzed (Plate 24).

Mitotic chromosome complement of *A. blitum* with 2n = 34 chromosomes

Somatic chromosome number	:	34
Karyotype formula	:	2M + 28nm + 4nsm(-)
Chromosome with secondary constriction	:	4
Range of chromosome length (RCL)	:	2.10 μm to 1.11 μm
Total chromosome length (TCL)	:	54.08 μm
Average chromosome length (ACL)	:	1.59 μm
Disparity index (DI)	:	30.84
Variation coefficient (VC)	:	17.41
TF value (%)	:	43.34
Karyotype asymmetry index (As K%)	:	56.65
Syi Index	:	75.55
Rec index	:	75.74
Intrachromosomal asymmetry index (A_1)	:	0.21
Interchromosomal asymmetry index (A_2)	:	0.17
Degree of asymmetry of karyotypes (A)	:	0.12
Asymmetry index AI	:	0.11

Table 14: Detailed karyomorphometric data of *A. blitum* with 2n = 34 chromosomes

No. of Chr.	c (µm)	s (µm)	l (µm)	R_1 (s/l)	R_2 (l/s)	I_1 (s/c %)	I_2 (l/c %)	Nature of PC
2*	2.10 ± 0.02	0.65 ± 0.01	1.45 ± 0.03	0.44 ± 0.02	2.23 ± 0.03	30.95 ± 0.02	69.04 ± 0.01	nsm(-)
2*	2.06 ± 0.04	0.55 ± 0.11	1.51 ± 0.05	0.36 ± 0.04	2.74 ± 0.04	26.69 ± 0.02	73.30 ± 0.05	nsm(-)
2	2.01 ± 0.04	0.95 ± 0.12	1.06 ± 0.15	0.89 ± 0.04	1.11 ± 0.11	47.26 ± 0.04	52.73 ± 0.05	nm
2	1.85 ± 0.17	0.85 ± 0.15	1.00 ± 0.00	0.85 ± 0.02	1.17 ± 0.11	45.94 ± 0.01	54.05 ± 0.01	nm
2	1.66 ± 0.04	0.79 ± 0.01	0.87 ± 0.04	0.90 ± 0.00	1.10 ± 0.07	47.59 ± 0.07	52.40 ± 0.05	nm
2	1.64 ± 0.00	0.79 ± 0.00	0.85 ± 0.00	0.92 ± 0.00	1.07 ± 0.00	48.17 ± 0.06	51.82 ± 0.06	nm
2	1.59 ± 0.01	0.65 ± 0.03	0.94 ± 0.02	0.69 ± 0.03	1.44 ± 0.01	40.88 ± 0.03	59.11 ± 0.04	nm
2	1.56 ± 0.01	0.78 ± 0.01	0.78 ± 0.05	1.00 ± 0.05	1.00 ± 0.05	50.00 ± 0.02	50.00 ± 0.01	M
2	1.52 ± 0.02	0.59 ± 0.02	0.93 ± 0.04	0.63 ± 0.02	1.57 ± 0.03	38.81 ± 0.00	61.18 ± 0.00	nm
2	1.52 ± 0.00	0.73 ± 0.00	0.79 ± 0.00	0.92 ± 0.00	1.08 ± 0.00	48.02 ± 0.00	51.97 ± 0.00	nm
2	1.50 ± 0.20	0.73 ± 0.11	0.77 ± 0.12	0.94 ± 0.15	1.05 ± 0.12	48.66 ± 0.12	51.33 ± 0.11	nm
2	1.49 ± 0.11	0.70 ± 0.11	0.79 ± 0.01	0.88 ± 0.03	1.12 ± 0.11	46.97 ± 0.12	53.02 ± 0.12	nm
2	1.46 ± 0.12	0.67 ± 0.15	0.79 ± 0.12	0.84 ± 0.11	1.17 ± 0.12	45.89 ± 0.13	54.10 ± 0.12	nm
2	1.38 ± 1.11	0.63 ± 1.11	0.75 ± 1.12	0.84 ± 0.09	1.19 ± 0.09	45.65 ± 0.08	54.34 ± 0.09	nm
2	1.30 ± 0.07	0.59 ± 0.06	0.71 ± 0.06	0.83 ± 0.07	1.20 ± 0.05	45.38 ± 0.07	54.61 ± 0.06	nm
2	1.29 ± 0.00	0.55 ± 0.01	0.74 ± 0.00	0.74 ± 0.00	1.34 ± 0.01	42.63 ± 0.01	57.36 ± 0.01	nm
2	1.11 ± 0.12	0.52 ± 0.11	0.59 ± 0.02	0.88 ± 0.05	1.13 ± 0.03	46.84 ± 0.03	53.15 ± 0.02	nm

Chr. – Chromosome, **c** – total length of chromosome, **s** – short arm length, **l** – long arm length, $\mathbf{R_1}$ – arm ratio 1, $\mathbf{R_2}$ – arm ratio 2, $\mathbf{I_1}$ – centromeric index 1, $\mathbf{I_2}$ – centromeric index 2, **PC** – primary constriction, * - chromosome with secondary constriction, M – median, nm – nearly median, nsm – nearly submedian

Mitotic chromosome complement of *A. caudatus* with 2n = 32 chromosomes

Somatic chromosome number	:	32
Karyotype formula	:	32nm
Chromosome with secondary constriction	:	4
Range of chromosome length (RCL)	:	1.76 µm to 0.86 µm
Total chromosome length (TCL)	:	38.86 µm
Average chromosome length (ACL)	:	1.21 µm
Disparity index (DI)	:	34.35
Variation coefficient (VC)	:	21.65
TF value (%)	:	44.10
Karyotype asymmetry index (As K%)	:	56
Syi Index	:	78
Rec index	:	69
Intrachromosomal asymmetry index (A_1)	:	0.21
Interchromosomal asymmetry index (A_2)	:	0.21
Degree of asymmetry of karyotypes (A)	:	0.11
Asymmetry index AI	:	0.12

Table 15: Detailed karyomorphometric data of *A. caudatus* with 2n = 32 chromosomes

No. of Chr.	c (µm)	s (µm)	l (µm)	R_1 (s/l)	R_2 (l/s)	I_1 (s/c %)	I_2 (l/c %)	Nature of PC
2*	1.76 ± 0.07	0.73 ± 0.07	1.03 ± 0.04	0.70 ± 0.06	1.41 ± 0.07	41.47 ± 0.06	58.52 ± 0.06	nm
2*	1.69 ± 0.00	0.72 ± 0.00	0.97 ± 0.00	0.74 ± 0.00	1.34 ± 0.00	42.60 ± 0.00	57.39 ± 0.00	nm
2	1.45 ± 0.01	0.68 ± 0.02	0.77 ± 0.01	0.88 ± 0.02	1.13 ± 0.02	46.89 ± 0.01	53.10 ± 0.01	nm
2	1.35 ± 0.00	0.58 ± 0.01	0.77 ± 0.00	0.75 ± 0.01	1.32 ± 0.00	42.96 ± 0.00	57.03 ± 0.00	nm
2	1.35 ± 0.11	0.61 ± 0.09	0.74 ± 0.09	0.82 ± 0.11	1.21 ± 0.11	45.18 ± 0.08	54.81 ± 0.11	nm
2	1.33 ± 0.06	0.62 ± 0.07	0.71 ± 0.09	0.87 ± 0.07	1.14 ± 0.07	46.61 ± 0.06	53.38 ± 0.06	nm
2	1.25 ± 0.08	0.53 ± 0.09	0.72 ± 0.03	0.73 ± 0.06	1.35 ± 0.05	42.40 ± 0.09	57.60 ± 0.03	nm
2	1.19 ± 0.04	0.50 ± 0.06	0.69 ± 0.01	0.72 ± 0.02	1.38 ± 0.01	42.01 ± 0.07	57.98 ± 0.01	nm
2	1.16 ± 0.00	0.45 ± 0.00	0.71 ± 0.00	0.63 ± 0.00	1.57 ± 0.00	38.79 ± 0.00	61.20 ± 0.00	nm
2	1.06 ± 0.04	0.45 ± 0.05	0.61 ± 0.04	0.73 ± 0.09	1.35 ± 0.07	42.45 ± 0.07	57.54 ± 0.04	nm
2	1.05 ± 0.11	0.48 ± 0.03	0.57 ± 0.05	0.84 ± 0.05	1.18 ± 0.04	45.71 ± 0.05	54.28 ± 0.05	nm
2	1.03 ± 0.00	0.48 ± 0.00	0.55 ± 0.00	0.87 ± 0.00	1.14 ± 0.00	46.60 ± 0.00	53.39 ± 0.00	nm
2	0.98 ± 0.07	0.46 ± 0.11	0.52 ± 0.04	0.88 ± 0.07	1.13 ± 0.04	46.93 ± 0.07	53.06 ± 0.04	nm
2	0.97 ± 0.00	0.41 ± 0.00	0.56 ± 0.00	0.73 ± 0.00	1.36 ± 0.00	42.26 ± 0.00	57.73 ± 0.00	nm
2	0.95 ± 0.04	0.46 ± 0.07	0.49 ± 0.11	0.93 ± 0.09	1.06 ± 0.05	48.42 ± 0.11	51.57 ± 0.05	nm
2	0.86 ± 0.09	0.41 ± 0.06	0.45 ± 0.04	0.91 ± 0.09	1.09 ± 0.04	47.67 ± 0.11	52.32 ± 0.09	nm

Chr. – Chromosome, **c** – total length of chromosome, **s** – short arm length, **l** – long arm length, **R_1** – arm ratio 1, **R_2** – arm ratio 2, **I_1** – centromeric index 1, **I_2** – centromeric index 2, **PC** – primary constriction, * - chromosome with secondary constriction, nm – nearly median

Mitotic chromosome complement of *A. dubius* with 2n = 64 chromosomes

Somatic chromosome number	:	64
Karyotype formula	:	56nm + 8nsm(-)
Chromosome with secondary constriction	:	8
Range of chromosome length (RCL)	:	2.24 μm to 1.03 μm
Total chromosome length (TCL)	:	94.12 μm
Average chromosome length (ACL)	:	1.47 μm
Disparity index (DI)	:	37
Variation coefficient (VC)	:	20.37
TF value (%)	:	42.77
Karyotype asymmetry index (As K%)	:	57.22
Syi Index	:	73.80
Rec index	:	65.65
Intrachromosomal asymmetry index (A_1)	:	0.20
Interchromosomal asymmetry index (A_2)	:	0.20
Degree of asymmetry of karyotypes (A)	:	0.12
Asymmetry index AI	:	0.14

Table 16: Detailed karyomorphometric data of *A. dubius* with 2n = 64 chromosomes

No. of Chr.	c (µm)	s (µm)	l (µm)	R_1 (s/l)	R_2 (l/s)	I_1 (s/c %)	I_2 (l/c %)	Nature of PC
2*	2.24 ± 0.03	0.67 ± 0.01	1.57 ± 0.01	0.42 ± 0.02	2.34 ± 0.03	29.91 ± 0.02	70.08 ± 0.01	nsm(-)
2*	2.22 ± 0.03	0.69 ± 0.05	1.53 ± 0.02	0.45 ± 0.03	2.21 ± 0.06	31.08 ± 0.08	68.91 ± 0.10	nsm(-)
2*	2.12 ± 0.00	0.74 ±0.01	1.38 ± 0.05	0.53 ± 0.05	1.86 ± 0.05	34.90 ± 0.03	65.09 ± 0.03	nsm(-)
2*	2.00 ± 0.07	0.56 ± 0.05	1.44 ± 0.06	0.38 ± 0.07	2.57 ± 0.07	28.00 ± 0.06	72.00 ± 0.10	nsm(-)
2	1.65 ± 0.12	0.75 ± 0.09	0.90 ± 0.03	0.83 ± 0.09	1.20 ± 0.05	45.45 ± 0.08	54.54 ± 0.09	nm
2	1.61 ± 0.05	0.70 ± 0.06	0.91 ± 0.08	0.76 ± 0.05	1.30 ± 0.06	43.47 ± 0.03	56.52 ± 0.05	nm
2	1.60 ± 0.06	0.66 ± 0.05	0.94 ± 0.09	0.70 ± 0.06	1.42 ± 0.06	41.25 ± 0.10	58.75 ± 0.08	nm
2	1.58 ± 0.00	0.70 ± 0.00	0.88 ± 0.00	0.79 ± 0.00	1.25 ± 0.00	44.30 ± 0.00	55.69 ± 0.00	nm
2	1.56 ± 0.07	0.75 ± 0.07	0.81 ± 0.03	0.92 ± 0.05	1.08 ± 0.05	48.07 ± 0.05	51.92 ± 0.10	nm
2	1.51 ± 0.03	0.65 ± 0.06	0.86 ± 0.06	0.75 ± 0.09	1.32 ± 0.07	43.04 ± 0.06	56.95 ± 0.05	nm
2	1.50 ± 0.08	0.64 ± 0.09	0.86 ± 0.08	0.74 ± 0.10	1.34 ± 0.05	42.66 ± 0.05	57.33 ± 0.08	nm
2	1.49 ± 0.00	0.63 ± 0.00	0.86 ± 0.00	0.73 ± 0.00	1.36 ± 0.00	42.28 ± 0.00	57.71 ± 0.00	nm
2	1.47 ± 0.12	0.70 ± 0.09	0.77 ± 0.19	0.90 ± 0.17	1.10 ± 0.12	47.61 ± 0.19	52.38 ± 0.17	nm
2	1.44 ± 0.05	0.63 ± 0.06	0.81 ± 0.05	0.77 ± 0.03	1.28 ± 0.06	43.75 ± 0.05	56.25 ± 0.08	nm
2	1.42 ± 0.09	0.64 ± 0.07	0.78 ± 0.09	0.82 ± 0.09	1.21 ± 0.05	45.07 ± 0.07	54.92 ± 0.10	nm
2	1.39 ± 0.06	0.68 ± 0.05	0.71 ± 0.08	0.95 ± 0.06	1.04 ± 0.10	48.92 ± 0.03	51.07 ± 0.03	nm
2	1.39 ± 0.11	0.66 ± 0.11	0.73 ± 0.05	0.90 ± 0.12	1.10 ± 0.11	47.48 ± 0.12	52.51 ± 0.11	nm
2	1.38 ± 0.06	0.68 ± 0.10	0.70 ± 0.07	0.97 ± 0.06	1.02 ± 0.10	49.27 ± 0.10	50.72 ± 0.10	nm
2	1.36 ± 0.05	0.58 ± 0.09	0.78 ± 0.07	0.74 ± 0.03	1.34 ± 0.03	42.64 ± 0.10	57.35 ± 0.08	nm
2	1.36 ± 0.12	0.60 ± 0.10	0.76 ± 0.12	0.78 ± 0.09	1.26 ± 0.07	44.11 ± 0.05	55.88 ± 0.05	nm
2	1.34 ± 0.06	0.65 ± 0.05	0.69 ± 0.10	0.94 ± 0.10	1.06 ± 0.12	48.50 ± 0.09	51.49 ± 0.07	nm
2	1.34 ± 0.10	0.59 ± 0.12	0.75 ± 0.12	0.78 ± 0.05	1.27 ± 0.03	44.02 ± 0.13	55.97 ± 0.09	nm

No. of Chr.	c (µm)	s (µm)	l (µm)	R_1 (s/l)	R_2 (l/s)	I_1 (s/c %)	I_2 (l/c %)	Nature of PC
2	1.30 ± 0.00	0.60 ± 0.00	0.70 ± 0.00	0.85 ± 0.00	1.16 ± 0.00	46.15 ± 0.00	53.84 ± 0.00	nm
2	1.30 ± 0.05	0.60 ± 0.11	0.70 ± 0.11	0.85 ± 0.07	1.16 ± 0.11	46.15 ± 0.09	53.84 ± 0.05	nm
2	1.26 ± 0.06	0.55 ± 0.05	0.71 ± 0.05	0.77 ± 0.08	1.29 ± 0.05	43.65 ± 0.05	56.34 ± 0.10	nm
2	1.25 ± 0.03	0.57 ± 0.08	0.68 ± 0.07	0.83 ± 0.03	1.19 ± 0.09	45.60 ± 0.03	54.40 ± 0.03	nm
2	1.24 ± 0.11	0.58 ± 0.11	0.66 ± 0.12	0.87 ± 0.11	1.13 ± 0.12	46.77 ± 0.11	53.22 ± 0.11	nm
2	1.23 ± 0.03	0.61 ± 0.05	0.62 ± 0.08	0.98 ± 0.10	1.01 ± 0.03	49.59 ± 0.09	50.40 ± 0.07	nm
2	1.21 ± 0.12	0.56 ± 0.07	0.65 ± 0.03	0.86 ± 0.12	1.16 ± 0.06	46.28 ± 0.10	53.71 ± 0.05	nm
2	1.15 ± 0.05	0.52 ± 0.10	0.63 ± 0.10	0.82 ± 0.05	1.21 ± 0.08	45.21 ± 0.03	54.78 ± 0.07	nm
2	1.12 ± 0.00	0.48 ± 0.00	0.64 ± 0.00	0.75 ± 0.00	1.33 ± 0.00	42.85 ± 0.00	57.14 ± 0.00	nm
2	1.03 ± 0.08	0.51 ± 0.10	0.52 ± 0.07	0.98 ± 0.03	1.01 ± 0.05	49.51 ± 0.06	50.48 ± 0.08	nm

Chr. – Chromosome, **c** – total length of chromosome, **s** – short arm length, **l** – long arm length, $\mathbf{R_1}$ – arm ratio 1, $\mathbf{R_2}$ – arm ratio 2, $\mathbf{I_1}$ – centromeric index 1, $\mathbf{I_2}$ – centromeric index 2, **PC** – primary constriction, * - chromosome with secondary constriction, nm – nearly median, nsm – nearly submedian

Mitotic chromosome complement of *A. hybridus* subsp. *hybridus* with 2n = 32 chromosomes

Somatic chromosome number	:	32
Karyotype formula	:	26nm + 6nsm(-)
Chromosome with secondary constriction	:	4
Range of chromosome length (RCL)	:	1.71 μm to 0.93 μm
Total chromosome length (TCL)	:	39.66 μm
Average chromosome length (ACL)	:	1.23 μm
Disparity index (DI)	:	29.54
Variation coefficient (VC)	:	18.09
TF value (%)	:	42.76
Karyotype asymmetry index (As K%)	:	57.18
Syi Index	:	75.71
Rec index	:	72.47
Intrachromosomal asymmetry index (A_1)	:	0.39
Interchromosomal asymmetry index (A_2)	:	0.18
Degree of asymmetry of karyotypes (A)	:	0.26
Asymmetry index AI	:	0.09

Table 17: Detailed karyomorphometric data of *A. hybridus* subsp. *hybridus* with 2n = 32 chromosomes

No. of Chr.	c (µm)	s (µm)	l (µm)	R_1 (s/l)	R_2 (l/s)	I_1 (s/c %)	I_2 (l/c %)	Nature of PC
2*	1.71 ± 0.00	0.46 ± 0.09	1.25 ± 0.01	0.36 ± 0.09	2.71 ± 0.02	26.9 ± 0.01	73.09 ± 0.09	nsm(-)
2*	1.70 ± 0.09	0.49 ± 0.02	1.21 ± 0.11	0.40 ± 0.01	2.46 ± 0.08	28.82 ± 0.02	71.17 ± 0.10	nsm(-)
2	1.39 ± 0.05	0.68 ± 0.12	0.71 ± 0.07	0.95 ± 0.07	1.04 ± 0.08	48.92 ± 0.10	51.07 ± 0.12	nm
2	1.38 ± 0.11	0.64 ± 0.08	0.74 ± 0.05	0.86 ± 0.09	1.15 ± 0.08	46.37 ± 0.07	53.62 ± 0.08	nm
2	1.32 ± 0.07	0.63 ± 0.01	0.69 ± 0.05	0.91 ± 0.02	1.09 ± 0.10	47.72 ± 0.11	52.27 ± 0.07	nm
2	1.29 ± 0.00	0.62 ± 0.00	0.67 ± 0.00	0.92 ± 0.00	1.08 ± 0.00	48.06 ± 0.00	51.93 ± 0.00	nm
2	1.26 ± 0.12	0.62 ± 0.11	0.64 ± 0.12	0.96 ± 0.10	1.03 ± 0.10	49.20 ± 0.12	50.79 ± 0.10	nm
2	1.19 ± 0.12	0.55 ± 0.11	0.64 ± 0.15	0.85 ± 0.09	1.16 ± 0.02	46.21 ± 0.10	53.78 ± 0.09	nm
2	1.15 ± 0.11	0.55 ± 0.12	0.60 ± 0.02	0.91 ± 0.10	1.09 ± 0.10	47.82 ± 0.05	52.17 ± 0.10	nm
2	1.13 ± 0.07	0.42 ± 0.08	0.71 ± 0.05	0.59 ± 0.09	1.69 ± 0.05	37.16 ± 0.10	62.83 ± 0.11	nsm(-)
2	1.12 ± 0.10	0.53 ± 0.01	0.59 ± 0.11	0.89 ± 0.12	1.11 ± 0.11	47.32 ± 0.08	52.67 ± 0.10	nm
2	1.11 ± 0.11	0.51 ± 0.09	0.59 ± 0.07	0.86 ± 0.07	1.15 ± 0.05	45.94 ± 0.12	53.15 ± 0.12	nm
2	1.07 ± 0.09	0.51 ± 0.10	0.56 ± 0.02	0.91 ± 0.05	1.09 ± 0.11	47.66 ± 0.10	52.33 ± 0.05	nm
2	1.05 ± 0.00	0.41 ± 0.00	0.64 ± 0.00	0.64 ± 0.00	1.56 ± 0.00	39.04 ± 0.00	60.95 ± 0.00	nm
2	1.03 ± 0.01	0.45 ± 0.08	0.58 ± 0.11	0.77 ± 0.02	1.28 ± 0.08	43.68 ± 0.10	56.31 ± 0.10	nm
2	0.93 ± 0.10	0.41 ± 0.10	0.52 ± 0.05	0.78 ± 0.10	1.26 ± 0.10	44.08 ± 0.05	55.91 ± 0.12	nm

Chr. – Chromosome, **c** – total length of chromosome, **s** – short arm length, **l** – long arm length, $\mathbf{R_1}$ – arm ratio 1, $\mathbf{R_2}$ – arm ratio 2, $\mathbf{I_1}$ – centromeric index 1, $\mathbf{I_2}$ – centromeric index 2, **PC** – primary constriction, * - chromosome with secondary constriction, nm – nearly median, nsm – nearly submedian

Mitotic chromosome complement of *A. hybridus* subsp. *cruentus* with 2n = 34 chromosomes

Somatic chromosome number	:	34
Karyotype formula	:	30nm + 4nsm (-)
Chromosome with secondary constriction	:	4
Range of chromosome length (RCL)	:	1.93 μm to 0.77 μm
Total chromosome length (TCL)	:	44.64 μm
Average chromosome length (ACL)	:	1.31 μm
Disparity index (DI)	:	42.96
Variation coefficient (VC)	:	22.96
TF value (%)	:	42.00
Karyotype asymmetry index (As K%)	:	58
Syi Index	:	72.36
Rec index	:	68.02
Intrachromosomal asymmetry index (A_1)	:	0.36
Interchromosomal asymmetry index (A_2)	:	0.22
Degree of asymmetry of karyotypes (A)	:	0.15
Asymmetry index AI	:	0.16

Table 18: Detailed karyomorphometric data of *A. hybridus* subsp. *cruentus* with 2n = 34 chromosomes

No. of Chr.	c (µm)	s (µm)	l (µm)	R_1 (s/l)	R_2 (l/s)	I_1 (s/c %)	I_2 (l/c %)	Nature of PC
2*	1.93 ± 0.12	0.77 ± 0.15	1.16 ± 0.11	0.66 ± 0.12	1.50 ± 0.12	39.89 ± 0.11	60.10 ± 0.13	nm
2*	1.85 ± 0.11	0.72 ± 0.04	1.13 ± 0.02	0.63 ± 0.10	1.56 ± 0.04	38.91 ± 0.05	61.08 ± 0.10	nm
2	1.65 ± 0.05	0.72 ± 0.03	0.93 ± 0.05	0.77 ± 0.11	1.29 ± 0.02	43.63 ± 0.10	56.36 ± 0.10	nm
2	1.50 ± 0.10	0.54 ± 0.07	0.96 ± 0.06	0.56 ± 0.02	1.77 ± 0.05	36.00 ± 0.07	64.00 ± 0.02	nsm(-)
2	1.44 ± 0.00	0.60 ± 0.00	0.84 ± 0.00	0.71 ± 0.00	1.40 ± 0.00	41.66 ± 0.00	58.33 ± 0.00	nm
2	1.40 ± 0.21	0.61 ± 0.09	0.79 ± 0.07	0.77 ± 0.07	1.29 ± 0.06	43.57 ± 0.05	56.42 ± 0.05	nm
2	1.36 ± 0.10	0.60 ± 0.05	0.76 ± 0.02	0.78 ± 0.11	1.26 ± 0.04	44.11 ± 0.06	55.88 ± 0.10	nm
2	1.33 ± 0.06	0.64 ± 0.04	0.69 ± 0.10	0.92 ± 0.03	1.07 ± 0.03	48.12 ± 0.05	51.87 ± 0.07	nm
2	1.26 ± 0.15	0.51 ± 0.11	0.75 ± 0.18	0.68 ± 0.15	1.47 ± 0.19	40.47 ± 0.18	59.52 ± 0.17	nm
2	1.21 ± 0.11	0.52 ± 0.03	0.69 ± 0.07	0.75 ± 0.02	1.32 ± 0.11	42.97 ± 0.10	57.02 ± 0.02	nm
2	1.19 ± 0.10	0.52 ± 0.05	0.67 ± 0.10	0.77 ± 0.05	1.28 ± 0.07	43.69 ± 0.05	56.30 ± 0.06	nm
2	1.15 ± 0.00	0.49 ± 0.00	0.66 ± 0.00	0.74 ± 0.00	1.34 ± 0.00	42.60 ± 0.00	57.39 ± 0.00	nm
2	1.13 ± 0.00	0.38 ± 0.00	0.75 ± 0.00	0.50 ± 0.00	1.97 ± 0.00	33.62 ± 0.00	66.37 ± 0.00	nsm(-)
2	1.12 ± 0.10	0.45 ± 0.02	0.67 ± 0.06	0.67 ± 0.04	1.48 ± 0.11	40.17 ± 0.03	59.82 ± 0.06	nm
2	1.07 ± 0.03	0.49 ± 0.10	0.58 ± 0.11	0.84 ± 0.03	1.18 ± 0.05	45.79 ± 0.10	54.20 ± 0.04	nm
2	0.96 ± 0.07	0.45 ± 0.10	0.51 ± 0.04	0.88 ± 0.11	1.13 ± 0.11	46.87 ± 0.04	53.12 ± 0.10	nm
2	0.77 ± 0.06	0.36 ± 0.03	0.41 ± 0.02	0.87 ± 0.07	1.13 ± 0.02	46.75 ± 0.10	53.24 ± 0.02	nm

Chr. – Chromosome, **c** – total length of chromosome, **s** – short arm length, **l** – long arm length, $\mathbf{R_1}$ – arm ratio 1, $\mathbf{R_2}$ – arm ratio 2, $\mathbf{I_1}$ – centromeric index 1, $\mathbf{I_2}$ – centromeric index 2, **PC** – primary constriction, * - chromosome with secondary constriction, nm – nearly median, nsm – nearly submedian

Mitotic chromosome complement of *A. spinosus* with 2n = 34 chromosomes

Somatic chromosome number	:	34
Karyotype formula	:	20nm + 4nsm(-) + 2nsm(+) + 2nst(-) + 6T
Chromosome with secondary constriction	:	4
Range of chromosome length (RCL)	:	2.43 µm to 1.34 µm
Total chromosome length (TCL)	:	57.68 µm
Average chromosome length (ACL)	:	1.69 µm
Disparity index (DI)	:	28.91
Variation coefficient (VC)	:	17.37
TF value (%)	:	34.18
Karyotype asymmetry index (As K%)	:	65.81
Syi Index	:	52.25
Rec index	:	69.81
Intrachromosomal asymmetry index (A_1)	:	0.41
Interchromosomal asymmetry index (A_2)	:	0.17
Degree of asymmetry of karyotypes (A)	:	0.33
Asymmetry index AI	:	0.14

Table 19: Detailed karyomorphometric data of *A. spinosus* with 2n = 34 chromosomes

No. of Chr.	c (µm)	s (µm)	l (µm)	R_1 (s/l)	R_2 (l/s)	I_1 (s/c %)	I_2 (l/c %)	Nature of PC
2*	2.43 ± 0.11	0.89 ± 0.13	1.54 ± 0.12	0.57 ± 0.09	1.73 ± 0.15	36.62 ± 0.09	63.37 ± 0.15	nsm(-)
2*	2.16 ± 0.06	0.76 ± 0.03	1.40 ± 0.01	0.54 ± 0.08	1.84 ± 0.10	35.18 ± 0.05	64.81 ± 0.06	nsm(-)
2	2.09 ± 0.11	0.95 ± 0.10	1.14 ± 0.15	0.83 ± 0.10	1.20 ± 0.11	45.45 ± 0.01	54.54 ± 0.10	nm
2	1.83 ± 0.00	0.87 ± 0.00	0.96 ± 0.00	0.90 ± 0.00	1.10 ± 0.00	47.54 ± 0.00	52.45 ± 0.00	nm
2	1.80 ± 0.10	0.73 ± 0.01	1.07 ± 0.09	0.68 ± 0.06	1.46 ± 0.09	40.55 ± 0.17	59.44 ± 0.01	nm
2	1.78 ± 0.09	0.83 ± 0.10	0.95 ± 0.15	0.87 ± 0.10	1.14 ± 0.11	46.62 ± 0.01	53.37 ± 0.11	nm
2	1.72 ± 0.06	0.26 ± 0.06	1.46 ± 0.01	0.17 ± 0.09	5.61 ± 0.01	15.11 ± 0.17	84.88 ± 0.10	nst(-)
2	1.60 ± 0.13	0.78 ± 0.17	0.82 ± 0.01	0.95 ± 0.13	1.05 ± 0.15	48.75 ± 0.13	51.25 ± 0.01	nm
2	1.59 ± 0.10	0.74 ± 0.01	0.85 ± 0.06	0.87 ± 0.01	1.14 ± 0.01	46.54 ± 0.13	53.45 ± 0.11	nm
2	1.55 ± 0.00	0.00 ± 0.00	1.55 ± 0.00	0.00 ± 0.00	∞	0.00 ± 0.00	100 ± 0.00	T
2	1.53 ± 0.11	0.29 ± 0.17	1.24 ± 0.15	0.23 ± 0.01	4.27 ± 0.09	18.95 ± 0.01	81.04 ± 0.06	nsm(+)
2	1.53 ± 0.10	0.70 ± 0.15	0.83 ± 0.10	0.84 ± 0.01	1.18 ± 0.13	45.75 ± 0.11	54.24 ± 0.01	nm
2	1.53 ± 0.00	0.00 ± 0.00	1.53 ± 0.00	0.00 ± 0.00	∞	0.00 ± 0.00	100 ± 0.00	T
2	1.50 ± 0.15	0.74 ± 0.10	0.76 ± 0.01	0.97 ± 0.17	1.02 ± 0.06	49.33 ± 0.01	50.66 ± 0.17	nm
2	1.47 ± 0.10	0.71 ± 0.09	0.76 ±0.13	0.93 ± 0.15	1.07 ± 0.01	48.29 ± 0.01	51.7 ± 0.09	nm
2	1.39 ± 0.10	0.61 ± 0.06	0.78 ± 0.10	0.78 ± 0.01	1.27 ± 0.13	43.88 ± 0.15	56.11 ± 0.06	nm
2	1.34 ± 0.00	0.00 ± 0.00	1.34 ± 0.00	0.00 ± 0.00	∞	0.00 ± 0.00	100 ± 0.00	T

Chr. – Chromosome, **c** – total length of chromosome, **s** – short arm length, **l** – long arm length, $\mathbf{R_1}$ – arm ratio 1, $\mathbf{R_2}$ – arm ratio 2, $\mathbf{I_1}$ – centromeric index 1, $\mathbf{I_2}$ – centromeric index 2, **PC** – primary constriction, * - chromosome with secondary constriction, nm – nearly median, nsm – nearly submedian, nst – nearly subterminal, T – terminal

Mitotic chromosome complement of *A. tricolor* with 2n = 34 chromosomes

Somatic chromosome number	:	34
Karyotype formula	:	8nm +18nsm(-) + 2nsm(+) + 6T
Chromosome with secondary constriction	:	4
Range of chromosome length (RCL)	:	2.34 µm to 0.99 µm
Total chromosome length (TCL)	:	47.32 µm
Average chromosome length (ACL)	:	1.39 µm
Disparity index (DI)	:	40.54
Variation coefficient (VC)	:	27.31
TF value (%)	:	29.33
Karyotype asymmetry index (As K%)	:	70.66
Syi Index	:	40.81
Rec index	:	59.47
Intrachromosomal asymmetry index (A_1)	:	0.53
Interchromosomal asymmetry index (A_2)	:	0.27
Degree of asymmetry of karyotypes (A)	:	0.40
Asymmetry index AI	:	0.35

Table 20: Detailed karyomorphometric data of *A. tricolor* with 2n = 34 chromosomes

No. of Chr.	c (µm)	s (µm)	l (µm)	R_1 (s/l)	R_2 (l/s)	I_1 (s/c %)	I_2 (l/c %)	Nature of PC
2*	2.34 ± 0.05	0.53 ± 0.04	1.81 ± 0.02	0.29 ± 0.04	3.41 ± 0.04	22.64 ± 0.04	77.35 ± 0.06	nsm(+)
2*	1.88 ± 0.08	0.48 ± 0.09	1.40 ± 0.06	0.34 ± 0.06	2.91 ± 0.11	25.53 ± 0.12	74.46 ± 0.10	nsm(-)
2	1.87 ± 0.11	0.54 ± 0.04	1.33 ± 0.06	0.40 ± 0.05	2.46 ± 0.05	28.87 ± 0.04	71.12 ± 0.06	nsm(-)
2	1.77 ± 0.09	0.76 ± 0.08	1.01 ± 0.08	0.75 ± 0.10	1.32 ± 0.10	42.93 ± 0.08	57.06 ± 0.11	nm
2	1.62 ± 0.09	0.58 ± 0.13	1.04 ± 0.06	0.55 ± 0.08	1.79 ± 0.11	35.80 ± 0.10	64.19 ± 0.04	nsm(-)
2	1.46 ± 0.13	0.49 ± 0.04	0.97 ± 0.08	0.50 ± 0.14	1.97 ± 0.10	33.56 ± 0.09	66.43 ± 0.11	nsm(-)
2	1.38 ± 0.11	0.40 ± 0.06	0.98 ± 0.05	0.40 ± 0.11	2.45 ± 0.06	28.98 ± 0.10	71.01 ± 0.05	nsm(-)
2	1.29 ± 0.05	0.48 ± 0.08	0.81 ± 0.11	0.59 ± 0.13	1.68 ± 0.11	37.20 ± 0.08	62.79 ± 0.13	nsm(-)
2	1.23 ± 0.09	0.59 ± 0.10	0.64 ± 0.11	0.92 ± 0.05	1.08 ± 0.09	47.96 ± 0.10	52.03 ± 0.08	nm
2	1.20 ± 0.01	0.00 ± 0.00	1.20 ± 0.00	0.00 ± 0.00	∞	0.00 ± 0.00	100 ± 0.00	T
2	1.16 ± 0.08	0.35 ± 0.08	0.81 ± 0.13	0.43 ± 0.08	2.31 ± 0.13	30.17 ± 0.10	69.82 ± 0.11	nsm(-)
2	1.14 ± 0.10	0.36 ± 0.13	0.78 ± 0.09	0.46 ± 0.10	2.16 ± 0.08	31.57 ± 0.06	68.42 ± 0.09	nsm(-)
2	1.13 ± 0.04	0.39 ± 0.09	0.74 ± 0.04	0.52 ± 0.10	1.89 ± 0.11	34.51 ± 0.11	65.48 ± 0.05	nsm(-)
2	1.10 ± 0.11	0.50 ± 0.10	0.60 ± 0.11	0.83 ± 0.06	1.20 ± 0.10	45.45 ± 0.05	54.54 ± 0.10	nm
2	1.07 ± 0.12	0.49 ± 0.11	0.58 ± 0.10	0.84 ± 0.10	1.18 ± 0.08	45.79 ± 0.10	54.20 ± 0.06	nm
2	1.03 ± 0.00	0.00 ± 0.00	1.03 ± 0.00	0.00 ± 0.00	∞	0.00 ± 0.00	100 ± 0.00	T
2	0.99 ± 0.00	0.00 ± 0.00	0.99 ± 0.00	0.00 ± 0.00	∞	0.00 ± 0.00	100 ± 0.00	T

Chr. – Chromosome, **c** – total length of chromosome, **s** – short arm length, **l** – long arm length, $\mathbf{R_1}$ – arm ratio 1, $\mathbf{R_2}$ – arm ratio 2, $\mathbf{I_1}$ – centromeric index 1, $\mathbf{I_2}$ – centromeric index 2, **PC** – primary constriction, * - chromosome with secondary constriction, nm – nearly median, nsm – nearly submedian, T – terminal

Mitotic chromosome complement of *A. viridis* with 2n = 32 chromosomes

Somatic chromosome number	:	32
Karyotype formula	:	12nm +18nsm(-) + 2T
Chromosome with secondary constriction	:	4
Range of chromosome length (RCL)	:	2.22 μm to 1.12 μm
Total chromosome length (TCL)	:	47.84 μm
Average chromosome length (ACL)	:	1.49 μm
Disparity index (DI)	:	32.93
Variation coefficient (VC)	:	21.51
TF value (%)	:	36.12
Karyotype asymmetry index (As K%)	:	63.83
Syi Index	:	56.84
Rec index	:	67.34
Intrachromosomal asymmetry index (A_1)	:	0.39
Interchromosomal asymmetry index (A_2)	:	0.21
Degree of asymmetry of karyotypes (A)	:	0.26
Asymmetry index AI	:	0.19

Table 21: Detailed karyomorphometric data of *A. viridis* with 2n = 32 chromosomes

No. of Chr.	c (µm)	s (µm)	l (µm)	R_1 (s/l)	R_2 (l/s)	I_1 (s/c %)	I_2 (l/c %)	Nature of PC
2*	2.22 ± 0.01	0.62 ± 0.04	1.60 ± 0.01	0.38 ± 0.04	2.58 ± 0.02	27.92 ± 0.01	72.07 ± 0.04	nsm(-)
2*	2.06 ± 0.12	0.57 ± 0.00	1.49 ± 0.11	0.38 ± 0.15	2.61 ± 0.11	27.66 ± 0.16	72.33 ± 0.15	nsm(-)
2	1.81 ± 0.03	0.89 ± 0.11	0.92 ± 0.06	0.96 ± 0.14	1.03 ± 0.11	49.17 ± 0.10	50.82 ± 0.03	nm
2	1.62 ± 0.05	0.77 ± 0.05	0.85 ± 0.11	0.90 ± 0.06	1.10 ± 0.13	47.53 ± 0.14	52.46 ± 0.06	nm
2	1.60 ± 0.15	0.54 ± 0.11	1.06 ± 0.13	0.50 ± 0.15	1.96 ± 0.06	33.75 ± 0.10	66.25 ± 0.11	nsm(-)
2	1.58 ± 0.06	0.59 ± 0.06	0.99 ± 0.14	0.59 ± 0.10	1.67 ± 0.03	37.34 ± 0.05	62.65 ± 0.10	nsm(-)
2	1.55 ± 0.01	0.50 ± 0.06	1.04 ± 0.01	0.48 ± 0.13	2.08 ± 0.10	32.25 ± 0.01	67.09 ± 0.05	nsm(-)
2	1.43 ± 0.03	0.68 ± 0.11	0.75 ± 0.06	0.90 ± 0.11	1.10 ± 0.05	47.55 ± 0.06	52.44 ± 0.10	nm
2	1.42 ± 0.13	0.45 ± 0.13	0.97 ± 0.05	0.46 ± 0.03	2.15 ± 0.10	31.69 ± 0.11	68.30 ± 0.03	nsm(-)
2	1.39 ± 0.09	0.56 ± 0.15	0.83 ± 0.13	0.67 ± 0.10	1.48 ± 0.15	40.28 ± 0.10	59.71 ± 0.05	nm
2	1.34 ± 0.06	0.46 ± 0.14	0.88 ± 0.05	0.52 ± 0.11	1.91 ± 0.13	34.32 ± 0.01	65.67 ± 0.14	nsm(-)
2	1.29 ± 0.01	0.46 ± 0.03	0.83 ± 0.01	0.55 ± 0.06	1.80 ± 0.05	35.65 ± 0.10	64.34 ± 0.10	nsm(-)
2	1.20 ± 0.13	0.57 ± 0.11	0.63 ± 0.10	0.90 ± 0.02	1.10 ± 0.01	47.50 ± 0.03	52.50 ± 0.11	nm
2	1.17 ± 0.11	0.43 ± 0.14	0.74 ± 0.03	0.58 ± 0.11	1.72 ± 0.06	36.75 ± 0.15	63.24 ± 0.10	nsm(-)
2	1.12 ± 0.10	0.55 ± 0.09	0.57 ± 0.10	0.96 ± 0.01	1.03 ± 0.11	49.10 ± 0.13	50.89 ± 0.06	nm
2	1.12 ± 0.00	0.00 ± 0.00	1.12 ± 0.00	0.00 ± 0.00	∞	0.00 ± 0.00	100 ± 0.00	T

Chr. – Chromosome, **c** – total length of chromosome, **s** – short arm length, **l** – long arm length, $\mathbf{R_1}$ – arm ratio 1, $\mathbf{R_2}$ – arm ratio 2, $\mathbf{I_1}$ – centromeric index 1, $\mathbf{I_2}$ – centromeric index 2, **PC** – primary constriction, * - chromosome with secondary constriction, nm – nearly median, nsm – nearly submedian, T – terminal

Table 22: Summary of karyomorphometric features of selected species of *Amaranthus*

Karyotype	No. of csc.	TCL (µm)	ACL (µm)	RCL (µm)	DI	VC	TF%	As K%	Syi	Rec	A_1	A_2	A	AI	Karyotype formula
A. blitum (2n = 34)	4	54.08	1.59	2.10 - 1.11	30.84	17.41	43.34	56.65	75.55	75.74	0.21	0.17	0.12	0.11	2M + 28nm + 4nsm(-)
A. caudatus (2n = 32)	4	38.86	1.21	1.76 – 0.86	34.35	21.65	44.10	56.00	78.00	69.00	0.21	0.21	0.11	0.12	32nm
A. dubius (2n = 64)	8	94.12	1.47	2.24 - 1.03	37	20.37	42.77	57.22	73.80	65.65	0.20	0.20	0.12	0.14	56nm + 8nsm(-)
A. hybridus subsp. *hybridus* (2n = 32)	4	39.66	1.23	1.71 – 0.93	29.54	18.09	42.76	57.18	75.71	72.47	0.39	0.18	0.26	0.09	26nm + 6nsm(-)
A. hybridus subsp. *cruentus* (2n = 34)	4	44.64	1.31	1.93 – 0.77	42.96	22.96	42.00	58.00	72.36	68.02	0.36	0.22	0.15	0.16	30nm + 4nsm(-)
A. spinosus (2n = 34)	4	57.68	1.69	2.43 – 1.34	28.91	17.37	34.18	65.81	52.25	69.81	0.41	0.17	0.33	0.14	20nm + 4nsm(-) + 2nsm(+) + 2nst(-) + 6T
A. tricolor (2n = 34)	4	47.32	1.39	2.34 – 0.99	40.54	27.31	29.33	70.66	40.81	59.47	0.53	0.27	0.40	0.35	8nm + 18nsm(-) + 2nsm(+) + 6T
A. viridis (2n = 32)	4	47.84	1.49	2.22 – 1.12	32.93	21.51	36.12	63.83	56.84	67.34	0.39	0.21	0.26	0.19	12nm + 18nsm(-) + 2T

csc. Chromosome with secondary constriction

3. MICROMORPHOLOGICAL CHARACTERIZATION

The presence and absence of different micromorphological characters were observed from the SEM studies and analysis of pollen grains, seed capsule and seed coat of different *Amaranthus* species (Tables 23-25; Plates 27-34). A comparative micromorphological analysis of different *Amaranthus* species was yet to be documented, thus the venture was undertaken for observing possible micro features under SEM. The observed micro features can be used to discriminate the species studied in the genus *Amaranthus*.

MICROMORPHOLOGICAL STUDIES OF POLLEN GRAINS

A comparative micromorphological study of *Amaranthus* pollen grains using acetolysis (Plate 27) and SEM (Table 23; Plates 28, 29) analysis revealed similarities and variations in both qualitative and quantitative parameters investigated. The result showed that all the studied species had poly pantoporate pollen, with many pores distributed on the surface. The number of pores observed in the visible aperture of each species was as follows: *A. blitum* (12), *A. caudatus* (20), *A. dubius* (32), A. *hybridus* subsp. *hybridus* (17), *hybridus* subsp. *cruentus* (22), *A. spinosus* (27), *A. tricolor* (22) and *A. viridis* (18). The aperture membrane ornamentation observed was scabrate for all the species, while its aggregation differed between species. Dense aggregations were observed in *A. dubius* (Plate 28 c1 & c2) and *A. spinosus* (Plate 29 b1& b2), while sparse aggregation was seen in all the other species, especially in *A. viridis* (Plate 29 d1 & d2). Various quantitative characters *viz*., polar axis length (P), equatorial length (E), P/E ratio and pore dimensions observed were represented in the Table 23. Pollen grains are small with a mean size range of 13.87 to 20.67 µm. Polar axis length (13.87 µm) and equatorial length (12.60 µm) was shortest in *A. blitum* and largest polar (20.67 µm) and equatorial (20.42 µm) length was observed in *A. dubius*.

Table 23. Pollen micromorphological characters of *Amaranthus* species

Species	Polar length (P)	Equatorial length (E)	P/E	Pollen shape	Pore diameter (µm)	Interpore distance (µm)	Pollen type	Surface sculpturing
A. blitum	13.87 ± 1.05	12.60 ± 0.05	1.10	Prolate-spheroidal	1.23 - 1.49	2. 62 - 2.75	Poly pantoporate	Scabrate
A. caudatus	20.50 ± 0.91	19.96 ± 0.15	1.02	Prolate-spheroidal	1.14 - 1.71	3.10 - 3.67	Poly pantoporate	Scabrate
A. dubius	20.67 ± 1.70	20.42 ± 1.50	1.01	Prolate-spheroidal	0.92 - 1.13	2. 28 - 2.73	Poly pantoporate	Scabrate
A. hybridus subsp. *hybridus*	19.33 ± 2.10	18.56 ± 1.80	1.04	Prolate-spheroidal	1.48 - 1.64	2.92 - 3.99	Poly pantoporate	Scabrate
A. hybridus subsp. *cruentus*	18.09 ± 0.50	16.75 ± 2.10	1.08	Prolate-spheroidal	0.96 - 1.20	2.65 - 3.06	Poly pantoporate	Scabrate
A. spinosus	18.17 ± 0.14	17.42 ± 2.00	1.04	Prolate-spheroidal	0.67 - 1.17	1.85 - 2.95	Poly pantoporate	Scabrate
A. tricolor	20.17 ± 0.60	19.82 ± 0.08	1.01	Prolate-spheroidal	0.98 - 1.44	1.77 - 2.77	Poly pantoporate	Scabrate
A. viridis	17.89 ± 0.72	17.31 ± 0.18	1.03	Prolate-spheroidal	1.13 - 1.82	2.74 - 2.98	Poly pantoporate	Scabrate

The P/E ratio obtained ranges from 1.01 – 1.10, based on the ratio the pollen shape in all the species was defined as prolate-spheroidal with a circular polar view. Pore dimensions varied between the studied species. Largest pore diameter was observed in *A. viridis* (1.82 µm) and smallest in *A. spinosus* (0.67 µm). The interpore distance was more in *A. hybridus* subsp. *hybridus* ranges from 2.92 to 3.99 µm and less in *A. tricolor*, where it ranges from 1.77 to 2.77 µm (Table 23). The pore ornamentation and aperture sculpturing in *A. viridis* showed some variations from other species studied.

MICROMORPHOLOGICAL STUDIES OF SEED CAPSULE

The micromorphological features of seed capsule of different *Amaranthus* species showed slight variations in size and capsule ornamentations (Table 24; Plates 30, 31). The capsule wall of *A. blitum* (Plate 30 a1-a3) and *A. viridis* (Plate 31 d1-d3) was found to be indehiscent and rupture irregularly with a size (length × width) of 2.19 × 1.23 and 2.53 × 1.26 mm respectively. The capsule ornamentation in *A. blitum* was uniform throughout the wall and showed entirely different pattern from other species. The capsule wall of *A. viridis* showed very strong rugose structure throughout. The capsule wall of all the other species of *Amaranthus* was found to be dehiscent with a prominent line of dehiscence. The capsule size of *A. caudatus*, *A. dubius*, *A. hybridus* subsp. *hybridus*, *A. hybridus* subsp. *cruentus*, *A. spinosus* and *A. tricolor* were 3.44 × 1.08, 2.99 × 0.98, 3.09 × 1.08, 3.74 × 1.02, 2.95 × 1.03 and 3.98 × 1.66 mm (Table 24) respectively and showed rugose or wrinkled pattern of wall ornamentations. The rugose pattern of ornamentation was uniform throughout the capsule of *A. caudatus* (Plate 30 b1-b3), *A. dubius* (Plate 30 c1-c3) and *A. hybridus* subsp. *hybridus* (Plate 30 d1-d3), whereas *A. hybridus* subsp. *cruentus* (Plate 31 a1-a3), *A. spinosus* (Plate 31 b1-b3) and *A. tricolor* (Plate 31 c1-c3) showed strong rugose above the line of dehiscence than the lower region of the capsule.

Table 24. Seed capsule micromorphological features of *Amaranthus* species

Species	Length (mm)	Width (mm)	Capsule rupturing
A. blitum	2.19 ± 0.05	1.23 ± 0.07	Indehiscent
A. caudatus	3.44 ± 0.07	1.08 ± 0.10	Dehiscent
A. dubius	2.99 ± 0.02	0.98 ± 0.18	Dehiscent
A. hybridus subsp. *hybridus*	3.09 ± 0.12	1.08 ± 0.20	Dehiscent
A. hybridus subsp. *cruentus*	3.74 ± 0.15	1.02 ± 0.22	Dehiscent
A. spinosus	2.95 ± 0.09	1.03 ± 0.10	Dehiscent
A. tricolor	3.98 ± 0.01	1.66 ± 0.11	Dehiscent
A. viridis	2.53 ± 0.07	1.26 ± 0.01	Indehiscent

MICROMORPHOLOGICAL STUDIES OF SEED COAT

Seed surface micromorphological features observed by SEM analysis revealed variations among different *Amaranthus* species studied. Variations are observed in the seed shape, size, weight, colour and spermoderm/testa ornamentations (Table 25; Plates 32-34). Seeds of *A. blitum* were found to be orbicular in shape with glossy surface and brownish black coloured. The length and width of the seed was 0.91 ± 0.05 and 0.79 ± 0.00 respectively. The length: width ratio was 1.15 ± 0.00. The weight in grams of 1000 seeds was 0.14 ± 0.05. Seeds showed prominent reticulation with polygonal and rectangular elevated areas on the spermoderm/testa (Plate 32 a; 33 a1 & a2).

Seeds of *A. caudatus* were elongated lenticular in shape with glossy surface and brownish black coloured. The length and width of the seed was 1.47 ± 0.01 and 1.01 ± 0.02 respectively. The length: width ratio was 1.45 ± 0.01. The weight in grams of 1000 seeds was 0.34 ± 0.12. Epidermal cells ornamentation shows prominent reticulation forming irregularly shaped areas on the spermoderm (Plate 32 b; 33 b1 & b2).

Seeds of *A. dubius* were lenticular in shape with glossy surface and black coloured. The length and width of the seed was 0.82 ± 0.00 and 0.79 ± 0.02 respectively. The length: width ratio was 1.03 ± 0.05. The weight in

grams of 1000 seeds was 0.28 ± 0.15. Seeds showed prominent reticulation forming hexagonal and polygonal areas on the spermoderm (Plate 32 c; 33 c1 & c2).

Seeds of *A. hybridus* subsp. *hybridus* were subglobose in shape with glossy surface and black coloured. The length and width of the seed was 0.98 ± 0.02 and 0.96 ± 0.02 respectively. The length: width ratio was 1.02 ± 0.07. The weight in grams of 1000 seeds was 0.46 ± 0.11. Seeds showed cross linked undulated thread like epidermal cells ornamentation forming polygonal meshes throughout the spermoderm (Plate 32 d; 33 d1 & d2).

Seeds of *A. hybridus* subsp. *cruentus* were elliptic-lenticular in shape with glossy surface and reddish brown coloured. The length and width of the seed was 0.84 ± 0.02 and 0.81 ± 0.08 respectively. The length: width ratio was 1.03 ± 0.00. The weight in grams of 1000 seeds was 0.22 ± 0.17. Seeds showed well defined reticulation forming rectangular and polygonal elevated areas mainly in the flange region and ill defined ornamentation in the central region of the spermoderm (Plate 32 e; 34 a1 & a2).

Seeds of *A. spinosus* were subglobose in shape with glossy surface and black coloured. The length and width of the seed was 0.92 ± 0.01 and 0.88 ± 0.06 respectively. The length: width ratio was 1.04 ± 0.04. The weight in grams of 1000 seeds was 0.16 ± 0.10. Seeds showed prominent reticulation forming hexagonal and polygonal cavities throughout the spermoderm (Plate 32 f; 34 b1 & b2).

Seeds of *A. tricolor* were elongated lenticular in shape with glossy surface and black coloured. The length and width of the seed was 1.38 ± 0.06 and 1.12 ± 0.00 respectively. The length: width ratio was 1.23 ± 0.05. The weight in grams of 1000 seeds was 0.82 ± 0.21. Seeds showed prominent reticulation forming hexagonal and polygonal areas throughout the spermoderm (Plate 32 g; 34 c1 & c2).

Table 25. Seed coat micromorphological characters of *Amaranthus* species

Species	Seed shape	Seed colour	Seed length (mm)	Seed width (mm)	Seed length: width (ratio)	1000 seeds weight (g)	Seed surface
A. blitum	Orbicular	Brownish black	0.91 ± 0.05	0.79 ± 0.00	1.15 ± 0.00	0.14 ± 0.05	Glossy
A. caudatus	Elongated lenticular	Brownish black	1.47 ± 0.01	1.01 ± 0.02	1.45 ± 0.01	0.34 ± 0.12	Glossy
A. dubius	Lenticular	Black	0.82 ± 0.00	0.79 ± 0.02	1.03 ± 0.05	0.28 ± 0.15	Glossy
A. hybridus subsp. *hybridus*	Subglobose	Black	0.98 ± 0.02	0.96 ± 0.02	1.02 ± 0.07	0.46 ± 0.11	Glossy
A. hybridus subsp. *cruentus*	Elliptic-lenticular	Reddish brown	0.84 ± 0.02	0.81 ± 0.08	1.03 ± 0.00	0.22 ± 0.17	Glossy
A. spinosus	Subglobose	Black	0.92 ± 0.01	0.88 ± 0.06	1.04 ± 0.04	0.16 ± 0.10	Glossy
A. tricolor	Elongated lenticular	Black	1.38 ± 0.06	1.12 ± 0.00	1.23 ± 0.05	0.82 ± 0.21	Glossy
A. viridis	Globose	Deep black	0.82 ± 0.04	0.85 ± 0.05	0.96 ± 0.01	0.28 ± 0.18	Rough

Seeds of *A. viridis* were globose in shape with rough surface and deep black coloured. The length and width of the seed was 0.82 ± 0.04 and 0.85 ± 0.05 respectively. The length: width ratio was 0.96 ± 0.01. The weight in grams of 1000 seeds was 0.28 ± 0.18. Seed surface ornamentation of *A. viridis* was entirely different from other species. Here the reticulate pattern of spermodem ornamentation is absent; instead the entire spermoderm is rugulate forming an irregular muriform arrangement (Plate 32 h; 34 d1 & d2).

4. PHYTOCHEMICAL CHARACTERIZATION

The phytochemical constituents present in different *Amaranthus* species studied were screened by using both qualitative and quantitative methodologies. The classes of compounds present were identified by preliminary screening and quantification was done for major classes. The volatile and non-volatile components present in the methanolic plant extract was screened by using GC/MS and HR-LC/MS techniques. A comparative phytochemical analysis was done to find out the chemical affinity among different *Amaranthus* species.

QUALITATIVE PHYTOCHEMICAL SCREENING

The result of the qualitative screening was based on colour formation and precipitation reactions, which indicated the presence and absence of various classes of phytochemical constituents. The qualitative tests revealed the presence of phenols, carbohydrates, proteins, flavonoids, alkaloids, terpenoids, triterpenoids, saponin, steroids, carotenoids, tannins, phlobatannins and cardiac glycosides. Quinones are absent in all the species except *A. viridis*. Anthraquinones are observed in *A. spinosus* alone, whereas *A. blitum* was devoid of saponins and phlobatannins. Phlobatannin was absent in *A. tricolor* (Table 26).

Table 26. Classes of phytochemical compounds observed in selected species of *Amaranthus* by preliminary screening

Sl No.	Class of compounds	Tests	AB	AC	AD	AH	AR	AS	AT	AV
1	Carbohydrates	Molisch's test	+	+	+	+	+	+	+	+
		Benedict's test	+	-	-	+	-	-	-	-
2	Proteins	Millen's test	+	+	+	+	+	+	+	+
3	Phenols	Ferric chloride test	+	+	+	+	+	+	+	+
		Phosphomolybdic acid test	+	+	+	+	+	+	+	+
4	Flavonoids	Alkaline reagent test	+	-	+	+	-	+	+	-
		Lead acetate solution test	+	+	+	+	+	+	+	+
5	Alkaloids	Mayer's test	+	+	+	+	+	+	+	+
		Wagner's test	+	+	+	+	+	+	+	+
6	Terpenoids	Salkowski's test	+	+	+	+	+	+	+	+
7	Triterpenoids	Extract + acetic anhydride + Con. H_2SO_4	+	+	+	+	+	+	+	+
8	Saponin	Foam test	-	+	+	+	+	+	+	+
9	Steroids	Liebermann Burchard test	+	+	+	+	+	+	+	+
10	Carotenoids	Chloroform extract + 85% H_2SO_4	+	+	+	+	+	+	+	+
11	Tannins	Braymer's test	+	+	+	+	+	+	+	+
12	Phlobatannins	Precipitation test	-	+	+	+	+	+	-	+
13	Cardiac glycosides	Keller kelliani's test	-	-	+	+	+	+	+	+
		Sodium nitoprusside test	+	+	+	+	+	+	+	+
14	Quinones	Extract + Con. HCl	-	-	-	-	-	-	-	+
15	Anthraquinones	Borntrager's test	-	-	-	-	-	+	-	-

+ : Presence; - : absence; AB – *A. blitum*; AC – *A. caudatus*; AD – *A. dubius*; AH – *A. hybridus* subsp. *hybridus*; AR – *A. hybridus* subsp. *cruentus*; AS – *A. spinosus*; AT – *A. tricolor*; AV – *A. viridis*

QUANTITATIVE ESTIMATION OF MAJOR PHYTOCOMPONENTS

Quantitative estimation of major phytocomponents such as phenols, flavonoids, terpenoids, alkaloids, tannins, carotenoids and saponin was performed in all the species and the results are summarized in Figures 8 to 11. Quantitative analysis revealed the occurrence of phenols and terpenoids in appreciable amount whereas flavonoids, alkaloids, tannins saponins and carotenoids in lesser amounts.

The total phenolic content in the extracts of *Amaranthus* were estimated using the Folin-Ciocalteu reagent and was expressed as gallic acid equivalents using the standard equation from the linear calibration curve ($y = 0.0068x - 0.007$, $R^2 = 0.997$; Fig. 3). Highest phenolic content (mg GAE/g DW) was observed in *A. hybridus* subsp. *hybridus* (248.78 ± 1.14), followed by *A. spinosus* (237.86 ± 0.53), *A. hybridus* subsp. *cruentus* (207.70 ± 0.97), *A. tricolor* (196.10 ± 0.38) *A. blitum* (177.93 ± 1.14), *A. viridis* (158.21 ± 0.28), *A. dubius* (125.39 ± 0.78) and *A. caudatus* (117.48 ± 1.12) (Fig. 8 a).

The total flavonoid content of the plant extracts was determined using the aluminium chloride colorimetric method. The flavonoid content was measured and expressed in terms of quercetin equivalent employing the standard equation obtained from the linear calibration curve ($y = 0.010x - 0.006$, $R^2 = 0.997$; Fig. 4). The total flavonoid content was estimated to be 9.54 ± 0.18 mg QE/g DW for *A. blitum*, 13.83 ± 0.04 for *A. caudatus*, 21.88 ± 0.10 for *A. dubius*, 19.88 ± 0.05 for *A. hybridus* subsp. *hybridus*, 15.67 ± 0.19 for *A. hybridus* subsp. *cruentus*, 17.12 ± 0.08 for *A. spinosus*, 17.67 ± 0.03 for *A. tricolor* and 10.62 ± 0.05 for *A. viridis*. The results revealed that highest flavonoid content was in *A. dubius* and lowest in *A. blitum*. The flavonoid content observed in *A. spinosus* and *A. tricolor* was more or less similar (Fig. 8 b).

The total terpenoid content in the extracts was expressed in terms of linalool equivalent employing the standard equation obtained from the linear calibration curve ($y = 0.004x + 0.003$, $R^2 = 0.999$; Fig. 5). The terpenoid content was estimated to be highest in *A. blitum* (168.48 ± 1.21 mg linalool/g DW) and lowest in *A. hybridus* subsp. *hybridus* (49.66 ± 1.06). The terpenoid content in other species observed was 76.47 ± 1.11 for *A. caudatus*, 132.63 ± 0.88 for *A. dubius*, 119.97 ± 0.91 for *A. hybridus* subsp. *cruentus*, 151.09 ± 0.74 for *A. spinosus*, 132.06 ± 1.42 for *A. tricolor* and 56.00 ± 1.18 for *A. viridis* (Fig. 9 a).

The total alkaloid content in the extracts was estimated and was expressed in percentage. Highest alkaloid content was observed in *A. spinosus* (34.60 ± 2.41) and lowest in *A. blitum* (8.18 ± 1.53). Alkaloid content observed in other species was 8.40 ± 0.86 (*A. caudatus*), 12 ± 0.60 (*A. dubius*), 9.40 ± 0.99 (*A. hybridus* subsp. *hybridus*), 13 ± 1.67 (*A. hybridus* subsp. *cruentus*), 15.60 ± 1.22 (*A. tricolor*) and 25.80 ± 1.61 (*A. viridis*) (Fig. 9 b).

The tannin content in the extracts was estimated using vanillin reagent and was expressed as mg tannic acid/g DW from the standard curve equation ($y = 0.078x + 0.003$, $R^2 = 0.999$; Fig. 6). The highest content was observed in *A. spinosus* (9.35 ± 0.25) and lowest in *A. caudatus* (4.06 ± 0.44). The tannin content in other extracts were 4.66 ± 0.18, 4.65 ± 0.12, 5.19 ± 0.34, 9.01 ± 0.97, 6.19 ± 0.06 and 6.26 ± 0.95 for *A. blitum*, *A. dubius*, *A. hybridus* subsp. *hybridus*, *A. hybridus* subsp. *cruentus*, *A. tricolor* and *A. viridis* respectively (Fig. 10 a).

The total carotenoid content in the extracts was estimated and expressed in terms of *β*-carotene equivalent employing the standard equation obtained from the linear calibration curve ($y = 0.271x + 0.045$, $R^2 = 0.997$; Fig. 7). The highest carotenoid content was observed in *A. blitum* (0.65 ±

0.00) and lowest in *A. hybridus* subsp. *cruentus* (0.16 ± 0.01). The carotenoid content observed in other species were 0.27 ± 0.07, 0.39 ± 0.00, 0.23 ± 0.05, 0.42 ± 0.02, 0.50 ± 0.00 and 0.31 ± 0.01 for *A. caudatus*, *A. dubius*, *A. hybridus* subsp. *hybridus*, *A. spinosus*, *A. tricolor* and *A. viridis* respectively (Fig. 10 b).

The total saponin content present in different *Amaranthus* species was estimated and results were expressed in percentage. Highest saponin content was observed in *A. spinosus* (27.76 ± 0.08) and lowest in *A. blitum* (1.66 ± 0.00). Saponin content observed in other species were 18.60 ± 0.15 (*A. caudatus*), 16.36 ± 0.06 (*A. dubius*), 13.30 ± 0.11 (*A. hybridus* subsp. *hybridus*), 21.33 ± 0.33 (*A. hybridus* subsp. *cruentus*), 20.20 ± 0.17 (*A. tricolor*) and 22.66 ± 0.08 (*A. viridis*) (Fig. 11).

The quantitative phytochemical analysis revealed the occurrence of phenols and terpenoids in considerable amounts in all the species studied whereas flavonoids, alkaloids saponin, tannin and carotenoid contents were also found to be significant, even though in lesser amounts.

PHYTOCHEMICAL ANALYSIS FOR THE IDENTIFICATION OF VOLATILE AND NON-VOLATILE COMPONENTS

Volatile and non-volatile phytocomponents present in the methanolic extract of different *Amaranthus* species was identified by GC/MS (Table 27; Figs 12-19, 20 (i) - 20 (xxii) and HR-LC/MS analysis (Table 28; Figs 21-28; 29 (i) – 29 (xii).

Gas chromatography-mass spectrometry (GC/MS) analysis

The phytochemical profile of the methanolic extracts of eight species of *Amaranthus* determined using GC/MS analysis revealed wide spectrum of compounds which are consolidated in Table 27. The gas chromatogram

obtained for the species are given in Figs 12-19 and the mass spectrum in Fig. 20 (i) – 20 (xxii). A total of 171 compounds were identified from the eight species which belonged to various classes like phenols, flavonoids, terpenoids, alkaloids, fatty acids, ketones, esters, sterols, vitamins *etc.*

A total of 28 compounds were identified from the methanolic extract of *A. blitum*. The major component detected were α-tocopherol (vitamin E) with highest area of 19.52%. Other major components obtained were palmitic acid ester (13.28%), 25,26-Dihydroelasterol (11.63%), tabersonine (9.09%), 3-Methyl-1-phenyl-2-azafluorenone (8.82%) and 1(22),7(16)-diepoxy-tricyclo[20.8.0.0(7,16)]triacontane (8.53%). The extract was rich in fatty acids and their esters like linolenic acid methyl ester (5.55%), linoleic acid methyl ester (4.55%), 14-methylheptadecanoic acid methyl ester (1.18%), palmitic acid (2.10%) and tricosanoic acid methyl ester (0.36%). Alkaloids were represented by maytansine (0.43%), 1-(1H-[1,2,4]Triazole-3-carbonyl)-piperidine (0.48%), tabersonine (9.09%) and 3-Methyl-1-phenyl-2-azafluorenone (8.82%). Terpenoid compounds obtained were 2,3-Dimethoxy-5-methyl-6-dekaisoprenyl-chinon (0.54%) and squalene (2.74%). Other classes of compounds obtained includes ketones [3,4-Dimethoxyacetophenone (0.42%) and 2-Butanone, 1-(2,3,6-trimethylphenyl) (0.22%)], ester [Acetic acid, (1,2,3,4,5,6,7,8-octahydro-3,8,8-trimethylnaphth-2-yl)methyl ester (0.12%)], amino acid derivative [n-acetyl-l-phenylalanine methyl ester (1.25%)], diterpene alcohol [phytol (5.32%)], triglyceride [Trilinolein (2.13%)] *etc*. (Table 27).

The methanolic extract of *A. caudatus* revealed 36 compounds, of which compounds with highest area percentage was represented by palmitic acid ester (25.25%), 7,22-Ergostadienol (16.34%), phytol (14.31%) and 1(22),7(16)-diepoxy-tricyclo[20.8.0.0(7,16)]triacontane (12.26%). Fatty acids and their esters obtained in addition to palmitic acid ester are lauric acid

(0.28%), estra-1,3,5(10)-trien-17.beta.-ol (0.33%), pentadecanoic acid methyl ester (2.60%), 6,9,12,15-Docosatetraenoic acid, methyl ester (2.16%), 14-methylheptadecanoic acid methyl ester (0.53%), linolenic acid methyl ester (3.82%) and tricosanoic acid methyl ester (0.17%). Two sesquiterpenoids detected includes α-cubebene (0.23%) and (E)-*β*-farnesene (0.29%). Ketones were represented by 1H-2-Indenone,2,4,5,6,7,7a-hexahydro-3-(1-methylethyl)-7a-methyl (1.40%), 5,9-Dimethyl-2-(1methylethyl) cyclodecanone (0.70%), beta-Ionone (0.29%), 4-(2,6,6-trimethyl-1,3-cyclohexadien-1-yl)butan-2-one (0.66%), 1-{2-[3-(2-Acetyloxiran-2-yl)-1,1-dimethylpropyl]cycloprop-2-enyl}ethanone (0.14%) and 1,10-Cycloeicosanedione (0.77%). Other classes of compounds obtained includes phenols [2-Methoxy-4-vinylphenol (0.31%) and 4-allyl-2-methoxyphenol (0.48%)], hydrocarbons [15-Ethenyl-15-methyl 1-oxacyclopentadecan-2-one (0.43%), 9,10-Dimethyltricyclo[4.2.1.1(2,5)]decane-9,10-diol (0.31%), 1-Hexacosene (0.51%) and tritetracontane (0.77%)], aliphatic aldehyde [pentadecanal (0.31%)] *etc*. (Table 27).

GC/MS analysis of the methanolic extract of *A. dubius* revealed the presence of 36 compounds. The prominent compounds were E,E,Z-1,3,12-Nonadecatriene-5,14-diol (27.34%), 25,26-Dihydroelasterol (14.38%), 4-Isopropyl-1 ,6-dimethyldecahydronaphthalene (11.93%), 1,5,5-Trimethyl-6-methylene-cyclohexene (11.44%) and 1(22),7(16)-diepoxy-tricyclo[20.8.0.0(7,16)]triacontane (6.50%). Fatty acids and their esters obtained were arachidic acid (0.27%), pentadecanoic acid methyl ester (1.38%), palmitic acid ester (7.06%), linolenic acid methyl ester (1.54%), palmitic acid (1.92%) and tricosanoic acid methyl ester (0.35%). Three sesquiterpenoids detected includes calamenene (0.34%), alpha-trans-bergamotenol (0.13%) and cis –Z- alpha-Bisabolene epoxide (0.20%). Other important compounds obtained were bornyl chloride (0.17%), 2-Methoxy-4-vinylphenol (0.18%), (Z)-9,17-Octadecadienal (0.68%), pentadecanal

(0.53%), 3,7,11-Trimethyl-1-dodecanol (0.27%), phytol (1.59%), 3,5,24-Trimethyltetracontane (1.15%), tritetracontane (0.73%) and squalene (3.33%) (Table 27).

A total of 45 compounds were identified from the methanolic extract of *A. hybridus* subsp. *hybridus*. The major compounds detected were phytol (14.39%), palmitic acid ester (12.53%), 1(22),7(16)-diepoxy-tricyclo[20.8.0.0(7,16)]triacontane (12.09%) and ergosta-7,22-dien-3-ol, (3.beta.,5.alpha.,22E) (9.94%). The phenolic compounds obtained include guaiacol (1.51%), 2-Methoxy-4-vinylphenol (4.91%), syringol (0.78%), eugenol (0.33%), cis-isoeugenol (0.35%) and 4-allyl-2-methoxyphenol (0.81%). Fatty acids and their esters obtained were linolenic acid methyl ester (5.69%), lauric acid (1.09%), 12-methyltridecanoic acid methyl ester (0.17%), 9-Octadecenoic acid, (2-phenyl-1,3-dioxolan-4-yl)methyl ester, cis- (0.60%), pentadecanoic acid methyl ester (3.43%), palmitic acid (1.25%), lignoceric acid methyl ester (0.56%) and hexacosanoic acid, methyl ester (0.46%). Other classes of compounds obtained included alkaloids [conhydrine (1.25%) and 2-cyclohexylpiperidine (3.34%)], ketones [dihydrocoumarone (1.51%), 1H-2-Indenone,2,4,5,6,7,7a-hexahydro-3-(1-methylethyl)-7a-methyl (0.05%), 5,9-Dimethyl-2-(1methylethyl)cyclodecanone (0.23%), 5,6,7,7a-tetrahydro-4,4,7a-trimethyl-2(4H)-benzofuranone (0.37%), 3,4-Dimethoxyacetophenone (1.42%) and tabanone (0.43%)], phenolic aldehyde [vanillin lactoside (0.20%)], sesquiterpenoid [thujopsene (1.09%)], steroidal alkaloid [oblonginine (1.93%)], fatty alcohols [1-Heptatriacontanol (1.64%) and 3,7,11-Trimethyl-1-dodecanol (0.41%)], triterpenoids [squalene (3.47%) and oleanolic acid (0.67%)] *etc*. (Table 27).

GC/MS analysis of the methanolic extract of *A. hybridus* subsp. *cruentus* revealed the presence of 30 compounds. The prominent compound obtained was a triglyceride, trilinolein (23.43%). Fatty acids and their esters

are other major components obtained which included linoleic acid methyl ester (0.79%), methyl palmitate (8.62%), palmitic acid ester (9.99%), palmitic acid (3.26%) and docosahexaenoic acid, 1,2,3-propanetriyl ester (1.15%). Phenols also form a major part of the extract, which included mequinol (4.33%), syringol (2.84%), beta-ionol (4.20%), o-eugenol (1.05%) and 4-allyl-2-methoxyphenol (2.02%). Ketones represented another important class of compounds which included 5,9-Dimethyl-2-(1methylethyl)cyclodecanone (2.33%), 4-(2,6,6-trimethyl-1,3-cyclohexadien-1-yl)butan-2-one (0.90%), 4-(1,5-dihydroxy-2,6,6-trimethylcyclohex-2-enyl) but-3-en-2-one (2.48%), 2,6-Naphthalenedione, octahydro-1,1,8a-trimethyl-, trans (1.07%) *etc*. Other important compounds obtained included Z,E-2,13-octadecadien-1-ol (3.41%), ingol 12-acetate (0.72%), phytol (3.62%), oleanolic acid (1.17%) *etc*. (Table 27).

The methanolic extract of *A. spinosus* revealed a total of 53 compounds, of which compounds with highest area percentage was represented by 1,5,5-Trimethyl-6-methylene-cyclohexene (15.92%), 3,5,24-Trimethyltetracontane (9.63%), chondrillasterol (8.27%), 1(22),7(16)-diepoxy-tricyclo[20.8.0.0(7,16)]triacontane (6.69%) and bis(2-Methylpropyl) ester 1,2-benzenedicarboxylic acid (6.21%). The extract contain terpenes and sesquiterpenes like 1,1,6-Trimethyl-1,2,3,4-tetrahydronaphthalene (0.63%), α-cuparenol (0.49%), squalene (4.31%), limonen-6-ol, pivalate (0.16%) and oleanolic acid (0.46%). The extract contained 1.53% of α-tocopherol (Vitamin E). Fatty acids and their esters obtained in abundance are methyl isolaurate (0.37%), 12-methyltridecanoic acid methyl ester (2.07%), oleic acid (0.35%), myristic acid ester (0.43%), pentadecanoic acid methyl ester (4.32%), palmitic acid ester (6.15%) and oleic acid ester (2.38%). Extract contained phenolic compounds such as guaiacol (0.64%), syringol (0.34%), (*E*)-2-Methoxy-5-(1-propenyl) phenol (0.19%) *etc*. Alkaloids obtained were conhydrine (1.28%) and 2-cyclohexylpiperidine (3.69%). Other important

classes of compounds obtained included flavonoid [Sinensetin (0.05%)], ketones [5,9-Dimethyl-2-(1methylethyl)cyclodecanone (1.92%), octahydro-4a-methyl-7-(1-methylethyl)-2(1H)- Naphthalenone (0.12%), beta-Ionone (0.35%), spiro[4.5]decan-7-one, 1,8-dimethyl-8,9-epoxy-4-isopropyl- (0.15%) *etc*.], diterpene alcohol [phytol (1.34%)], fatty alcohol [1-Heptatriacontanol (0.91%)], sterols [2,3,16-trihydroxy-5-pregnan-17(R)-20-yl acetate (0.68%), pregna-3, 5-dien-20-one (1.09%) and 9,19-Cyclolanost-24-en-3- ol,acetate (0.54%)], hydrocarbons [tritetracontane (4.86%), 1-Chlorohexadecane (0.23%) and 1,3,8-p-Menthatriene (0.52%)] *etc*. (Table 27).

GC/MS analysis of the methanolic extract of *A. tricolor* revealed the presence of 29 compounds. The prominent compound obtained were dasycarpidan-1-methanol, acetate (ester) (23.29%), Z,Z-3,15-Octadecadien-1-ol acetate (16.11%), tetracontane-1,40-diol (10.98%), phytol (6.98%) and S-[2-[N,N-Dimethylamino]N,N-dimethylcarbamoyl thiocarbohydroximate (5.29%). Extract was found to be rich in phenolic compounds like guaiacol (0.86%), 2-Methoxy-4-vinylphenol (4.57%), syringol (0.82%), trans-isoeugenol (0.52%) and 4-allyl-2-methoxyphenol (0.89%). Fatty acids and their esters found in the extract were lauric acid (1.28%), 12-methyltridecanoic acid methyl ester (0.19%), pentadecanoic acid methyl ester (2.34%), linolenic acid methyl ester (4.39%), palmitic acid (3.80%) and palmitic acid methyl ester (1%). Few terpenoid compounds obtained were 2,3-Dimethoxy-5-methyl-6-dekaisoprenyl-chinon (0.25%) and squalene (3.62%). Ketones like dihydrocoumarone (3.7%), 4-Hydroxy-.beta.-ionone (0.5%), 5,6,7,7a-tetrahydro-4,4,7a- trimethyl-2(4H)-benzofuranone (0.77%), tabanone (0.31%) *etc*. were also found in the extract (Table 27).

The extract of *A. viridis* also showed the presence of potent compounds with palmitic acid ester (27.77%) being the major one. A total of 44 compounds were detected from this extract. The compound tabersonine, a

terpenoid indol alkaloid was also occupied a major portion of the extract with 13.54%. The amount of hydrocarbon 1(22),7(16)-diepoxy-tricyclo[20.8.0.0(7,16)]triacontane, diterpene alcohol phytol, triterpenoid squalene and sterol chondrillasterol were also noteworthy with 9.17, 7.0, 5.43 and 4.48% respectively. The extract contained terpenes like anethofuran (0.15%), cadinene (0.50%) and 2,3-Dimethoxy-5-methyl-6-dekaisoprenyl-chinon (0.52%). One flavonoid detected was sinensetin (0.03%). Phenolic compounds obtained from the extract were 2-Methoxy-4-vinylphenol (0.85%), syringol (0.31%) and 4-allyl-2-methoxyphenol (0.59%). Extract was found to be rich in fatty acids and their esters *viz*., lauric acid (0.65%), 12-methyltridecanoic acid methyl ester (0.16%), arachidic acid (0.65%), methyl isopalmitate (4.54%), 14-methylheptadecanoic acid methyl ester (0.99%) and linolenic acid methyl ester (2.91%). The extract contained ketones like 4-ketoisophorone (0.30%), 3,4-Dimethoxyacetophenone (0.79%), 4-(2,6,6-trimethyl-1,3-cyclohexadien-1-yl)butan-2-one (0.62%), tabanone (0.35%) *etc*. Few alkaloids obtained included tetrahydro-22-desoxy-tomatillidine (0.16%), N-Ethyl-desoxy-veratramine (3.49%) and conessine (1.70%) (Table 27).

GC/MS analysis of eight species of *Amaranthus* revealed an array of bioactive compounds. Fatty acids, terpenes, phenolic compounds, alkaloids and ketones were the predominant classes observed. Occurrence of flavonoids in small amounts was observed in all the extracts. Phytol, a diterpene alcohol and palmitic acid, a fatty acid are the common compounds found in all the species studied.

Table 27. Phytochemical constituents detected in the methanolic extracts of selected species of *Amaranthus* by GC/MS analysis

Sl. No.	RT (min.)	Compounds	Class	Peak area (%)							
				AB	AC	AD	AH	AR	AS	AT	AV
1	6.973	Oblonginine	Steroidal alkaloid	-	-	-	1.93	-	-	-	-
2	11.468	Guaiacol	Phenol	-	-	-	1.51	-	0.64	0.86	-
3	11.508	Mequinol	Phenol	-	-	-	-	4.33	-	-	-
4	12.728	Methyl nicotinate	Organic compound	-	-	-	-	1.55	0.23	-	-
5	12.829	4-ketoisophorone	Ketone	-	-	-	-	-	-	-	0.30
6	13.392	Desulfogluconasturtiin	Organic compound	-	-	-	-	-	-	-	0.18
7	13.521	Conhydrine	Alkaloid	-	-	-	1.25	-	1.28	-	-
8	13.717	Methyl salicylate	Organic ester	-	-	-	0.09	-	-	-	-
9	13.796	Anethofuran	Monoterpene	-	-	-	-	-	-	-	0.15
10	14.011	Sinensetin	Flavonoid	-	-	-	-	-	0.05	-	0.03
11	14.183	Dihydrocoumarone	Ketone	-	-	-	1.51	-	-	3.7	-
12	14.330	3-ethyl-4-methyl-1H-pyrrole-2,5-dione	N-containing compound	-	-	-	0.44	-	0.22	-	0.28
13	14.359	α-cubebene	Sesquiterpenoid	-	0.23	-	-	-	-	-	-
14	14.490	21-Deoxy-16-methoxy-22α-methyl-4,25-secoobscurinervan	Organic compound	-	-	-	-	-	0.02	-	-
15	14.665	1,1,4a-trimethyl-3,4,4a,5,6,7-hexahydro-2(1H)-naphthalenone	Organic compound	-	0.50	0.38	-	-	-	-	-
16	14.805	Bicyclo(3.3.1)nonan-2-one,1-methyl-9-(1-methylethylidene)	Organic compound	-	0.39	-	-	0.85	-	-	-
17	14.849	Methyl isolaurate	Fatty acid methyl ester	-	-	-	-	-	0.37	-	-
18	14.870	9-Oximino-2,7-diethoxyfluorene	Hydrocarbon	-	-	-	-	-	-	-	0.06

19	14.998	1H-2-Indenone,2,4,5,6,7,7a-hexahydro-3-(1-methylethyl)-7a-methyl	Ketone	-	1.40	0.61	0.05	2.24	-	-	-
20	15.143	Bornyl chloride	Terpenoid	-	-	0.17	-	-	-	-	-
21	15.217	Indolizine	Heterocyclic aromatic compound	-	-	-	-	-	-	-	0.34
22	15.254	Indole	Aromatic organic compound	-	-	-	-	4.56	0.51	-	-
23	15.317	2,6-dimethyl-2,5-heptadien-4-one	Organic compound	-	0.82	1.81	0.76	-	0.86	-	0.56
24	15.383	2-Methoxy-4-vinylphenol	Phenol	-	0.31	0.18	4.91	-	-	4.57	0.85
25	15.385	Carvacrol	Monoterpenoid	0.24	-	-	-	-	-	-	-
26	15.437	1,7-Dimethyl-4-(1-methylethyl)-Spiro[4.5]dec-6-en-8-one	Organic compound	-	0.08	-	-	-	-	-	-
27	15.488	exo-2-Hydroxycineole	Organic compound	-	-	-	-	-	-	-	0.12
28	15.558	2-Methyl-4-(2,6,6-trimethylcyclohex-1-enyl)but-2-en-1-ol	Alcohol	-	0.10	0.14	-	-	-	-	-
29	15.646	Tetrahydro-22-desoxy-tomatillidine	Steroidal alkaloid	-	-	-	-	-	-	-	0.16
30	15.713	3,4,4-Trimethyl-2-cyclopenten-1-one	Hydrocarbon	-	-	-	-	-	-	-	0.21
31	15.742	4-(2,2,6-Trimethyl-bicyclo[4.1.0]hept-1-yl)-butan-2-one	Miscellaneous	-	-	-	-	-	0.31	-	-
32	15.749	5,9-Dimethyl-2-(1methylethyl)cyclodecanone	Ketone	-	0.70	-	0.23	2.33	1.92	-	-
33	15.816	Syringol	Phenol	-	-	-	0.78	2.84	0.34	0.82	0.31
34	15.817	1-Naphthalenol, decahydro-1,4a-dimethyl-7-(1-methylethylidene)	Organic compound	-	-	0.15	-	-	-	-	-
35	15.848	Eugenol	Phenol	-	-	-	0.33	-	-	-	-
36	15.911	1,2-Dihydro-1,1,6-trimethylnaphthalene	Organic compound	0.57	-	-	-	-	-	-	-
37	15.918	1,1,6-trimethyl-1,2-dihydronaphthalene	Organic compound	-	0.24	-	-	-	-	0.49	0.11
38	15.923	Calamenene	Sesquiterpenoid	-	-	0.34	-	-	-	-	-
39	15.925	1,1,6-Trimethyl-1,2,3,4-tetrahydronaphthalene	Terpenoid	-	-	-	-	-	0.63	-	-

40	16.023	2-Methyl-3-[(1S,2S)-1,3,3-trimethyl-2-(2-hydroxyethyl)cyclohexyl]tetrahydrofuran	Organic compound	-	-	-	-	-	0.22	-	-
41	16.164	2-cyclohexylpiperidine	Heterocyclic amine/ Alkaloid	-	-	-	3.34	-	3.69	-	-
42	16.296	1-(3,6,6-Trimethyl-1,6,7,7a-tetrahydrocyclopenta[c]pyran-1-yl)ethanone	Miscellaneous	-	-	-	0.25	-	-	-	-
43	16.321	Tricyclo[6.3.0.0(1,5)]undecan-4-one, 5,9-dimethyl-	Hydrocarbon	-	-	0.17	-	-	-	-	-
44	16.389	Vanillin lactoside	Phenolic aldehyde	-	-	-	0.20	-	-	-	0.10
45	16.510	6,8-Nonadien-2-one,6-methyl-5(1-methyl-ethylidene)	Organic compound	-	-	-	-	-	-	1.21	-
46	16.588	2,3-dibromo-8-phenyl-*p*-menthane	Monoterpene hydrocarbon	-	-	-	-	-	-	-	0.82
47	16.592	1,3,8-p-Menthatriene	Alkene	-	-	-	-	-	0.52	-	-
48	16.653	Thujopsene	Sesquiterpenoid	-	-	-	1.09	-	-	-	-
49	16.659	4-Hydroxy-.beta.-ionone	Ketone	-	-	-	-	-	-	0.5	-
50	16.670	Beta-ionol	Phenol	-	-	-	-	4.20	-	-	-
51	16.728	1-(1,5-Dimethyl-4-hexenyl)-4-methylbenzene	Organic compound	-	-	-	-	-	2.25	-	-
52	16.855	cis-isoeugenol	Phenol	-	-	-	0.35	-	-	-	0.32
53	16.858	trans-isoeugenol	Phenol	-	-	-	-	-	-	0.52	-
54	16.860	(*E*)-2-Methoxy-5-(1-propenyl) phenol	Phenol	-	-	-	-	-	0.19	-	-
55	16.872	o-eugenol	Phenol	-	-	-	-	1.05	-	-	-
56	16.997	2-Thiaadamantan-4-ol	Alcohol	-	-	-	-	-	0.20	-	-
57	17.145	beta-Ionone	Ketone	-	0.29	-	-	-	0.35	-	-
58	17.274	15-Ethenyl-15-methyl 1-oxacyclopentadecan-2-one	Hydrocarbon	-	0.43	-	-	-	-	-	-
59	17.278	Octahydro-4a-methyl-7-(1-methylethyl)-2(1H)-Naphthalenone	Ketone	-	-	-	-	-	0.12	-	-
60	17.286	Z,E-2,13-octadecadien-1-ol	Alcohol	-	-	-	-	3.41	-	-	-
61	17.325	Maytansine	Alkaloid	0.43	-	-	-	-	-	-	-
62	17.379	Cadinene	Sesquiterpene	-	-	-	-	-	-	-	0.50

63	17.381	Spiro[4.5]decan-7-one, 1,8-dimethyl-8,9-epoxy-4-isopropyl-	Sesquiterpene ketone	-	-	-	-	-	0.15	-	-
64	17.399	4,6,10,10-Tetramethyl-5-oxatricyclo[4.4.0.0(1,4)]dec-2-en-7-ol	Alcohol	-	-	-	-	0.83	-	-	-
65	17.439	Cyclopropanebutanoic acid,2-[[2-[[2-[[2-pentylcyclopropyl]methyl]cyclopropyl]methyl]cyclopropyl] methyl]-methyl ester	Ester	-	-	-	0.98	-	-	-	-
66	17.454	1-Benzoylamino-5-piperidinyl-1-phenylpentane	Organic compound	0.44	-	-	-	-	-	-	-
67	17.517	7-Dehydrocholesteryl isocaproate	Sterol	-	-	-	-	-	-	-	1.01
68	17.658	1H-2-Indenol, 2,3,4,5,6,7-hexahydro-1-(2-hydroxy-2-methylpropyl)	Phenol	-	-	-	-	-	0.14	-	-
69	17.720	5,6,7,7a-tetrahydro-4,4,7a- trimethyl-2(4H)-benzofuranone	Ketone	-	-	-	0.37	-	0.21	0.77	0.44
70	17.827	Lauric acid	Fatty acid	-	0.28	-	1.09	-	-	1.28	0.65
71	17.868	3-tert-butyl-4-hydroxyanisole	Ether	-	-	-	-	-	0.65	-	-
72	17.907	3,4-Dimethoxyacetophenone	Ketone	0.42	-	-	1.42	-	-	-	0.79
73	17.971	4-(2,6,6-trimethyl-1,3-cyclohexadien-1-yl)butan-2-one	Ketone	-	0.66	-	-	0.90	0.51	0.6	0.62
74	17.979	Benzene,1-methyl-3-[(1 methylethylidene)cyclopropyl]-	Organic compound	-	-	0.47	-	-	-	-	-
75	18.063	2-Butanone, 1-(2,3,6-trimethylphenyl)	Ketone	0.22	-	-	-	-	-	-	-
76	18.082	1-Chlorohexadecane	Alkane	-	-	-	-	-	0.23	-	-
77	18.095	(E)-*β*-farnesene	Sesquiterpenoid	-	0.29	-	-	-	-	-	-
78	18.096	alpha-trans-bergamotenol	Sesquiterpenoid	-	-	0.13	-	-	-	-	-
79	18.102	Linoleic acid methyl ester	Fatty acid methyl ester	-	-	-	-	0.79	-	-	-
80	18.334	1-(1H-[1,2,4]Triazole-3-carbonyl)-piperidine	Alkaloid	0.48	-	-	-	-	-	-	-
81	18.385	9-(3,3-Dimethyloxiran-2-yl)-2,7-dimethylnona-2,6-dien-1-ol	Organic compound	-	0.50	-	-	-	-	-	-
82	18.447	Tabanone	Aromatic ketone	-	-	-	0.43	-	-	0.31	0.35
83	18.455	Limonen-6-ol, pivalate	Terpenoid	-	-	-	-	-	0.16	-	-

84	18.459	Acetic acid,10,11-dihydroxy-3,7,11-trimethyl dodeca-2,6-dienyl ester	Terpenoid ester	-	-	0.21	-	-	-	-	-
85	18.464	Acetic acid,(1,2,3,4,5,6,7,8-octahydro-3,8,8-trimethylnaphth-2-yl)methyl ester	Ester	0.12	-	-	0.46	-	-	-	0.21
86	18.468	Cis –Z- alpha-Bisabolene epoxide	Oxygenated sesquiterpenoid	-	-	0.20	-	-	-	-	-
87	18.494	4-(1,5-dihydroxy-2,6,6-trimethylcyclohex-2-enyl) but-3-en-2-one	Ketone	-	-	-	-	2.48	-	-	-
88	18.656	4,4,5,8-Tetramethylchroman-2-ol	Alcohol	-	-	-	-	-	-	0.61	-
89	18.726	6-(3-Hydroxy-but-1-enyl)-1,5,5-trimethyl-7-oxabicyclo[4.1.0]heptan-2-ol	Alcohol	-	-	0.07	-	-	-	-	-
90	18.743	Ingol 12-acetate	Diterpenoid	-	-	-	-	0.72	-	-	-
91	18.863	1b,4a-Epoxy-2H cyclopenta[3,4]cyclopropa[8,9]cycloundec[1,2-b]oxiren-5(6H)-one, 7-(acetyloxy)decahydro-2,9,10-trihydroxy-3,6,8,8,10a-pentamethyl	Organic compound	0.08	-	-	-	-	-	-	-
92	18.908	(Z)-9,17-Octadecadienal	Aldehyde	-	-	0.68	-	-	-	-	-
93	18.931	1-{2-[3-(2-Acetyloxiran-2-yl)-1,1-dimethylpropyl]cycloprop-2-enyl}ethanone	Ketone	-	0.14	-	1.34	3.75	0.96	0.74	1.70
94	18.999	4-allyl-2-methoxyphenol	Phenol	-	0.48	-	0.81	2.02	-	0.89	0.59
95	19.033	Pentadecanal	Aliphatic aldehyde	-	0.31	0.53	-	-	-	-	-
96	19.037	n-acetyl-l-phenylalanine methyl ester	Amino acid derivative	1.25	-	-	-	-	-	-	-
97	19.057	α-cuparenol	Sesquiterpenoid	-	-	-	-	-	0.49	-	-
98	19.068	12-methyltridecanoic acid methyl ester	Fatty acid methyl ester	-	-	-	0.17	-	2.07	0.19	0.16
99	19.167	Benzyl benzoate	Aromatic hydrocarbon	-	-	-	-	-	0.74	-	-
100	19.282	3,9.beta.;14,15-Diepoxypregn-16-en-20-one, 3,11.beta.,18-triacetoxy	Organic compound	0.06	-	-	-	-	0.42	-	-

101	19.363	Estra-1,3,5(10)-trien-17.beta.-ol	Fatty acid	-	0.33	-	-	-	-	-	-
102	19.367	9-Octadecenoic acid, (2-phenyl-1,3-dioxolan-4-yl)methyl ester, cis-	Fatty acid methyl ester	-	-	-	0.60	-	-	-	-
103	19.377	Palmitic acid methyl ester	Fatty acid methyl ester	-	-	-	-	-	-	1.00	-
104	19.427	9,10-Dimethyltricyclo[4.2.1.1(2,5)]decane-9,10-diol	Hydrocarbon	-	0.31	-	-	-	-	-	-
105	19.442	Oleic acid	Fatty acid	-	-	-	-	-	0.35	-	-
106	19.504	Arachidic acid	Fatty acid	-	-	0.27	-	-	-	-	0.65
107	19.504	3,7,11-Trimethyl-1-dodecanol	Fatty alcohol	-	-	0.27	0.41	-	-	-	-
108	19.509	P-propiolic acid, 3-(1-hydroxy-2-isopropyl-5-methylcyclohexyl)	Organic compound	0.22	-	-	-	-	-	-	-
109	19.651	(1,5,5,8-Tetramethyl-bicyclo[4.2.1]non-9-yl] acetic acid	Miscellaneous	-	-	-	0.48	0.70	-	-	-
110	19.656	Myristic acid ester	Fatty acid ester	-	-	-	-	-	0.43	-	-
111	19.718	2,3-Dimethoxy-5-methyl-6-dekaisoprenyl-chinon	Terpenoid	0.54	-	-	-	0.41	-	0.25	0.52
112	19.731	N-Ethyl-desoxy-veratramine	Alkaloid	-	-	-	-	-	-	-	3.49
113	19.809	Propanoic acid	Carboxylic acid	0.13	-	-	-	-	-	-	-
114	19.833	Acetic acid, chloro-, octadecyl ester	Ester	-	-	-	-	-	-	0.36	-
115	19.917	**E,E,Z-1,3,12-Nonadecatriene-5,14-diol**	Miscellaneous	-	-	27.34	-	-	-	-	-
116	19.936	6, 10, 14-trimethyl-2-pentadecanone	Ketone	-	-	-	-	-	-	-	1.0
117	19.992	bis(2-Methylpropyl) ester 1,2-benzenedicarboxylic acid	Ester	-	-	-	-	-	6.21	-	-
118	20.068	2,6-Naphthalenedione, octahydro-1,1,8a-trimethyl-, trans	Ketone	-	-	-	-	1.07	-	-	-
119	20.184	Oxirane, 2,2-dimethyl-3-(3,7,12,16,20-pentamethyl-3,7,11,15,19-heneicosapentaenyl)-, (all-E)-	Miscellaneous	-	-	-	0.27	2.42	-	-	-
120	20.206	4-Isopropyl-1 ,6-dimethyldecahydronaphthalene	Hydrocarbon	-	-	11.93	-	-	-	-	-
121	20.459	2(1H)-Benzocyclooctenone,decahydro-10a-methyl-,trans	Miscellaneous	-	-	-	0.42	-	-	-	-
122	20.505	Methyl isopalmitate	Fatty acid ester	-	-	-	-	-	-	-	4.54
123	20.507	Methyl palmitate	Fatty acid methyl ester	-	-	-	-	8.62	-	-	-
124	20.509	3-Methyl-1-phenyl-2-azafluorenone	Alkaloid	8.82	-	-	-	-	-	-	-
125	20.509	Pentadecanoic acid methyl ester	Fatty acid methyl ester	-	2.60	1.38	3.43	-	4.32	2.34	-

126	20.646	Isophytol	Diterpene alcohol	-	0.49	-	-	-	-	-	-
127	20.864	Dasycarpidan-1-methanol, acetate (ester)	Ester	-	-	-	-	-	-	23.29	-
128	20.900	Palmitic acid ester	Fatty acid ester	13.28	25.25	7.06	12.53	9.99	6.15	-	27.77
129	21.633	Linoleic acid methyl ester	Fatty acid methyl ester	4.55	-	-	-	-	-	-	-
130	21.664	6,9,12,15-Docosatetraenoic acid, methyl ester	Fatty acid methyl ester	-	2.16	-	-	-	-	-	-
131	21.724	Phytol	Diterpene alcohol	5.32	14.31	1.59	14.39	3.62	1.34	6.98	7.00
132	21.732	Tetracontane-1,40-diol	Hydrocarbon	-	-	-	-	-	-	10.98	-
133	21.743	Tabersonine	Terpenoid indol alkaloid	9.09	-	-	-	-	-	-	13.54
134	21.747	1,5,5-Trimethyl-6-methylene-cyclohexene	Hydrocarbon	-	-	11.44	-	-	15.92	-	-
135	21.805	14-methylheptadecanoic acid methyl ester	Fatty acid ester	1.18	0.53	-	-	-	-	-	0.99
136	22.034	1(22),7(16)-diepoxy-tricyclo[20.8.0.0(7,16)]triacontane	Hydrocarbon	8.53	12.26	6.50	12.09	0.92	6.69	-	9.17
137	22.038	Z,Z-3,15-Octadecadien-1-ol acetate	Alcohol	-	-	-	-	-	-	16.11	-
138	22.059	Oleic acid ester	Fatty acid ester	-	-	-	-	-	2.38	-	-
139	22.092	Linolenic acid methyl ester	Fatty acid methyl ester	5.55	3.82	1.54	5.69	-	-	4.39	2.91
140	22.114	Nonanoic acid, 9-(3-hexenylidenecyclopro-	Fatty acid derivative	-	-	-	-	-	-	3.53	-
141	22.149	1-Heptatriacontanol	Fatty alcohol	-	-	-	1.64	-	0.91	-	0.30
142	22.814	1-Hentetracontanol	Alcohol	-	-	-	-	-	2.28	-	-
143	23.155	1,10-Cycloeicosanedione	Ketone	-	0.77	-	-	-	-	-	-
144	23.200	5-Methyl-Z-5-docosene	Hydrocarbon	-	-	0.37	-	-	-	-	-
145	23.639	2,3,16-trihydroxy-5-pregnan-17(R)-20-yl acetate	Sterol	-	-	-	-	-	0.68	-	-
146	23.831	S-[2-[N,N-Dimethylamino]N,N-dimethylcarbamoyl thiocarbohydroximate	Organic compound	-	-	-	3.32	4.40	-	5.29	4.55
147	23.991	1-Hexacosene	Hydrocarbon	-	0.51	-	-	-	-	-	-
148	23.999	2-Hexyl-1-octanol	Alcohol	-	-	0.62	-	-	-	-	-
149	24.003	Pregna-3, 5-dien-20-one	Sterol	-	-	-	-	-	1.09	-	-
150	24.209	Palmitic acid	Fatty acid	2.10	-	1.92	1.25	3.26	-	3.80	-

151	24.593	2-Hexyl-1-decanol	Alcohol	-	0.52	-	-	-	-	-	-
152	24.810	Tricosanoic acid methyl ester	Fatty acid methyl ester	0.36	0.17	0.35	-	-	-	-	-
153	25.223	3,5,24-Trimethyltetracontane	Hydrocarbon	-	-	1.15	-	-	9.63	-	-
154	25.399	Trilinolein	Triglyceride	2.13	-	-	-	23.42	-	-	-
155	25.422	Lignoceric acid methyl ester	Fatty acid methyl ester	-	-	-	0.56	-	-	-	-
156	25.434	Tetracosanoic acid methyl ester	Ester	-	-	0.67	-	-	-	-	-
157	25.831	Docosahexaenoic acid, 1,2,3-propanetriyl ester	Fatty acid ester	-	-	-	-	1.15	-	-	-
158	25.835	Tritetracontane	Hydrocarbon	-	0.77	0.73	-	-	4.86	-	-
159	25.849	1,54-Dibromotetrapentacontane	Hydrocarbon	-	-	0.92	-	-	-	-	-
160	25.879	Conessine	Steroidal alkaloid	-	-	-	-	-	-	-	1.70
161	25.963	6,10,14,18,22-Tetracosapentaen-2-ol,3bromo-2,6,10,15,19,23-hexamethyl-,(all-E)-	Terpenoid	-	10.71	-	-	-	-	-	-
162	25.985	Squalene	Triterpenoid	2.74	-	3.33	3.47	-	4.31	3.62	5.43
163	26.788	Hexacosanoic acid, methyl ester	Fatty acid methyl ester	-	-	-	0.46	-	-	-	-
164	28.420	9,19-Cyclolanost-24-en-3- ol,acetate	Sterol	-	-	-	-	-	0.54	-	-
165	28.830	α-tocopherol	Vitamin E	19.52	-	-	-	-	1.53	-	-
166	30.480	Oleanolic acid	Triterpenoid	-	-	-	0.67	1.17	0.46	-	-
167	31.247	Ergosta-7,22-dien-3-ol, (3.beta.,5.alpha.,22E)	Miscellaneous	-	-	-	9.94	-	-	-	-
168	31.282	7,22-Ergostadienol	Sterol	-	16.34	-	-	-	-	-	-
169	31.340	Chondrillasterol	Sterol	-	-	-	-	-	8.27	-	4.48
170	31.426	25,26-Dihydroelasterol	Sterol	11.63	-	14.38	-	-	-	-	-
171	32.835	Urs-12-en-24-oic acid, 3-oxo-, methyl ester, (+)-	Ester	-	-	-	2.29	-	-	-	-

RT – Retention time; AB – *A. blitum*; AC – *A. caudatus*; AD – *A. dubius*; AH – *A. hybridus* subsp. *hybridus*; AR – *A. hybridus* subsp. *cruentus*; AS – *A. spinosus*; AT – *A. tricolor*; AV – *A. viridis*

High-resolution liquid chromatography mass spectrometry (HR-LC/MS)

HR-LC/MS analysis of all the eight species of *Amaranthus* was done to detect the presence of phytoconstituents which were not revealed through GC/MS. The components are listed in Table 28. A total of 96 compounds were identified from all the species, which includes phenols, flavonoids, alkaloids, fatty acid derivatives, amino acids and their derivatives, peptides, vitamins, glycosides, sesquiterpenoids, tetranortriterpenoids, phospholipids, ketones *etc*. The liquid chromatograms are given in Figs 21-28 and mass spectra in Figs 29 (i) - 29 (xii).

The methanolic extract of *A. blitum* revealed a total of 23 compounds, which includes amino acid derivatives, peptides, phospholipids, triterpenoids and alkaloids. An amino acid leucine was also identified. The extract contained pantothenic acid (vitamin B_5), non-protein amino acids like DL-ornithine, L-2-aminoadipic acid and 2S-aminoheptanoic acid, α-amino acids like O-acetylserine and homoserine, amino acid derivatives like 4-hydroxy-L-threonine and methyl N-(a-methylbutyryl) Glycine. The extract of *A. caudatus* had 27 compounds, which included choline, carnitine, 4-hydroxy-L-threonine, a fatty acid derivative 9-amino-nonanoic acid, an alkaloid ecgonine, an aromatic carboxylic acid benzoic acid, tetranortriterpenoids like 2,6-dihydrofissinolide and swietenine, ketone like 3,7-epoxycaryophyllan-6-one, phospholipid such as phytosphingosine and peptides like Thr-Gly, Lys-Gly-Pro, Asn-Val-Ile, Leu-Ile *etc*. The methanolic extract of *A. dubius* revealed 21 compounds, which included amino acid derivatives like betaine, hydroxylysine and lyxosylamine, lipid dihydrosphingosine, tetranortriterpenoid swietenine, alkaloids like rescinnamine and cuscohygrine, fatty acid nervonic acid and peptides like Arg-Pro-Pro, Leu-Ser-Val, Asn-Asn-Asn, Ile-Leu-Leu *etc*. Pantothenic acid (Vitamin B_5) was also obtained from *A. dubius*. Thirty eight compounds were obtained from the extract of *A. hybridus* subsp. *hybridus* and the important ones being, fatty acids and derivatives like 9-amino-nonanoic acid, 12-hydroxy-10-octadecynoic acid and omega-hydroxy myristic acid, amino acid derivatives like dimethylglycine,

tyramine and methyl N-(a-methylbutyryl) glycine, sesquiterpenoids such as dihydrocaryophyllen-5-one and punctaporin B, tetranortriterpenoid swietenine, α-amino acids like O-acetylserine and 1-aminocyclopropane-1-carboxylic acid, cardiac glycoside such as peruvoside, lipids like phytosphingosine and dihydrosphingosine. Flavonoids like dihydromyricetin and rutin was also found in the extract. Alkaloids like ambelline, alpha-erythroidine and beta-carboline were also found (Table 28).

A total of 25 compounds were identified from the methanolic extract of *A. hybridus* subsp. *cruentus* which included choline, 1,4-dideoxy-1,4-imino-D-arabinitol, carnitine, 4-hydroxy-L-threonine, D-glucoheptose *etc*. Alpha-amino acids like homoserine and O-acetylserine was also found. Fatty acid derivative like 9-amino-nonanoic acid, non-protein amino acids like 2S-aminoheptanoic acid, L-2-Aminoadipic acid and many peptides such as Lys-Gly-Pro, Asn-Val-Ile, Leu-Ile *etc*. were also obtained from the extract. Norharman and cuscohygrine were the two alkaloids obtained. Seventeen compounds were obtained from *A. spinosus*, which included choline, 1,4-dideoxy-1,4-imino-D-arabinitol, 4-hydroxy-L-threonine, homoserine, O-acetylserine, leucine, isonicotinamide, 2-propyl-, dihydrosphingosine, swietenine, cuscohygrine, khivorin *etc*. The methanolic extract of *A. tricolor* and *A. viridis* revealed a total of 21 compounds each. The compounds obtained from the extract of *A. tricolor* were a sesquiterpenoid artemisinin, an amino compound lyxosylamine, amino acid leucine, alkaloid cuscohygrine, fatty acid docosanedioic acid, tetranortriterpenoid swietenine and peptides like Thr-Pro-Asp, Ala-Glu-His, Ile-Leu-Leu *etc*. Compounds like carnitine, 4-hydroxy-L-threonine, 3-methyl-2,5-dioxo-3- pyrrolidineacetic acid, leucine, punctaporin B, 2,6-dihydrofissinolide, 6-deoxotyphasterol, dihydrosphingosine, swietenine, cuscohygrine, docosanedioic acid *etc*. were obtained from the methanolic extract of *A. viridis*.

The HR-LC/MS analysis of the methanolic extract revealed many bioactive compounds of which a tetranortriterpenoid compound, swietenine was found to be the common compound found in all the species (Table 28).

Table 28. Phytochemical constituents detected in the methanolic extracts of selected species of *Amaranthus* by HR-LC/MS analysis

Sl No.	RT (min.)	Compounds	Mass	Formula	Class	AB	AC	AD	AH	AR	AS	AT	AV
1	0.497	omega-hydroxy myristic acid	244.200	$C_{14}H_{28}O_3$	Fatty acid derivative	-	-	-	+	-	-	-	-
2	0.679	Pro-Ile	228.145	$C_{11}H_{20}N_2O_3$	Peptide	-	-	-	+	-	-	-	-
3	0.681	Dimethylglycine	103.062	$C_4H_9NO_2$	Amino acid derivative	-	-	-	+	-	-	-	-
4	0.765	Tyramine	137.083	$C_8H_{11}NO$	Amine derivative	-	-	-	+	-	-	-	-
5	0.769	1-Aminocyclopropane-1-carboxylic acid	101.047	$C_4H_7NO_2$	α-amino acid	-	-	-	+	-	-	-	-
6	0.909	Normetanephrine	183.088	$C_9H_{13}NO_3$	Catecholamine	-	-	-	+	-	-	-	-
7	0.938	2-methoxy-4-vinylphenol	120.056	C_8H_8O	Phenol	-	-	-	+	-	-	-	-
8	3.334	Indole-3-ethanol	161.083	$C_{10}H_{11}NO$	Aromatic alcohol	-	-	-	+	-	-	-	-
9	3.954	beta-Carboline	168.068	$C_{11}H_8N_2$	Alkaloid	-	-	-	+	-	-	-	-
10	4.213	2-Methylene-5-(2,5-dioxotetrahydrofuran-3-yl)-6-oxo-10,10dimethyl bicyclo[7:2:0]undecane	304.164	$C_{18}H_{24}O_4$	Organic compound	-	-	-	+	-	-	-	-
11	4.525	Choline	104.106	$C_5H_{14}NO$	Vitamin	+	+	-	-	+	+	+	-
12	4.868	Thr-Gly	176.078	$C_6H_{12}N_2O_4$	Peptide	-	+	-	-	-	-	-	+
13	5.064	Betaine	118.085	$C_5H_{12}NO_2$	Amino acid derivative	-	-	+	-	-	-	-	-
14	5.209	1,4-dideoxy-1,4-imino-D-arabinitol	133.074	$C_5H_{11}NO_3$	Alcohol	-	-	+	-	+	+	-	-
15	5.423	Carnitine	162.111	$C_7H_{16}NO_3$	Quaternary ammonium amino acid	-	+	+	-	+	-	+	+
16	5.504	Dihydromyricetin	320.051	$C_{15}H_{12}O_8$	Flavonoid	-	-	-	+	-	-	-	-
17	5.505	Rutin	610.149	$C_{27}H_{30}O_{16}$	Flavonoid	-	-	-	+	-	-	-	-
18	5.608	Methyl N-(a-	173.103	$C_8H_{15}NO_3$	Amino acid derivative	+	-	-	+	-	-	-	-

Sl No.	RT (min.)	Compounds	Mass	Formula	Class	AB	AC	AD	AH	AR	AS	AT	AV
		methylbutyryl) glycine											
19	5.624	4-Hydroxy-L-threonine	135.052	$C_4H_9NO_4$	Amino acid derivative	+	+	-	-	+	+	+	+
20	5.730	Benzenemethanol,2-(2-aminopropoxy)-3-methyl	196.109	$C_{11}H_{16}O_3$	Organic compound	-	-	-	+	-	-	-	-
21	5.773	Artemisinin	282.141	$C_{15}H_{22}O_5$	Sesquiterpenoid	-	-	-	-	-	-	+	-
22	5.807	D-Glucoheptose	210.071	$C_7H_{14}O_7$	Sugar	-	-	-	-	+	-	-	-
23	6.817	Thr-Arg-Tyr	438.221	$C_{19}H_{30}N_6O_6$	Peptide	-	-	-	+	-	-	-	-
24	7.090	Dihydrocaryophyllen-5-one	220.180	$C_{15}H_{24}O$	Sesquiterpenoid	-	-	-	+	-	-	-	-
25	7.127	Ambelline	331.140	$C_{18}H_{21}NO_5$	Alkaloid	-	-	-	+	-	-	-	-
26	7.466	Homoserine	119.059	$C_4H_9NO_3$	α-amino acid	+	-	-	-	+	+	-	-
27	7.516	Ile-Asp-Asp	361.151	$C_{14}H_{23}N_3O_8$	Peptide	-	-	-	+	-	-	-	-
28	7.816	Lys-Lys-Thr	375.239	$C_{16}H_{33}N_5O_5$	Peptide	-	-	-	+	-	-	-	-
29	8.003	alpha-Erythroidine	273.135	$C_{16}H_{19}NO_3$	Alkaloid	-	-	-	+	-	-	-	-
30	8.245	O-Acetylserine	147.051	$C_5H_9NO_4$	α-amino acid	+	-	-	+	+	+	+	-
31	8.801	Hydroxylysine	162.101	$C_6H_{14}N_2O_3$	Amino acid derivative	-	-	+	-	-	-	-	-
32	8.924	Pro-Arg-Val	370.231	$C_{16}H_{30}N_6O_4$	Peptide	-	-	-	+	-	-	-	-
33	9.579	9-amino-nonanoic acid	173.140	$C_9H_{19}NO_2$	Fatty acid derivative	-	+	-	+	+	-	-	-
34	10.087	Pro-Thr	216.109	$C_9H_{16}N_2O_4$	Peptide	+	-	-	-	+	-	-	-
35	10.172	Ecgonine	185.1042	$C_9H_{15}NO_3$	Alkaloid	-	+	-	-	-	-	-	-
36	10.200	2S-aminoheptanoic acid	145.110	$C_7H_{15}NO_2$	Non-protein amino acid	+	-	-	-	+	-	+	-
37	10.427	Val-Arg-Gly	330.203	$C_{13}H_{26}N_6O_4$	Peptide	-	-	-	+	-	-	-	-
38	10.641	Thr-Phe	266.1280	$C_{13}H_{18}N_2O_4$	Peptide	-	+	-	-	-	-	-	-
39	10.952	Gly-Ala-Val	245.136	$C_{10}H_{19}N_3O_4$	Peptide	-	-	-	-	+	-	-	-
40	10.981	L-2-Aminoadipic acid	161.067	$C_6H_{11}NO_4$	Non-protein amino acid	+	-	-	-	+	-	-	-
41	11.032	DL-Ornithine	132.088	$C_5H_{12}N_2O_2$	Non-protein amino	+	-	-	-	-	-	-	-

Sl No.	RT (min.)	Compounds	Mass	Formula	Class	AB	AC	AD	AH	AR	AS	AT	AV
					acid								
42	11.138	3-methyl-2,5-dioxo-3-Pyrrolidineacetic acid	171.051	$C_7H_9NO_4$	Alkaloid	-	+	-	-	-	-	-	+
43	11.173	Pantothenic acid	278.132	$C_{11}H_{22}N_2O_4S$	Vitamin B_5	+	-	+	-	-	-	-	-
44	11.182	Lyxosylamine	149.069	$C_5H_{11}NO_4$	Amino compound	+	-	+	-	-	-	+	-
45	11.792	4-heptanone	114.106	$C_7H_{14}O$	Ketone	-	-	-	+	-	-	-	-
46	11.795	Gly-Pro-Lys	300.176	$C_{13}H_{24}N_4O_4$	Peptide	+	-	+	-	-	-	-	+
47	11.800	Lys-Gly-Pro	300.1766	$C_{13}H_{24}N_4O_4$	Peptide	-	+	-	-	+	-	-	-
48	11.972	Leu-Leu	244.175	$C_{12}H_{24}N_2O_3$	Peptide	-	-	+	-	-	+	+	-
49	12.292	Asn-Val-Ile	344.202	$C_{15}H_{28}N_4O_5$	Peptide	-	+	-	+	+	-	-	-
50	12.293	Lys-Ser-Lys	361.228	$C_{15}H_{31}N_5O_5$	Peptide	+	+	+	-	+	+	+	+
51	12.335	Ala-Ala-Leu	273.167	$C_{12}H_{23}N_3O_4$	Peptide	-	-	-	-	-	-	+	-
52	12.339	Ile-Arg-Ala	358.232	$C_{15}H_{30}N_6O_4$	Peptide	-	-	-	+	-	-	-	-
53	12.450	N-Carboxyethyl-gamma-aminobutyric acid	175.083	$C_7H_{13}NO_4$	Non-protein amino acid derivative	+	-	-	-	-	-	-	-
54	12.716	Lys-Gln-Leu	387.2444	$C_{17}H_{33}N_5O_5$	Peptide	-	+	-	-	-	-	-	-
55	12.737	Leu-Ile	244.176	$C_{12}H_{24}N_2O_3$	Peptide	+	+	-	-	+	-	-	+
56	13.099	Norharman	168.067	$C_{11}H_8N_2$	Alkaloid		-	-	-	+	-	-	-
57	13.172	Leucine	131.092	$C_6H_{13}NO_2$	Amino acid	+	-	+	-	-	+	+	+
58	13.173	Isonicotinamide, 2-propyl-	164.095	$C_9H_{12}N_2O$	Amide	+	-	-	-	-	+	+	-
59	13.397	Arg-Arg-Gln	458.269	$C_{17}H_{34}N_{10}O_5$	Peptide	-	-	-	+	+	-	+	-
60	13.600	Ser-Lys-Lys	361.230	$C_{15}H_{31}N_5O_5$	Peptide	-	-	+	-	-	-	-	-
61	13.615	Ile-Leu-Leu	357.259	$C_{18}H_{35}N_3O_4$	Peptide	+	+	+	-	+	+	+	+
62	13.780	12-hydroxy-10-octadecynoic acid	296.2344	$C_{18}H_{32}O_3$	Fatty acid	-	-	-	+	-	-	-	-
63	13.784	Punctaporin B	252.1723	$C_{15}H_{24}O_3$	Sesquiterpenoid	-	-	-	+	-	-	-	+
64	14.020	Benzoic acid	122.0360	$C_7H_6O_2$	Aromatic carboxylic	-	+	-	-	-	-	-	-

Sl No.	RT (min.)	Compounds	Mass	Formula	Class	AB	AC	AD	AH	AR	AS	AT	AV
					acid								
65	14.383	Embelin	294.1827	$C_{17}H_{26}O_4$	Glycoside	-	-	-	+	-	-	-	-
66	16.373	Arg-Ile-Asp	402.2238	$C_{16}H_{30}N_6O_6$	Peptide	-	-	-	+	-	-	-	-
67	16.697	Asn-Asn-Asn	360.136	$C_{12}H_{20}N_6O_7$	Peptide	-	+	+	-	-	-	-	+
68	17.159	Ala-Phe-Pro	355.150	$C_{14}H_{21}N_5O_6$	Peptide	-	-	-	-	-	+	-	-
69	17.159	Ala-Glu-His	355.150	$C_{14}H_{21}N_5O_6$	Peptide	+	-	-	-	-	-	+	-
70	17.186	Phe-Ala-Pro	333.169	$C_{17}H_{23}N_3$	Peptide	-	-	-	-	-	-	-	+
71	17.367	2,6-dihydrofissinolide	514.2586	$C_{29}H_{38}O_8$	Tetranortriterpenoid	-	+	-	-	-	-	-	+
72	17.656	3,7-Epoxycaryophyllan-6-one	236.1766	$C_{15}H_{24}O_2$	Ketone	-	+	-	-	-	-	-	-
73	18.094	Calotropin	532.2685	$C_{29}H_{40}O_9$	Cardenolide	-	-	-	+	-	-	-	-
74	18.117	Leu-Ser-Val	317.192	$C_{14}H_{27}N_3O_5$	Peptide	-	-	+	-	-	-	-	+
75	18.322	Phe-Arg-Thr	422.224	$C_{19}H_{30}N_6O_5$	Peptide	-	-	-	-	+	-	-	-
76	18.899	Thr-Pro-Asp	331.138	$C_{13}H_{21}N_3O_7$	Peptide	-	-	-	-	-	-	+	-
77	19.484	Phe-Val-Leu	377.232	$C_{20}H_{31}N_3O_4$	Peptide	-	-	-	-	-	+	-	-
78	19.486	6-Deoxotyphasterol	434.373	$C_{28}H_{50}O_3$	Brassinosteroid precursor	-	-	-	-	-	-	-	+
79	19.855	Peruvoside	548.2995	$C_{30}H_{44}O_9$	Cardiac glycoside	-	-	-	+	-	-	-	-
80	20.903	Phytosphingosine	317.2910	$C_{18}H_{39}NO_3$	Sphingolipid	+	+	-	+	-	-	-	-
81	21.021	Dihydrosphingosine	301.295	$C_{18}H_{39}NO_2$	Sphingolipid	-	-	+	+	-	+	-	+
82	21.141	Thr-Arg-Phe	422.2255	$C_{19}H_{30}N_6O_5$	Peptide	-	+	-	-	-	-	-	-
83	21.833	Swietenine	568.268	$C_{32}H_{40}O_9$	Tetranortriterpenoid	+	+	+	+	+	+	+	+
84	23.546	Ajmaline	308.1972	$C_{20}H_{26}N_2O_2$	Alkaloid	-	+	-	-	-	-	-	-
85	24.456	Arg-Pro-Pro	368.216	$C_{16}H_{28}N_6O_4$	Peptide	-	-	+	-	-	-	-	-
86	24.862	Lys-His-Cys	386.1711	$C_{15}H_{26}N_6O_4S$	Peptide	-	+	-	-	-	-	-	-
87	24.961	Ala-Pro-Asp	301.128	$C_{12}H_{19}N_3O_6$	Peptide	-	-	-	-	-	-	-	+
88	25.207	Cuscohygrine	224.186	$C_{13}H_{24}N_2O$	Alkaloid	+	+	+	-	+	+	+	+
89	25.253	Docosanedioic acid	370.315	$C_{22}H_{42}O_4$	Fatty acid	-	-	-	-	-	-	+	+
90	25.289	Nervonic acid	366.344	$C_{24}H_{46}O_2$	Fatty acid	-	-	+	-	-	-	-	-

Sl No.	RT (min.)	Compounds	Mass	Formula	Class	AB	AC	AD	AH	AR	AS	AT	AV
91	26.216	Arg-Pro-Gly	328.1872	$C_{13}H_{24}N_6O_4$	Peptide	-	+	-	-	-	-	-	-
92	26.435	Methyl dihydrojasmonate	226.155	$C_{13}H_{22}O_3$	Organic compound	+	+	+	-	+	+	-	-
93	26.449	4-methyl-decanoic acid	186.162	$C_{11}H_{22}O_2$	Fatty acid derivative	-	-	-	-	-	-	+	+
94	26.878	Arg-Phe-Gln	449.2388	$C_{20}H_{31}N_7O_5$	Peptide	-	+	-	-	-	-	-	-
95	30.191	Khivorin	586.275	$C_{32}H_{42}O_{10}$	Organic compound	-	-	-	+	+	+	+	-
96	33.344	Rescinnamine	634.298	$C_{35}H_{42}N_2O_9$	Alkaloid	-	-	+	-	+	-	-	-

RT: retention time; AB – *A*. *blitum*; AC – *A*. *caudatus*; AD – *A*. *dubius*; AH – *A*. *hybridus* subsp. *hybridus*; AR - *A*. *hybridus* subsp. *cruentus*; AS – *A*. *spinosus*, AT – *A*. *tricolor*; AV – *A*. *viridis*; + presence; - absence

PROXIMATE COMPOSITION ANALYSIS

Proximate composition analysis of different *Amaranthus* species was investigated using standard analytical methods in order to assess the nutritional potential. The percentage content of protein, lipid, carbohydrate, ash and dietary fibre was determined and represented in Fig. 30. The results showed that, protein, ash (mineral) and dietary fibre is present in highest quantity in all the species rather than carbohydrate and lipid. The results of proximate composition of different *Amaranthus* species showed high moisture content [*A. blitum* (84.73 ± 0.08), *A. caudatus* (86.43 ± 0.13), *A. dubius* (84.03 ± 0.03), *A. hybridus* subsp. *hybridus* (83.26 ± 0.08), *A. hybridus* subsp. *cruentus* (84.76 ± 0.06), *A. spinosus* (87.70 ± 0.05), *A. tricolor* (82.63 ± 0.08) and *A. viridis* (79.60 ± 0.15). Comparatively highest moisture content was observed for *A. spinosus* and lowest for *A. viridis*. The crude protein content was found to be highest in *A. tricolor* (31.8 ± 0.18), followed by *A. blitum* (19.4 ± 0.08), *A. dubius* (18.8 ± 0.21), *A. caudatus* (18 ± 0.18), *A. viridis* (13.5 ± 0.09), *A. hybridus* subsp. *hybridus* (12 ± 0.12), *A. spinosus* (12 ± 0.11) and *A. hybridus* subsp. *cruentus* (6.4 ± 0.12). Significant amount of ash content was present in all the species, of which highest content was found in *A. hybridus* subsp. *cruentus* (21.31 ± 0.07) and lowest in *A. caudatus* (17.53 ± 0.07). Almost equal percentage of ash content was observed for *A. blitum* (20.57 ± 0.06), *A. dubius* (20 ± 0.20), *A. hybridus* subsp. *hybridus* (20.83 ± 0.04) and *A. viridis* (20.75 ± 0.03). Ash content of *A. spinosus* and *A. tricolor* was 19.87 ± 0.03 and 19.72 ± 0.04 respectively. Appreciable amount of dietary fibre content was observed in all the *Amaranthus* species studied. The crude fibre content in *A. blitum*, *A. caudatus*, *A. dubius*, *A. hybridus* subsp. *hybridus*, *A. hybridus* subsp. *cruentus*, *A. spinosus*, *A. tricolor* and *A. viridis* was 6.14 ± 0.02, 6.25 ± 0.005, 7.67 ± 0.01, 5.26 ± 0.006, 8.57 ± 0.01, 6.25 ± 0.005, 7.27 ± 0.01 and 6.46 ± 0.006 respectively.

Amaranthus species showed negligible crude fat content, that ranges from 2.01 ± 0.05 (*A. tricolor*) to 2.82 ± 0.08 (*A. dubius*). Lipid content observed in other species was 2.61 ± 0.05 (*A. blitum*), 2.42 ± 0.06 (*A. caudatus*), 2.42 ± 0.02 (*A. hybridus* subsp. *hybridus*), 2.22 ± 0.07 (*A. hybridus* subsp. *cruentus*), 2.63 ± 0.09 (*A. spinosus*) and 2.02 ± 0.01 (*A. viridis*). The carbohydrate content was also very low in all the species, which ranges from 1.92 ± 0.01 (*A. blitum*) to 7.40 ± 0.005 (*A. viridis*). Carbohydrate content in other species was 7.0 ± 0.02 (*A. caudatus*), 2.61 ± 0.01 (*A. dubius*), 1.98 ± 0.04 (*A. hybridus* subsp. *hybridus*), 2.25 ± 0.01 (*A. hybridus* subsp. *cruentus*), 2.82 ± 0.02 (*A. spinosus*) and 3.8 ± 0.06 (*A. tricolor*). The calorific value (Kcal/100 g) of different *Amaranthus* species was estimated to be 76.03, 89.16, 78.79, 56.60, 42.22, 61.36, 107.98, 76.26 Kcal/100 g for *A. blitum*, *A. caudatus*, *A. dubius*, *A. hybridus* subsp. *hybridus*, *A. hybridus* subsp. *cruentus*, *A. spinosus*, *A. tricolor* and *A. viridis* respectively. Comparatively high calorific value for *A. tricolor* and low value for *A. hybridus* subsp. *cruentus* was observed.

ELEMENTAL ANALYSIS (ICP-AES)

Analysis of mineral elements *viz*., Na, K, Ca, Mg, Fe, Zn, P and Mn in different *Amaranthus* species revealed their presence in all the species in varying concentrations (Fig. 31 a, b). The results showed that all the species of *Amaranthus* contained higher proportions of macro-minerals such as K, Ca, Mg and P and lower proportions of micro-minerals/trace minerals such as Fe, Mn, and Zn. But the macro-mineral Na was found in low percentage in all the species studied.

Among the macro-minerals K content was highest in all the species, which ranges from 18.76% (*A. tricolor*) to 25.19% (*A. hybridus* subsp. *hybridus*). K content in other species was 22.40% in *A. blitum*, 20.55% in *A. caudatus*, 21.68% in *A. dubius*, 21.23% in *A. hybridus* subsp. *cruentus*,

22.53% in *A. spinosus* and 24.66% in *A. viridis*. Following K, Ca is found in highest percentage in all the species, of which *A. hybridus* subsp. *cruentus* showed highest quantity of 18.71%, whereas *A. hybridus* subsp. *hybridus* and *A. bitum* showed lower percentages of 10.72% and 10.90% respectively. Ca content observed in other species was 17.77% in *A. dubius*, 16.51% in *A. tricolor*, 14.86% in *A. viridis*, 13.86% in *A. caudatus* and 12.77% in *A. spinosus*. Following Ca, the macro-mineral, Mg is found in highest percentage in all the species studied, of which *A. blitum* showed higher content of 10.62% and *A. hybridus* subsp. *cruentus* showed least amount of 4.35%. Percentage Mg content observed in other species such as *A. caudatus*, *A. spinosus*, *A. hybridus* subsp. *hybridus*, *A. tricolor*, *A. dubius* and *A. viridis* was 8.14, 7.36, 6.94, 6.84, 5.40 and 5.30% respectively. P was the next element found in highest percentage in all the species studied. Among the species, highest P content was observed in *A. tricolor* (2.85%) followed by *A. blitum* (2.60%), *A. caudatus* (2.46%), *A. hybridus* subsp. *cruentus* (2.27%), *A. spinosus* (2.24%), *A. hybridus* subsp. *hybridus* (1.88%), *A. dubius* (1.80%) and *A. viridis* (1.42%) (Fig. 31 a). Comparatively low percentage of Na was observed in all the species of *Amaranthus* studied and was 0.95% in *A. tricolor*, 0.40% in *A. hybridus* subsp. *cruentus*, 0.39% in *A. dubius*, 0.38% in *A. caudatus*, 0.34% in *A. blitum*, 0.28% in *A. viridis*, 0.24% in *A. hybridus* subsp. *hybridus* and 0.23% in *A. spinosus* (Fig. 31 b).

Among the trace minerals, Fe was found in significantly higher proportions than Mn and Zn. The Fe content ranges from 0.14% (*A. blitum*) to 0.89% (*A. tricolor*). The percentage Fe content in *A. caudatus*, *A. dubius*, *A. hybridus* subsp. *hybridus*, *A. hybridus* subsp. *cruentus*, *A. spinosus* and *A. viridis* was 0.37, 0.31, 0.191, 0.199, 0.296 and 0.293% respectively. Micro-minerals such as Mn and Zn were also found in all the species, even though in lesser amounts. Compared to Zn, Mn content was higher in all the species except in *A. hybridus* subsp. *cruentus* and *A. viridis*. Among the eight species,

highest Mn content was in *A. hybridus* subsp. *hybridus* (0.085%) and lowest in *A. viridis* (0.034%). Mn content in other species was 0.069% in *A. dubius*, 0.054% in *A. caudatus* and *A. spinosus*, 0.045% in *A. tricolor*, 0.040% in *A. blitum* and 0.038% in *A. hybridus* subsp. *cruentus*. Highest Zn content was found in *A. hybridus* subsp. *cruentus* (0.052%), followed by *A. dubius* (0.038%), *A. caudatus* (0.037%), *A. viridis* (0.035%), *A. hybridus* subsp. *hybridus* (0.031%), *A. tricolor* (0.024%), *A. spinosus* (0.022%) and least amount in *A. blitum* (0.021%) (Fig. 31 b).

The Ca/P ratio obtained for different *Amaranthus* species was greater than one, which were as follows; 4.19 for *A. blitum*, 5.61 for *A. caudatus*, 9.82 for *A. dubius*, 5.68 for *A. hybridus* subsp. *hybridus*, 8.21 for *A. hybridus* subsp. *cruentus*, 5.70 for *A. spinosus*, 5.77 for *A. tricolor* and 10.41 for *A. viridis*. The Na/K ratio obtained was less than one for all the species, which were as follows; 0.01 for *A. blitum*, *A. caudatus*, *A. dubius*, *A. hybridus* subsp. *cruentus*, *A. spinosus* and *A. viridis*, 0.009 for *A. hybridus* subsp. *hybridus* and 0.05 for *A. tricolor*.

DISCUSSION

Amaranth is an American native plant historically cultivated by Mayans, Aztecs and Incas. A few decades ago *Amaranthus* was re-emerged as a most promising alternative crop not only for its high nutritional value, but also due to its nutraceutical properties (Barba de la Rosa et al., 2010). Since then research has been focused on various aspects of *Amaranthus* species. The genus *Amaranthus* includes about 70 species **(**Espitia-Rangel et al., 2012) and have different centres of domestication and origin, being widely distributed in North America (Canada, United States), Central America (Mexico, Guatemala) and the South American Andes (Peru, Bolivia, Ecuador), where also the greatest genetic diversity is found. Most of the species have high economic value, some of them being cultivated from prehistoric times for their grains (*A. caudatus, A. cruentus* and *A. hypochondriacus*) or leaves (*A. blitum*, *A. dubius* and *A. tricolor*). Some species are useful as colourful ornamentals **(**Brenner et al., 2010). Amaranth, especially the grain type is one of the staple food sources of many under-developed countries. Both grain and vegetable amaranths are very promising food crops in arid region, due to their resistance to heat, draught, diseases and pests. In addition, the nutritional value of both the seeds and leaves is excellent. Most of the amaranth species are potent medicinal sources and their therapeutic properties are being explored from ancient period itself.

Amaranthus species have great morphological variability due to the occurrence of frequent interspecific hybridization, which results in many hybrids with indistinguishable morphological features, that makes their identification difficult. The genus *Amaranthus* is still poorly understood, being widely considered as a difficult genus. Currently the taxonomic problems are far from being clarified especially because of the widespread

nomenclatural disorders caused chiefly by repeated misapplication of names (Costea et al., 2001). Hence precise identification is necessary before moving into further studies for getting accurate results, which was attempted in the present study through molecular analysis of eight species of *Amaranthus viz*., *A. blitum*, *A. caudatus*, *A. dubius*, *A. hybridus* subsp. *hybridus*, *A. hybridus* subsp. *cruentus*, *A. spinosus*, *A. tricolor* and *A. viridis* (Plate 1). In addition to molecular studies, to get better clarity in species identity, some other aspects such as chromosome architecture, micromorphological features and phytochemical composition was also undertaken. Since the knowledge of chromosome number and karyomorphology have been an aid in revealing the relationships between taxa, a study was done to determine the chromosome number of the selected species of *Amaranthus* with the hope that the information might be helpful in future biosystematic investigation of the genus. Micromorphological studies of the pollen grains, seed capsule and seed coat using SEM analysis revealed common and unique micromorphological features of the species studied. Chemotaxonomy, provides a qualitative account of plant secondary metabolites, which is another well accredited technique used for plant identification. This involves the characterization of plants based on their chemical composition, as many plants have unique secondary metabolites in them, which can be used as specific markers (Joshi et al., 2011). GC/MS and HR-LC/MS analysis were carried out to unravel the phytochemical constituents in the plant materials.

According to Onyango (2010), improvement of amaranths through research and development could produce an easy and cost-effective way of eliminating malnutrition and promoting people's health as well as achieving food security. But there are still gaps in knowledge of some popular amaranths and there are confusions in the nomenclature of species, for instance, of the hybridus complex while comprehensive nutritional profiles are yet to be compiled (Grubben & Denton, 2004). Moreover, little is known

on the breeding potentials particularly of wild relatives that can be promoted for sustainable utilization (Achigan-Dako et al., 2014). A comparative analysis of the proximate composition and mineral elements present in the aerial parts of eight *Amaranthus* species of the present study revealed their nutritional potential.

1. MOLECULAR CHARACTERIZATION

DNA barcoding involves the generation of DNA sequencing data from particular genetic regions in an organism and the use of these sequence data is to identify or "barcode" that organism and distinguish it from other species (Schori & Showalter, 2011). The phylogenetic relationship and taxonomic delimitation in the genus *Amaranthus* are still not resolved with extreme clarity. But classification of the genus *Amaranthus* into three subgenera *viz*., Acnida, Amaranthus and Albersia based on morphological parameters, especially inflorescence and floral characteristics is quite acceptable (Das, 2012 a).

In the present study phylogenetic analysis is used to authenticate species identity of the collected *Amaranthus* species thereby distinguishing them from other similar species, as it is difficult morphologically alone to differentiate due to the occurrence of many natural interspecific hybrids. Along with the species identity, the present study is also focused to characterize the molecular profile of the eight species as it is not much studied so far. Good quality DNA isolated from the plant species yielded distinct bands of rbcL, matK and ITS region of rDNA sequences after PCR amplification. The well resolved sequences (Plates 2-4) from the three gene regions with less ambiguity was subjected to phylogenetic tree construction (Plates 5-7) from which molecular identity of the species studied were confirmed. The tree-based criterion results in the clustering of species and their conspecifics at distinct nodes with high support values and revealed the

monophyly of the genus *Amaranthus* with a congruent topology for ML and BI analysis.

DNA barcoding is a method for identifying an organism based on sequence data from one to several gene regions (Schori & Showalter, 2011). Barcoding has multiple applications and has been used for ecological surveys (Dick & Kress, 2009), cryptic taxon identification (Lahaye et al., 2008), confirmation of medicinal plant samples (Xue & Li, 2011) *etc*. Several chloroplast gene regions are typically used as plant barcodes, such as maturase K (matK) and ribulose 1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL). The spacer between tRNA-His and photosystem II protein D1 (*trnH-psbA* spacer) and the nuclear internal transcribed spacer (ITS), are also widely used (Chen et al., 2010; Gao et al., 2010 a; Fu et al., 2011). Barcoding works by matching sequence data from a query sample (an unknown specimen) to a reference sequence (from a voucher specimen) (Schori & Showalter, 2011). A match of 100% between a query sequence and a reference sequence is unambiguous and indicated that, each base pair is exactly matched. The match might be along a part of the gene region that is highly conserved, with little to no variation among many species. In tree-based analysis, genus identification was considered successful when the unknown sample formed a monophyletic group together with all members of a single genus, with bootstrap support of >70% (Oshingboye et al., 2017). Depending on this statement, the tree-based method adopted in the present study for species authentication was successful since the query samples formed a single cluster together with its conspecifics, with a strong bootstrap support of >90%. Several challenges to the successful implementation of plant DNA barcoding are presented and discussed so far. Despite these challenges, DNA barcoding has the potential to uniquely identify plants and provide quality control and standardization of the plant material supplied to the pharmaceutical industry (Schori & Showalter, 2011).

DNA barcoding is currently a widely used and effective tool that enables rapid and accurate identification of plant species, however, none of the available loci works across all species because single-locus DNA barcodes lack adequate variations in closely related taxa (Li et al., 2015 b). There is now a growing acceptance that the genetic barcoding of land plants requires a multi-locus approach (Chase et al., 2005, 2007; Newmaster et al., 2006). The selection of loci to feature in a multi-locus barcode requires a balance between conflicting factors. Newmaster et al. (2006) also considered familiarity and the value of considerable existing sequence resources when suggesting the combination of rbcL with other plastid loci as part of a multi-locus barcode. Kress and Erickson (2007) evaluated a subset of the loci and primers with several other loci on the basis of amplification success and sequence variability. They proposed that psbA-trnH should be combined with part of the rbcL gene (rbcL-a), with the former providing the sequence variation for species identification and the latter providing a less variable taxonomic 'anchor'.

An attempt by CBOL Plant Working Group (2009) in order to fix standard DNA barcodes for land plant identification recommend the 2-locus combination of rbcL + matK as the plant barcode. They compared the performance of 7 leading candidate plastid DNA regions *viz*., atpF–atpH spacer, matK, rbcL, rpoB, rpoC1 gene, psbK–psbI and trnH–psbA spacer. Based on assessments of recoverability, sequence quality and levels of species discrimination they found out the suitability of rbcL and matK gene regions as a standard 2-locus barcode. According to them an ideal DNA barcode should be routinely retrievable with a single primer pair, be amenable to bidirectional sequencing with little requirement for manual editing of sequence traces and provide maximal discrimination among species. In contradiction to the above results, Roy et al. (2010) and Fu et al. (2011) observed that matK and rbcL are not always useful as barcodes for certain groups of plants (*Berberis* and

Tetrastigma). A similar observation was also made by Gielly and Taberlet (1994), regarding the coding and non coding genes of chloroplast DNA to resolve plant phylogenies and showed that rbcL does not contain enough information to resolve relationships between closely related genera, such as *Hordeum, Triticum* and *Aegilops*. The present study also observed less variable regions in the rbcL and matK sequences obtained when compared to ITS region of rDNA sequences. Recent barcoding studies have placed high emphasis on the use of whole-chloroplast genome sequencing which is now more readily available as a consequence of improving sequencing technologies (Li et al., 2015 b).

During the last decades, molecular markers have proven to be powerful tools for assessing genetic variation within and among populations of plants (Sammour et al., 2012). Previous efforts to address phylogenetic relationships within Amaranthaceae include morphological studies and molecular analyses of chloroplast genes (rbcL, matK, atpB, trnK, intergenic trnL - trnF), nuclear ribosomal RNA genes (ITS1 and ITS2) and variability studies by using RAPD, AFLP, ISSR, PCR-RFLP and isozyme markers. Genetic markers are essential tools for modern plant breeding research programs (Eathington et al., 2007). They are particularly important for germplasm conservation, characterization, and in breeding applications, such as marker-assisted selection (Maughan et al., 2011). One of the important criteria for an ideal DNA barcode region is efficient recovery of good quality sequence data (CBOL Plant Working Group, 2009). Based on this, the results of the present study revealed that the three gene regions showed good amplification and sequencing success, indicating the potential of rbcL, matK and ITS region of rDNA gene markers for phylogenetic studies in *Amaranthus*.

Authentic identification is crucial for sustainable management of endangered plants. The plastid-encoded rbcL gene sequence is the most

frequently used one for plant phylogenetic analyses. In a major attempt, 499 species of seed plants, representing all major taxonomic groups, were studied using rbcL gene sequences (Chase et al., 1993). Phylogenetic relationships of plants under Dipsacales (Donoghue et al., 1992) and closely related genera of *Aegilops, Hordeum* and *Triticum* (Gielly & Taberlet, 1994) were determined using rbcL gene sequences. The effectiveness of the core plant barcode regions (rbcL and matK) and a supplemental ribosomal DNA (ITS2) were examined using 900 specimens, representing 312 species of vascular plants. The sequencing success for rbcL gene is reportedly high for most plant species (Kuzmina et al., 2012). The present study also revealed high sequencing success for rbcL and matk genes for all the plant species compared to ITS, but it contain more variable regions than the others. Geologically ancient DNA of *Hymenaea protera* (Fabaceae) from Miocene fossils was suspected as a contaminant from *Arabidopsis thaliana*. Plastid rbcL gene sequences were used to differentiate the suspected samples from each other (Rosselló, 2013). Plant plastid rbcL loci provides greater success in PCR-amplification (Bafeel et al., 2011) and for obtaining a clean sequence (Kuzmina et al., 2012) as well. SNPs (Single nucleotide polymorphisms) in the rbcL region were used for the identification of plant species (Kim et al., 2012).

Amaranthus is considered as a difficult genus from the taxonomic point of view. Frequent outcrossing and hybridization has caused broadening of the *Amaranthus* gene pool and has created a large number of morphotypes or accessions (Das, 2012 b). Molecular genetic cluster analysis could help define the genetic similarities among *Amaranthus* species and indicate which species are likely to produce fit interspecies hybrids. Molecular data, specifically DNA sequences, have received a great deal of attention as a potential source of "phylogenetically informative" characters that are putatively less ambiguous than non- molecular characters (Chase et al., 1993).

Previous molecular genetic analyses of *Amaranthus* species have been designed to determine ancestors of domestic species (Sun et al., 1999). Interspecies hybridization has also been reported in wild *Amaranthus* populations. Grant (1959 b) described the karyotypes of putative interspecies *Amaranthus* hybrids.

In DNA barcoding, a short DNA sequence is used as a molecular marker for identifying the diversity that exists among plant and animal species. An internal transcribed spacer (ITS) region of nuclear ribosomal cistron is another most commonly used sequence locus for plant molecular systematic investigations **(**Kress et al., 2005). Each rDNA gene is separated by several hundred base pairs referred to as the internal transcribed spacer (ITS1 and ITS2). These regions are more variable in nucleotide sequence than the rDNA genes, which are highly conserved. The ITS regions of the nuclear rDNA may vary among species within a genus or among populations (White et al., 1990). Furthermore, 1,000 to 10,000 copies of rDNA are present in each plant cell (Jorgensen & Cluster, 1988), making it easy to detect and amplify. An ideal DNA barcoding marker for taxonomic classification should be fast-evolving to allow classification at the species level but must also contain highly conserved priming sites and be highly reliable for DNA amplification and sequencing (Taberlet et al., 2006). The ITS2 region meets the expected criteria of a global DNA barcode. Han et al. (2013) demonstrated the potential of the ITS2 minibarcode for DNA barcoding analyses. ITS2 showed high sequence variability among 12861 samples from 8313 species. Their analysis supports the use of the ITS2 minibarcode as a "universal DNA barcode" for the rapid identification of medicinal materials and specimens. Analysis of ITS regions of nuclear ribosomal DNA (rDNA) showed that 12 of 92 *Amaranthus* accessions collected and identified by weed scientists were misidentified. ITS primers have proven to be a useful source of information for the resolution of phylogenetic relationships at the species level (Popa et

al., 2010). However, taxonomic confusion remains among closely related taxa such as *A. cruentus*, *A. caudatus* and *A. hypochondriacus*, and their putative wild progenitors, *A. hybridus*, *A. quitensis* and *A. powellii*. Low internal transcribed spacer divergence in these taxa resulted in poorly resolved phylogeny (Xu & Sun, 2001). But RAPD (Random Amplified Polymorphic DNA) and isozyme markers resolved the genetic variability and evolutionary relationships in grain amaranths (Chan & Sun, 1997).

Along with nuclear genes, many chloroplast and mitochondrial genes are also been utilized for studying sequence variation at genus level. Chloroplast DNA (cpDNA) is a molecular marker that is widely used for taxon identification (Clegg & Zurawski, 1992). The cpDNA has an independent genome that encodes several proteins, which are protein related photosynthetic and housekeeping genes. The cpDNA encode 30-50 tRNA genes and 100 other proteins. The genes that encode proteins are divided into several kinds, *viz*., splicing factor genes (rpoB, rpoC1, rpoC2, rpsl6 and matK*)* and protein related photosynthetic genes (rbcL, afpB, psaA and petB) (Baumgartner et al., 1993; Sugiura, 1995; De Las Rivas et al., 2002). Among these genes, rbcL gene sequence have been analysed by various workers to address plant systematics **(**Chase et al., 1993). The rbcL gene encodes ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (Ellis, 1979). The rbcL sequence can be used for cogeneric analysis (Kress et al., 2005) and is a core locus in chloroplast genome (Newmaster et al., 2006).

The matK gene of chloroplast is 1500 bp long, located within the intron of the trnK and codes for maturase like protein, which is involved in Group II intron splicing. The two exons of the trnK gene that flank the matK were lost, leaving the gene intact in the event of splicing. The gene contains high substitution rates within the species and is emerging as potential candidate to study plant systematics and evolution **(**Notredame et al., 2000). It

is another emerging gene with potential contribution to plant molecular systematics and evolution (Hilu & Liang, 1997). The matK-trnK gene complex is commonly used for plant evolution studies and forms the solution at various taxonomic levels (Ito et al., 1999). The matK gene has ideal size, high rate of substitution, large proportion of variation at nucleic acid level at first and second codon position, low transition/transversion (Ts/Tv) ratio and shows the presence of mutationally conserved sectors. These features of matK gene are exploited to resolve family and species level relationships. Polymorphism of cpDNA especially trnK, matK and intergenic trnL-trnF regions has been used to study the phylogeny of various plants (Wolfe et al., 1987). Genetic variation in *A. spinosus* was analyzed based on PCR-sequencing of cpDNA, genes that encode tRNA (trnL intron), splicing factor protein (matK) and protein related to photosynthesis (rbcL) (Fatinah et al., 2013). The results showed that *A. spinosus* variant from tropical zone have revealed a high genetic variability with Ts/Tv approximately 0.5-1.19 and conserved sequence >70%. The high genetic variability is caused by local adaptation and gene flow among species.

A comparative analysis of the relationship between species of Amaranthaceae and the closely related family such as Chenopodiaceae, Portulacaceae and Caryophyllaceae by sequencing of the chloroplast matK coding and non-coding regions was done by Ogundipe and Chase (2009). Phylogenetic analysis of the matK sequences alone and in combination using maximum parsimony methods produced monophyletic lineage of Amaranthaceae and Chenopodiaceae. Giannasi et al. (1992), Rettig et al. (1992) and Cuénoud et al. (2002) in their studies included more than two taxa of Amaranthaceae and Chenopodiaceae in their sampling and both families were resolved as sister families. Manhart and Rettig (1994) and Cuénoud et al. (2002) identified Amaranthaceae as nested within Chenopodiaceae. Müller and Borsch (2005) using matK/trnK in their study on Amaranthaceae and

Chenopodiaceae concluded that this lineage is monophyletic, with Achatocarpaceae and Caryophyllaceae being successive sisters. Modern genetics-based classification schemes, considered Amaranthaceae and Chenopodiaceae as sister groups that form the most basal branch of the order Caryophyllales (Borsch et al., 2001). APG (1998, 2003) supported this recommendation and merged Chenopodiaceae in Amaranthaceae based on the molecular studies. The Maximum Likelihood dendrogram of rbcL and ITS region of rDNA resolved in the present study also agree with the above report, since in both the trees the genus *Chenopodium* is positioned in the same cluster in which *Amaranthus* belongs.

Genetic diversity is the variation of individual genotypes within and among species. It is an important trait for long-term survival of species and enables a population to adapt to new conditions brought by environmental change (Hamrick & Godt, 1996). Over evolutionary time, such among-population genetic differences can accumulate and result in the development of a new species. Knowledge of the amount and distribution of genetic variability within a species is vital to plant breeders because it is an important consideration when selecting germplasm to be included in a breeding program. Also, it is helpful for geneticists managing plant genetic resources and provides information for designing sampling protocols (Yu et al., 2001). All together genetic diversity studies are essential for providing information for propagation, domestication and breeding programs as well as conservation of genetic resources for plant species (Sammour et al., 2012). Estimation of genetic variability are based on morphological, cytological, biochemical and molecular traits. However, the estimation of genetic variability based on morphological and cytological traits alone has the disadvantage of being influenced by both environmental and genetic factors and may therefore not provide an accurate measure (Basu et al., 2004).

Members of amaranth family have variations in morphological characters, which may be due to high genetic diversity, differences of growing type and differences on the type of adaptations (Kulakow, 1987; Jacobsen & Mujica, 2003). The phylogeny developed based on morphological features has limitation in data accuracy because of high subjectivity and it cannot determine taxa up to their species level (Stussey, 1990). Molecular markers can be used for phylogenetic analysis and can determine up to infraspecific level (Fatinah et al., 2012). RAPD is one of the molecular markers that can be used in the study of phylogeny. RAPD is used for polymorphism detection and it is now possible to survey a large number of loci and ascribes unambiguous taxonomic and genetic relationships among different taxa (Williams et al., 1990; Mondini et al., 2009).

A study by Fatinah et al. (2012) on genetic diversity among six genera of amaranth family based on both morphological and molecular markers (RAPD) resulted in high number and percentage of polymorphic bands that revealed genomic DNA variation in accordance with phenotypic variation detected. The study concluded that based on DNA polymorphism detected by RAPD, amaranth family can be classified into two sub-families namely Amaranthoideae and Gomphrenoideae. Phylogeny inferences with UPGMA method indicated that the two sub-families formed a well supported paraphyletic clade.

Little is known about the genetic or evolutionary origin of grain *Amaranthus*, and without such knowledge scientific breeding, especially making use of biotechnological methods, is not possible (Sammour et al., 2012). Studies on chromosome number and hybrid fertility (Gupta & Gudu, 1991; Pal & Khoshoo, 1972), RAPD, restriction site variation of chloroplast and nuclear DNA (Lanoue et al., 1996) have already clarified some aspects regarding genetic diversity and evolutionary relationships among grain

amaranths and their wild relatives. These studies have allowed the development of some hypotheses about the geographical origin of species and establish phylogenetic links between them (Popa et al., 2010).

Mandal and Das (2002) demonstrated a high level of genetic similarity between *A. hypochondriacus* and *A. caudatus*. RAPD analysis was performed to study the genetic diversity in three grain amaranths *viz*., *A. hypochondriacus*, *A. caudatus* and *A. cruentus* comprising a total of 17 accessions. The RAPD profile developed from primer 1 and 2 indicated a strong possibility of a single common progenitor of these three grain amaranth species. Genetic variation and genetic relationships of a total of 23 species and 60 populations of cultivated and wild amaranths were performed using isozyme and RAPD markers **(**Chan, 1996). High levels of interspecific and intraspecific variations were found between the investigated species and populations. The polymorphism assays clarified the relationships of grain amaranths (*A. caudatus*, *A. cruentus* and *A. hypochondriacus*) and their putative ancestors (*A. hybridus*, *A. powellii* and *A. quitensis*), and the results point toward a monophyletic origin of the grain amaranths. Isozyme analysis has been used for over 60 years for various purposes in biology, such as to delineate phylogenetic relationships, to estimate genetic variability and taxonomy, to identify cultivars and genes and to study population genetics and developmental biology **(**Sammour et al., 2007; El-Esawi, 2008; Rahman, 2001).

Phylogenetic relationship of grain amaranths and their wild relatives and taxonomic confusion that exists among three cultivated grain amaranths, *A. cruentus*, *A. caudatus* and *A. hypochondriacus*, and their putative wild progenitors, *A. hybridus*, *A. quitensis* and *A. powellii* was re-examined using ITS, AFLP (Amplified Fragment Length Polymorphism) and ISSR (Inter Simple Sequence Repeat) markers (Xu & Sun, 2001). Low ITS divergence in

these taxa resulted in poorly resolved phylogeny. However, extensive polymorphisms exist at AFLP and ISSR loci both within and among species. In phylogenetic trees based on either AFLP or ISSR or the combined data sets, nearly all intraspecific accessions can be placed in their corresponding species clades, indicating that these taxa are well-separated species. The AFLP trees share many features in common with the ISSR trees, showing a close relationship between *A. caudatus* and *A. quitensis*, placing *A. hybridus* in the same clade as all grain amaranths, and indicating that *A. powellii* is the most divergent taxon in the *A. hybridus* species complex. This study has demonstrated that both AFLP and double-primer fluorescent ISSR have a great potential for generating a large number of informative characters for phylogenetic analysis of closely related species, especially when ITS diversity is insufficient.

The present study strongly supports previous interpretations regarding interrelationships among grain amaranths, *viz*., *A. caudatus*, *A. cruentus* and *A. hypochondriacus*, since the phylogenetic trees from rbcL, matK and ITS region of rDNA showed the occurrence of 3 grain amaranth species in a single subcluster with node support of 60%, 95% and 67% respectively. Most of the study yielded a common inference that all grain amaranths have evolved from the weedy progenitor, *A. hybridus*. Grain species showed close relation with *A. hybridus* than other weedy species (Das, 2016).

Several species of *Amaranthus* are considered as weeds. Ten *Amaranthus* species occur as major weeds throughout the U. S. which includes monoecious species such as *A. retroflexus*, *A. hybridus*, *A. powellii*, *A. spinosus*, *A. albus* and *A. blitoides* and dioecious species *viz*., *A. rudis*, *A. tuberculatus*, *A. palmeri* and *A. arenicola* (Great Plains Flora Association, 1986; Horak et al., 1994). These weeds are annual herbs and prolific seed producers. They compete with crops and may cause yield reductions and

harvest problems (Klingman & Oliver, 1994; Knezevic et al., 1997; Murphy et al., 1996). For many years, several weedy Amaranthaceae species were identified incorrectly as *A. retroflexus* or *A. hybridus*, and much of the early literature improperly identified these species (Wax, 1995). Many of the species exhibit similar morphological characteristics, especially in the vegetative stage, making it difficult to distinguish between them (Horak et al., 1994). The great morphological variation within species and the possibility that some of the species may intercross and form hybrids with intermediate morphological traits (Murray, 1940; Sauer, 1967) add to identification difficulties. Precise identification of the species is important for herbicide selection as well as for biological and ecological studies. DNA analysis has been used for species identification (Transue et al., 1994). It allows for precise identification without the variation associated with morphological traits or environmental conditions. An identification system utilizing DNA analysis for species of *Amaranthus* would be useful for gene flow, hybridization and phylogeny studies among and within the weedy species (Wetzel et al., 1999).

Wetzel et al. (1999) re-initiated to develop a molecular marker identification system utilizing restriction enzyme analysis of amplified ribosomal DNA (rDNA) for the identification of weedy amaranth species, commonly referred to as pigweeds. A set of PCR markers was developed to distinguish 10 species of pigweeds. Restriction-site variation utilizing five endonucleases within the ITS region of the rDNA allowed for the positive identification of the species. According to them these markers are useful for biological and ecological studies of the genus.

The genetic relationships among 8 weedy *Amaranthus* species with 141 specimens representing 98 accessions were studied by Wassom and Tranel (2005). Weedy *Amaranthus* species frequently cause economically

significant reductions in crop yields. Accurate identification of *Amaranthus* species is important for efficient weed control, but *Amaranthus* species can interbreed, which might cause difficulty when identifying hybrid-derived specimens. To determine which of the several economically important weedy amaranth species are most genetically similar, and thus most likely to produce viable hybrids, they performed AFLP studies. The study resulted in the grouping of weedy amaranth species into four principal clusters composed of palmer amaranth (*A. palmeri*) and spiny amaranth (*A. spinosus*); powell amaranth (*A. powellii*), redroot pigweed (*A. retroflexus*) and smooth pigweed (*A. hybridus*); waterhemp (*A. tuberculatus*) and sandhills amaranth (*A. arenicola*); and tumble pigweed (*A. albus*). This generally agrees with the classification of Mosyakin and Robertson (1996), which divides *Amaranthus* into three subgenera: Acnida, composed only of dioecious species, including palmer amaranth, waterhemp and sandhills amaranth; Amaranthus, including powell amaranth, redroot pigweed and smooth pigweed; and Albersia, including tumble pigweed. The cluster analysis provided evidence suggesting hybridization among powell amaranth, redroot pigweed and smooth pigweed. Further investigations using molecular analysis of the ribosomal ITS region supported this notion (Wassom & Tranel, 2005).

The present study also agrees with the close genetic relationship among weedy amaranth species. The phylogram from ITS region of rDNA showed the occurrence of weedy amaranth species such as *A. arenicola* and *A. tuberculatus* in one subcluster and *A. palmeri* together with *A. spinosus*, of the present study in another subcluster. The other weedy species *viz*., *A. powellii* and *A. retroflexus* together with *A. hybridus* subsp. *hybridus* of the present study were placed in adjoining clades with higher node support. The close clustering of *A. palmeri* and *A. albus* in the matK tree and *A. retroflexus*, *A. spinosus* and *A. hybridus* in the rbcL tree further confirmed the close genetic similarity among weedy amaranth species.

The grouping of monoecious (*A. spinosus*) and dioecious (*A. palmeri*) amaranth species in a single cluster with a high bootstrap support of 87% observed in the phylogram of ITS region of rDNA was unexpected. The same observation was reported in a previous study by Wassom and Tranel (2005), while studying the genetic relationships among 8 weedy *Amaranthus* species. According to Murray (1940), although spiny amaranth is typically monoecious, its flowers unlike those of the other monoecious amaranth species are spatially separated (male flowers are borne terminally toward the top of the plant and female flowers are borne lower on the plant in leaf axils). According to Mosyakin and Robertson (1996) spiny amaranth might be in a transition stage towards developing dioecious flowering condition, indicating a possible genetic relationship with the dioecious palmer amaranth.

Herbal products available to consumers in the market may be contaminated or substituted with alternative plant species and fillers that are not listed on the labels. According to the World Health Organization, the adulteration of herbal products is a threat to consumer safety. Many studies are ongoing to investigate herbal product integrity and authenticity with the goal of protecting consumers from health risks associated with product substitution and contamination. There are limitations for identifying the species of the various ingredients used in herbal products. This is because the diagnostic morphological features of the plants on which the current Linnaean taxonomic system is based cannot typically be assessed from powdered or otherwise processed biomaterials. As a result, the marketplace is prone to contamination and possible product substitution, which dilute the effectiveness of otherwise useful remedies, lowering the perceived value of all related products because of a lack of consumer confidence in them (Newmaster et al., 2013).

Herbal product substitution has been documented for many individual medicinal plant species (Techen et al., 2004; Song et al., 2009; Srirama et al., 2010) and nutraceuticals (Jaakola et al., 2010; Bruni et al., 2010). Although there is limited research available, the frequency of product mislabeling in herbal products has been estimated at 14% to 33% from previous studies (Stoeckle et al., 2011; Baker, 2012; Wallace et al., 2012). DNA barcoding has been used to test a number of natural products in a rapid, accurate and cost-effective manner (Stoeckle et al., 2011; Wallace et al., 2012; Sui et al., 2011). For example, Srirama et al. (2010) has demonstrated the efficiency of DNA barcoding in identifying adulterants in the raw drug trade of *Phyllanthus* (Euphorbiaceae). *Phyllanthus* is one of the most important groups of species traded as a raw herbal drug which is used for the treatment of liver disorders (Ved & Goraya, 2007). This study showed that plant DNA barcoding can effectively discriminate *Phyllanthus* species, and hence can be used to resolve species admixtures in the raw drug trade samples. There are several phenotypically similar species that could easily be misidentified and mixed within herbal products. Over the last 3 years, studies of Chinese plant medicine (Gao et al., 2010 b; Chen et al., 2010; Guo et al., 2011; Li et al., 2011; Xue & Li, 2011; Pang et al., 2012) have documented the potential scope and magnitude of marketplace substitution using DNA barcoding. Currently there is a gap in our understanding to the extent of herbal product substitution, contamination and use of fillers. Also, there are no studies that have used a standard reference material (SRM) herbal barcode library and barcode regions that facilitate better species resolution (Newmaster et al., 2013).

A study conducted by Newmaster et al. (2013) detected contamination and substitution in North American herbal products with the help of DNA barcoding. They recovered DNA barcodes from most herbal products (91%) and all leaf samples (100%), with 95% species resolution using a tiered

approach (rbcL + ITS2). Most (59%) of the products tested contained DNA barcodes from plant species not listed on the labels. Most of the herbal products tested were of poor quality, including considerable product substitution, contamination and use of fillers. The study suggested that the herbal industry should embrace DNA barcoding for authenticating herbal products through testing of raw materials used in manufacturing products. The use of an SRM DNA herbal barcode library for testing bulk materials could provide a method for 'best practices' in the manufacturing of herbal products.

Correct genotype identification is important to evaluate the genetic diversity of amaranth species. Identification and preservation of germplasm are necessary for maintaining genetic diversity, studying local genetic material in order to choose ecotypes having high nutritional interest in their place of origin and initiating breeding programs (Perez- Gonzalez, 2001). At its peak, the Rodale Research Center collection (RRC, Pennsylvania, USA) contained approximately 1,400 accessions of *Amaranthus*, mostly represented by *A. cruentus*, *A. hypochondriacus*, *A. caudatus*, *A. tricolor* and *A. dubius* (Brenner et al., 2010). The *Amaranthus* germplasm collection of the Plant Introduction Station (USDA) includes approximately 3,000 accessions, and half of the species in the genus (Brenner et al., 2010) and species in high numbers include *A. hypochondriacus*, *A. caudatus*, *A. cruentus*, *A. hybridus* and *A. tricolor*. The National Botanical Research Institute (NBRI, India) has one of the best qualitative collections of amaranth germplasm in the world with over 2,500 accessions referable to 20 species (Mathews, 2001). The World Vegetable Center (AVRDC) holds a collection of circa 520 acessions of 18 amaranth species (http[23]). Other germplasm collections are held by at least 60 different groups or institutions, although most of these tend to have fewer than 100 entries (Brenner et al., 2010).

Molecular phylogenetics applies a combination of molecular and statistical techniques to infer evolutionary relationships among organisms or genes. The primary objective of molecular phylogenetic studies is to recover the order of evolutionary events and represent them in evolutionary trees that graphically depict relationships among species or genes over time. Phylogenetics infers trees from observations about existing organisms using morphological, physiological and molecular characteristics (Dowell, 2008). Three DNA sequence based markers rbcL, matK (chloroplast) and ITS (nuclear) were explored in the present study. The sequence data were subjected to pairwise distance and Bayesian Inference analyses. Irrespective of the method used for tree construction, all three markers gives similar tree topologies having 2 major clusters one with species of the genus *Amaranthus* and the other with remaining out-groups used (Plates 5-7). Comparatively, ITS region of rDNA sequences (Plate 4) showed greatest number of polymorphic sites. Closely related species of *Amaranthus*, species of grain amaranths and weedy amaranths shared high sequence similarity and thereby rightly positioned in single/adjoining clusters. All together the present phylogenetic characterization revealed the monophyletic clustering of collected *Amaranthus* species together with their conspecifics, which depicts the species relatedness and identity.

2. CYTOGENETICAL CHARACTERIZATION

Karyomorphological study has been undertaken to characterize eight species of the genus *Amaranthus*. Karyosystematics is regarded as one of the most relevant parameter in the evaluation of the genetic relationship and divergence among species or populations (Guerra, 2008). Survey of the earlier literatures have revealed that different workers attempted to report the chromosome number of different species of *Amaranthus* but in most cases, the reports were limited to chromosome count only. In the present study the

species have been shown to be characterized by well defined and morphologically separable karyotypes. Although there are some similarities in the chromosome morphology, the species under study have been shown to differ from each other in detailed karyomorphometric features. Karyotype analysis has been proved to be very effective for assessing taxonomic relationships in many cases (Cristina et al., 2005; Yuan & Yang, 2006; Bonasora et al., 2013; Mandáková & Lysak, 2008; Gao et al., 2012). Comparative karyotype analysis of closely related species has been performed in many cases to explain patterns and directions of chromosomal evolution and to deduce the evolutionary role of karyotype changes (Vanzela et al., 2000; Shan et al., 2003). Considering the importance of karyotype analysis, the present study was carried out to investigate the effectiveness of karyotype data for the assessment of future phylogenetic studies in the genus *Amaranthus*.

MITOTIC CHROMOSOME STUDIES

Karyotypic analysis is one of the most important and established criteria to determine the interrelationship among the plant groups. The data on chromosome number and comparative karyomorphology is very significant to comprehend the genome structure, its organization and evolution within the genus at inter- and intra specific levels (Stace, 2000; Cao, 2003). The eight species of *Amaranthus viz*., *A. blitum*, *A. caudatus*, *A. dubius*, *A. hybridus* subsp. *hybridus*, *A. hybridus* subsp. *cruentus*, *A. spinosus*, *A. tricolor* and *A. viridis* (Plate 1) were subjected to chromosome analysis through karyomorphological studies and marked differences in chromosome counts and microscopic aspects (length, centromere position) were observed. The chromosome number was found to be 2n = 34 for *A. blitum*, *A. hybridus* subsp. *cruentus*, *A. spinosus* and *A. tricolor*, 2n = 32 for *A. caudatus*, *A. hybridus* subsp. *hybridus* and *A. viridis* and 2n = 64 for *A. dubius*. The

chromosome counts observed was in agreement with previous reports (Table 1). Chromosomal studies dealing with somatic chromosome number determination and its ploidy level are of fundamental consideration for any given species in understanding the basic structure of the genetic complement (Chakraborti et al., 2010). The species *A. dubius* [2n=64 (n = 32)] is the only tetraploid species observed. Chromosome numbers resulting from variations due to polyploidy and dysploidy have significant role in phylogeny and karyotype evolution (Guerra, 2000). Cytogenetics of *A. dubius* is doubtful and it was believed that *A. dubius* is a natural hybrid of *A. hybridus* and *A. spinosus*. However, Pal and Khoshoo (1965) have provided evidence that *A. spinosus* could not have been the progenitor species for *A. dubius*. Ranade et al. (1997) supported the statement and provided evidences from a genome analysis study of *Amaranthus* using RAPD and also mentioned about the close similarity of *A. caudatus* with *A. dubius* rather than *A. spinosus*. The present molecular characterization study using three gene regions showed the close clustering of *A. hybridus* subsp. *hybridus*, *A. caudatus* and *A. cruentus* with *A. dubius* rather than *A. spinosus*.

Polyploidy has been ubiquitous in plant evolution and is thought to be an important engine of biodiversity that facilitates speciation, adaptation and range expansion. Polyploid species can exhibit higher ecological tolerance than their progenitor species. For allotetraploid species, this higher tolerance is often attributed to the existence of heterosis, resulting from entire genome duplication. However, multiple origins of allopolyploid species may further promote their ecological success by providing genetic variability in ecological traits underlying local adaptation and range expansion (Meimberg et al., 2009).

Basic chromosome number is an important character to determine the systematic position of a taxon at higher taxonomic level (Raven, 1975). Both

primary and secondary base numbers are involved in the evolution of *Amaranthus* species. Previous data have indicated that the genus *Amaranthus* is dibasic as it showed two basic chromosome numbers such as x = 8 and 9 (Turner, 1994; Grant 1959 c, b, a). The karyotype of eight species revealed in the present study showed somatic chromosome numbers of 2n = 32, 34 and 64 (Plates 8-24) and three possible gametic chromosome numbers such as n = 16, 17 and 32 which was further confirmed by the haploid chromosome complements observed in the meiotic cells (Plate 25). Three gametic numbers *viz*., n = 14, 16 and 17 were reported in the genus *Amaranthus* previously. Pal et al. (1982) suggested that the gametic number n = 17 has evolved from n = 16 through primary trisomy. Greizerstein and Poggio (1992) supported this hypothesis through the analysis of meiotic behaviour of species and interspecific hybrids. The new gametic number n = 14 was reported previously by Pal et al. (2000) in *A. tenuifolius* (2n = 28). The reports of Pal et al. (2000) was confirmed by a recent cytogenetic study in *A. blitum* (2n = 28) by Srivastava and Roy (2014). The reduction in chromosome number from n = 16 to n = 14 was suggested to be due to aneuploidy or dysploid reduction.

In the present investigation, the genus *Amaranthus* seems to be dibasic with the primary base numbers (x_1) 8 and 9. The diploid chromosome number, 2n = 32 obtained for *A. caudatus*, *A. hybridus* subsp. *hybridus* and *A. viridis* might have originated from secondary basic chromosome number of x_2 = 16. Doubling of primary base number x_1 = 8 by protoautoploidy leads to the formation of secondary base number x_2 = 16, from which the normal chromosome number of 2n = 32 might have originated (Plate 26). The doubling of 2n = 32 might have resulted in the formation of the tetraploid (2n = 64) condition observed in *A. dubius*.

The chromosome number 2n = 34 obtained for *A. blitum*, *A. hybridus* subsp. *cruentus*, *A. spinosus* and *A. tricolor* might have originated by three

possible ways. Doubling of primary base number $x_1 = 8$ by protoautoploidy leads to the formation of secondary base number $x_2 = 16$, which undergoes an ascending dysploidy leading to an increase in a chromosome in the secondary basic set forming $x_2 = 17$ from which the normal chromosome number of 2n = 34 might have originated (Plate 26).

Doubling of primary base number $x_1 = 9$ by protoautoploidy leads to the formation of secondary base number $x_2 = 18$, which undergoes a descending dysploidy leading to a decrease in a chromosome in the secondary basic set forming $x_2 = 17$ from which the normal chromosome number of 2n = 34 might have originated (Plate 26).

Joining of the two primary base numbers $x_1 = 8$ and $x_1 = 9$ leads to the formation of the secondary base number $x_2 = 17$ by amphiploidy and there after doubling of $x_2 = 17$ forms cells with 2n = 34 chromosomes. Amphiploid origin of basic chromosome number is a comparatively primitive condition (Jones, 1978). Phylogenetic scheme showing the probable evolution of chromosome numbers in the *Amaranthus* species investigated was represented in Plate 26. These mechanisms either singly or together might have contributed to the formation of normal chromosome complements observed in *Amaranthus* species during the course of evolution (Plate 26).

The chromosome number for *Amaranthus* species is normally 2n = 32 (n = 16), but occasionally it is 34 (n = 17). It has been observed that both gametic number (n = 17 and 16) has been reported for the same species (Grant, 1959 c; Pal & Khoshoo, 1972; Pal et al*.,* 1982). In addition to chromosome number, karyotypic work has demonstrated that *Amaranthus* chromosomes are small sized (Grant, 1959 a; Grant, 1959 c; Greizerstein & Poggio, 1994); possess a single NOR locus and demonstrate a wide variety of C banding patterns putatively due to heterochromatin content (Greizerstein & Poggio, 1994). In a study by Bonasora et al. (2013), the distribution and

variability of constitutive heterochromatin were detailed using DAPI-CMA3 banding technique. The position of the nucleolus organizer region (NOR) was observed using Ag-NOR banding (active loci) and fluorescent *in situ* hybridization (rDNA-FISH) in four *Amaranthus* species. Cytogenetic analyses, as a description of chromosome number and size, as well as the banding pattern and position of the centromere, frequently contribute to the understanding of evolution in plants (Shan et al., 2003) and to the elucidation of factors that have been involved in the evolutionary diversification of the taxon (Pedrosa et al., 2000; Vilatersana et al., 2000).

Apart from the earlier reports on the chromosome counts, the karyomorphometric studies in detail have not yet been attempted in the genus. It may be due to the small chromosome size as well as indistinguishable primary constriction. This is evident from the morphometric details of the karyotype in the species studied, wherein the range of chromosome length of each chromosome complement does not exceed 2.5 µm. The ACL of all the eight species ranged between 1.21 µm – 1.69 µm which established the small size of chromosomes (Table 22). In comparison the size of chromosome showed a decrease in the order of *A. spinosus* > *A. tricolor* > *A. dubius* > *A. viridis* > *A. blitum* > *A. hybridus* subsp. *cruentus* > *A. caudatus* > *A. hybridus* subsp. *hybridus*. The differences in the chromosome length and volume may be attributed to differential spiralization and condensation of chromosomes along with the content of protein and DNA (Ramachandran, 2012). According to Grant (1959 c) the small size of the chromosomes has made detailed karyomorphological studies impractical. The size variation observed in the somatic chromosome complements of *Amaranthus* species reveals a tendency towards asymmetry. Stebbins (1971) suggested that asymmetrical karyotypes are more advanced than symmetrical ones in relation to phylogeny and evolutionary processes. Karyotypic asymmetry is involved in the speciation process, and symmetrical karyotypes are the more ancestral condition

(Rodrigues et al., 2012). Since chromosomes are not rigidly stable structures, chromosome variations play an important role during evolution in all species (Souzad & Benko-Iseppon, 2004).

A. blitum

The somatic chromosome number was found to be 2n = 34 (Plate 8, 9; Plate 24 a) with a gametic number of n = 17 (Plate 25 a). The chromosome length ranged between 2.10 µm to 1.11 µm summing upto a total of 54.08 µm in length (Table 22). The various parameters of DI, VC, TF%, As K%, Syi and Rec indices, A_1, A_2 and A indices and AI index were calculated and was found to be 30.84, 17.41, 43.34, 56.65, 75.55, 75.74, 0.21, 0.17, 0.12 and 0.11 which was used to determine the asymmetry index as well as evolutionary progress of the species.

A. caudatus

The somatic chromosome number was found to be 2n = 32 (Plate 10, 11; Plate 24 b) with a gametic number of n = 16 (Plate 25 b). The chromosome length ranged between 1.76 µm to 0.86 µm summing upto a total of 38.86 µm in length (Table 22). The various parameters of DI, VC, TF%, As K%, Syi and Rec indices, A_1, A_2 and A indices and AI index were calculated and was found to be 34.35, 21.65, 44.10, 56, 78, 69, 0.21, 0.21, 0.11 and 0.12.

A. dubius

The somatic chromosome number was found to be 2n = 64 (Plate 12, 13; Plate 24 c) with a gametic number of n = 32 (Plate 25 c). The chromosome length ranged between 2.24 µm to 1.03 µm summing upto a total of 94.12 µm in length (Table 22). The various parameters of DI, VC, TF%, As K%, Syi and Rec indices, A1, A_2 and A indices and AI index were

calculated and was found to be 37, 20.37, 42.77, 57.22, 73.80, 65.65, 0.20, 0.20, 0.12 and 0.14.

A. hybridus* subsp. *hybridus

The somatic chromosome number was found to be 2n = 32 (Plate 14. 15; Plate 24 d) with a gametic number of n = 16 (Plate 25 d). The chromosome length ranged between 1.71 µm to 0.93 µm summing upto a total of 39.66 µm in length (Table 22). The various parameters of DI, VC, TF%, As K%, Syi and Rec indices, A_1, A_2 and A indices and AI index were calculated and was found to be 29.54, 18.09, 42.76, 57.18, 75.71, 72.47, 0.39, 0.18, 0.26 and 0.09.

A. hybridus* subsp. *cruentus

The somatic chromosome number was found to be 2n = 34 (Plate 16, 17; Plate 24 e) with a gametic number of n = 17 (Plate 25 e). The chromosome length ranged between 1.93 µm to 0.77 µm summing upto a total of 44.64 µm in length (Table 22). The various parameters of DI, VC, TF%, As K%, Syi and Rec indices, A_1, A_2 and A indices and AI index were calculated and was found to be 42.96, 22.96, 42, 58, 72.36, 68.02, 0.36, 0.22, 0.15 and 0.16.

A. spinosus

The somatic chromosome number was found to be 2n = 34 (Plate 18, 19; Plate 24 f) with a gametic number of n = 17 (Plate 25 f). The chromosome length ranged between 2.43 µm to 1.34 µm summing upto a total of 57.68 µm in length (Table 22). The various parameters of DI, VC, TF%, As K%, Syi and Rec indices, A_1, A_2 and A indices and AI index were calculated and was found to be 28.91, 17.37, 34.18, 65.81, 52.25, 69.81, 0.41, 0.17, 0.33 and 0.14.

A. tricolor

The somatic chromosome number was found to be 2n = 34 (Plate 20, 21; Plate 24 g) with a gametic number of n = 17 (Plate 25 g). The chromosome length ranged between 2.34 µm to 0.99 µm summing upto a total of 47.32 µm in length (Table 22). The various parameters of DI, VC, TF%, As K%, Syi and Rec indices, A_1, A_2 and A indices and AI index were calculated and was found to be 40.54, 27.31, 29.33, 70.66, 40.81, 59.47, 0.53, 0.27, 0.40 and 0.35.

A. viridis

The somatic chromosome number was found to be 2n = 32 (Plate 22, 23; Plate 24 h) with a gametic number of n = 16 (Plate 25 h). The chromosome length ranged between 2.22 µm to 1.12 µm summing upto a total of 47.84 µm in length (Table 22). The various parameters of DI, VC, TF%, As K%, Syi and Rec indices, A_1, A_2 and A indices and AI index were calculated and was found to be 32.93, 21.51, 36.12, 63.83, 56.84, 67.34, 0.39, 0.21, 0.26 and 0.19.

There are differences in karyotype formula and asymmetry indices among species. The karyotype formulae obtained for *A. blitum*, *A. caudatus*, *A. dubius*, *A. hybridus* subsp. *hybridus*, *A. hybridus* subsp. *cruentus*, *A. spinosus*, *A. tricolor* and *A. viridis* was 2M + 28nm + 4nsm(-), 32nm, 56nm + 8nsm(-), 26nm + 6nsm(-), 30nm + 4nsm(-), 20nm + 4nsm(-) + 2nsm(+) + 2nst(-) + 6T, 8nm + 18nsm(-) + 2nsm(+) + 6T and 12nm + 18nsm(-) + 2T. The chromosomes of *A. caudatus* showed symmetrical karyotype since all the chromosomes possessed nearly median centromere with relatively equal arm length, whereas karyotype formula of *A. tricolor* and *A. viridis* showed more numbers of chromosomes with nearly sub-median centromeres rather than median and nearly median, which indicates its asymmetric karyotype.

Karyotype asymmetry is a good expression for the general morphology of karyotype in plants. Changes in morphological characters of the genome have been frequently related to evolution in higher plants (Zarco, 1986). Karyotype studies were principally based on the fact that symmetrical karyotypes are more primitive than asymmetrical ones. Primitiveness of karyotypes are expressed by the presence of symmetrical karyotype, having longer chromosomes, median centromeres with chromosome arms of equal size and low basic chromosome numbers while the more advanced karyotype features depicts asymmetrical karyotypes, shorter chromosomes, submedian or other centromere, unequal length of chromosome arms and higher basic chromosome numbers. Karyotype asymmetry can be considered to be the driving force behind speciation since symmetrical karyotype represents ancestral condition in evolution (Stebbins, 1971). Hence parameters determining asymmetry of karyotype can be used to interpret evolutionary status of a species. According to Gunjan and Roy (2010), karyotype asymmetry can be explained by shifting of the centromeric position from median to subterminal or through differences in relative size between individual chromosomes.

Various parameters used to assess the karyotype asymmetry or symmetry and evolutionary status of the species includes DI, VC, TF%, As K%, Syi-Rec indices, A_1-A_2 indices, A and AI index (Table 22) along with a population dispersion plot based on A_1-A_2 parameters (Fig. 2). These parameters described the karyotype asymmetry on the basis of variations in chromosome length and centromeric position. Low values of TF%, Syi and Rec indices indicate increasing asymmetry of a karyotype whereas an increase in As K%, A_1, A_2, A and AI values also point towards an asymmetric karyotype.

Disparity index (DI) values are employed for assessing the karyotype asymmetry of the species studied. DI values represent the chromosome size variations. The low values of DI indicate homogeneity of chromosomes whereas high values represent the heterogeneous assemblage of chromosomes (Mohanty et al., 1991). Based on DI value, the heterogeneous assemblage of chromosomes were more prominent in *A. hybridus* subsp. *cruentus* (42.96) followed by *A. tricolor* (40.54), *A. dubius* (37), *A. caudatus* (34.35), *A. viridis* (32.93), *A. blitum* (30.84), *A. hybridus* subsp. *hybridus* (29.54) and *A. spinosus* (28.91). The intermediary DI values might be an indication of the occurrence of the species in an evolutionary path.

The minute variations among chromosome complements can be determined from the values of variation coefficient (VC). Variations in chromosome size and its distribution are very well established by VC parameter. Low VC values depict homogeneity and primitiveness of the karyotype (Stebbins, 1959). Based on VC value, the heterogeneous chromosomes were more prominent in *A. tricolor* (27.31) whereas homogeneous chromosomes were more prominent in *A. spinosus* (17.37) as observed for DI. The VC values were also found to be intermediary as in DI, which represents all the species are in an evolutionary path.

The total forma percentage (TF%) or mean centromeric index has been proposed by Huziwara (1962) and it is a frequently used method to evaluate the karyotype symmetry/asymmetry and the karyotypic relationship between species (Costa & Forni-Martins, 2003). TF% provides data to infer about karyotype affinity in related taxa and it measures the extent of centromeric changes in a species. The changes in centromeric position of the species studied leads to variations in TF% values. The TF% of 50 depicts absolute symmetry of a karyotype, which indicates median centromeres for all the chromosomes. Absolute asymmetry of karyotype can be attributed to a TF%

of zero, which implies terminal centromeres for all the chromosomes (Kapoor, 1977). Thus the reduction in TF% value leads to increase in karyotype asymmetry. The TF% obtained for the studied taxa was as follows, *A. tricolor* (29.33), *A. spinosus* (34.18), *A. viridis* (36.12), *A. hybridus* subsp. *cruentus* (42), *A. hybridus* subsp. *hybridus* (42.76), *A. dubius* (42.77), *A. blitum* (43.34), *A. caudatus* (44.10). As far as the TF% is concerned the most asymmetric karyotype is for *A. tricolor* as it recorded lowest TF% value whereas most symmetric karyotype for *A. caudatus* with highest TF% as it showed all the chromosomes in the karyotype with nearly median centromere (32nm). Increasing asymmetry of the karyotype of *A. tricolor* occur either through the shift of centromere position from median/submedian to terminal or subterminal, or through the accumulation of differences in the relative size between the chromosomes of the complement, thus making the karyotype more heterogeneous (Stebbins, 1971). Here the karyotype of *A. tricolor* showed 2n = 34 chromosomes, which include 8 nm, 18 nsm(-), 2nsm(+) and 6 T chromosomes with a size range of 2.34 – 0.99 µm. The predominance of nearly submedian and terminal centromeres rather than median and nearly median might have been the reason for the asymmetric karyotype observed in *A. tricolor*, which marks the path of evolution of the species from a primitive to advanced one. The low TF% values of *A. spinosus* and *A. viridis* also highlights the nearly submedian, nearly subterminal and terminal type of chromosomes that results in asymmetric karyotype. All the other species of *Amaranthus* showed a TF% of above 40, which represents the predominance of sub-metacentric chromosomes with apparent symmetry.

Greilhuber and Speta (1976) developed two indices, the index of karyotype symmetry and the index of chromosomal size resemblance, to evaluate karyotype asymmetry. These two indices were later called by Venora et al. (2002) as the Syi index and the Rec index, respectively. The values of these indices theoretically ranged from 0 to 100. The Syi value indicates the

ratio of the mean length of the short arms against the mean length of the long arms in a chromosome set. The Rec index expresses the mean of the ratios of the length of each chromosome (CLi) to that of the longest one (LC) (Paszko, 2006). Both indices have been used to estimate karyotype asymmetry and to discuss the relationships between *Amaranthus* species. The highest karyotype asymmetry of *A. tricolor* was supported by the positive correlation between TF% and Syi-Rec indices, because both Syi (40.81) and Rec (59.47) value obtained for *A. tricolor* was found to be lowest among the species studied, which highlights its asymmetric karyotype. Following *A. tricolor*, low Syi index was observed for *A. spinosus* (52.25) then for *A. viridis* (56.84) and all other species showed higher values above 70 indicating a shift toward symmetric karyotype. As far as the TF% and Syi index are concerned the most symmetric karyotype was observed in *A. caudatus*, but Rec index depicted the most symmetric karyotype for *A. blitum*, which means that individual parameters cannot produce a conclusive remark in karyotype asymmetry studies. Hence combinations of various parameters are required to identify the karyotype asymmetry as well as evolutionary advancement within a genus (Neelamkavil, 2015).

Karyotype asymmetry with respect to the position of centromere in a chromosome complement was also studied by using As K%, A_1, A_2, A and AI indices. Increasing asymmetry depicts increase in the values of As K%, A_1, A_2, A and AI indices. Arano (1963) introduced the karyotype asymmetry index, the As K%, which was used to determine the phylogenetic relations and chromosome evolution between and within the species of a genus. The As K% index is expressed by the ratio of the sum of the lengths of the long arms of individual chromosomes to the total length of the chromosome complement (Arano, 1963). Analysis revealed that As K% index shows a negative correlation with the TF% and Syi-Rec indices. Examination of chromosome morphology revealed that highest karyotype asymmetry was observed for *A.*

tricolor (70.66) and most symmetrical karyotype was for *A. hybridus* subsp. *hybridus* (57.18). Following *A. tricolor* the highest asymmetric karyotype was found for *A. spinosus* (65.81), then for *A. viridis* (63.83) and all the other species showed As K% value below 60. The higher asymmetry of the karyotypes of *A. tricolor*, *A. spinosus* and *A. viridis* when compared to other species highlights their evolutionary advancement.

Zarco (1986) provided an alternative method for measuring karyotype asymmetry by using quantification and graphic representation. He proposed two numerical parameters to estimate karyotype asymmetry, the intrachromosomal asymmetry index (A_1) and the interchromosomal asymmetry index (A_2). This new method can detect even there are slight differences in karyotype asymmetry when karyograms and idiograms are available (Zarco, 1986). Karyotype asymmetry for the relations between the chromosome arms has been estimated using A_1 index and their values ranging from zero to one. The A_1 index does not depend on chromosome number or chromosome size. The equation is formulated in order to obtain lower values when chromosomes tend to be metacentric (Zarco, 1986). The A_1 index obtained for eight *Amaranthus* species are as follows; *A. tricolor* (0.53), *A. spinosus* (0.41), *A. viridis* (0.39), *A. hybridus* subsp. *hybridus* (0.39), *A. hybridus* subsp. *cruentus* (0.36), *A. blitum* (0.21), *A. caudatus* (0.21) and *A. dubius* (0.20). From the results it was clear that the highest intrachromosomal asymmetric karyotype was for *A. tricolor* as it recorded the highest value and the most symmetric karyotype was for *A. dubius*. Following *A. tricolor* higher intrachromosomal asymmetry was observed for the karyotypes of *A. spinosus*, *A. viridis* and *A. hybridus* subsp. *hybridus* respectively.

Karyotype asymmetry due to relations between sizes of different chromosomes has been estimated using Pearson's dispersion coefficient (A_2), that is, the ratio between the standard deviation and the mean of chromosome

length for each sample. The interchromosomal asymmetry index (A_2), provides an easy way of estimating chromosome length, which does not depend on chromosome number (Zarco, 1986). The interchromosomal asymmetry index observed for different *Amaranthus* species was 0.27 for *A. tricolor*, 0.22 for *A. hybridus* subsp. *cruentus*, 0.21 for *A. viridis* and *A. caudatus*, 0.20 for *A. dubius*, 0.18 for *A. hybridus* subsp. *hybridus*, 0.17 for *A. blitum* and *A. spinosus*. Among the species, *A. tricolor* showed highest asymmetry with respect to chromosome length (A_2) in the karyotype as it recorded higher value. Whereas, asymmetry with respect to chromosome length was least in *A. blitum* and *A. spinosus*. The degree of karyotype asymmetry (A) values was found to be 0.12 for *A. blitum* and *A. dubius*, 0.11 for *A. caudatus*, 0.26 for *A. hybridus* subsp. *hybridus* and *A. viridis*, 0.15 for *A. hybridus* subsp. *cruentus*, 0.33 for *A. spinosus* and 0.40 for *A. tricolor*. Comparatively highest A value was observed for *A. tricolor*, followed by *A. spinosus*, *A. viridis* and *A. hybridus* subsp. *hybridus*. Based on A_1-A_2 and A indices it was revealed that *A. tricolor* possess the most asymmetric karyotype and all other species showed intermediate values. The scatter plot based on A_1-A_2 parameters also represented the highest degree of asymmetry for *A. tricolor* and intermediate condition for all the other species which means that *A. tricolor* is the most evolved species among the eight *Amaranthus* species studied (Fig. 2).

A new index, asymmetry index (AI) was developed by Paszko (2006), which has the advantage of allowing a high degree of precision and sensitivity when assessing karyotype asymmetry. Higher values of the AI index are considered to indicate higher levels of karyotypic heterogeneity (Meng et al., 2014). In the present study the AI value ranged from 0.09 to 0.35. The higher AI value of *A. tricolor* further confirmed its highest asymmetric karyotype among the species studied and the intermediate values obtained for other species indicates they are in a path of evolutionary advancement.

Karyotype asymmetry indices have been widely used to make assumptions about the mechanisms of chromosomal evolution in plants (Paszko, 2006). Stebbins (1971), writing about karyotype asymmetry, referred the Russian school of comparative karyotype morphology, led by Levitsky (1931), who developed the concept of symmetry vs. asymmetry (Paszko, 2006). Various quantitative parameters used in the present study resulted in the establishment of detailed karomorphometric data of the species studied. The karyotype images, karyograms and idiograms developed can serve as a useful tool for the cytological identity of each species. Three somatic numbers *viz*., 2n = 32, 34 and 64 was observed with predominance of nearly median centromere for all the species except *A. tricolor* and *A. viridis*, which showed more numbers of nearly submedian chromosomes. Telocentric chromosomes are observed in *A. spinosus*, *A. tricolor* and *A. viridis*. All the chromosomes in *A. caudatus* were of sub-metacentric type. One pair of metacentric chromosome was found in the karyotype of *A. blitum*. Karyotypes of all the species showed four chromosomes with secondary constriction except in the tetraploid plant *A. dubius*, which showed eight chromosomes with secondary constriction. Thus, the present study sheds light on the distinct karyotype patterns of the eight species of *Amaranthus*, which helps not only in the authentic identification of different species, but also augment our understanding of the intra-generic relationships and the structural changes that lead to the diversification of the genus (Dutta & Bandyopadhyay, 2014).

Karyotype evolution is an important aspect in evolutionary processes and considered as an isolating mechanism in speciation and has their own evolutionary trends independent of genetic evolution (Imai et al., 2001). Therefore, karyotype evolution generally tends towards an increasing number and terminal-centromeric chromosomes. The opposite tendency, the reduction of chromosome number and formation of median centromeric chromosomes are primitive (Imai et al., 2001). In the present investigation, a comparision was

made between the karyotypes of eight species of the genus *Amaranthus* to interpret the karyomorphological interrelations among them. This is one of the few attempts to make a detailed karyomorphological study in the genus. Karyomorphometric and asymmetric analysis based on chromosome number, position of centromere and variations in chromosome size within and between species revealed the karyotype asymmetry/symmetry together with their probable evolutionary status. The intermediary values obtained for all the parameters provide evidence for the role of the species in evolution wherein the eight species are in a path of evolutionary advancement. The short sized chromosomes obtained for all the species itself indicated its advanced status. By analyzing the obtained data it can be confirmed that the eight species of *Amaranthus* studied are not in a primitive stage of evolution.

The lowest values of TF%, Syi and Rec indices and highest values of VC, As K%, A_1, A_2, A and AI indices obtained for *A. tricolor* among the species studied confirmed its highest asymmetric karyotypic status. A comparative analysis of the karyotype of the eight *Amaranthus* species based on the karyomorphometric and asymmetric indices revealed that *A. tricolor* possess more heterogeneous karyotype than other species, which led to the conclusion that *A. tricolor* is the most evolved species among the eight *Amaranthus* species investigated. Differences in karyomorphological details between individuals with the same chromosome number were observed indicating the ongoing evolutionary process even at the microlevel (Neelamkavil, 2015).

A previous cytological investigation of 14 samples that belong to 6 species of *Amaranthus* [*A. viridus, A. sylvestris, A. graeccizans, A. hypochondriacus, A. cruentus* and *A. chlorostachys* (*hybridus*)] revealed that the genus is diploid with 2n = 32 and 34. Karyotypes are mainly comprised of many metacentric chromosomes and few sub metacentric ones **(**Al-Turki et

al., 2000; Hamoud et al., 1994). But the present study showed more number of chromosomes having nearly median centromere rather than median type. In addition to nearly submedian, nearly subterminal and terminal type of centromeres was also observed. In the previous study, noticeable variation among accessions in the number of chromosomes in each type was observed and accordingly *A. viridis* was the most advanced species amongst all the investigated taxa. The present study also supports the asymmetric karyotype of *A. viridis*, but the most advanced species observed was *A. tricolor* among the eight *Amaranthus* species studied. As per their study *A. viridis* exhibits the diploid number 2n = 34 and the shortest haploid genome length with more karyotypic activity, concerning chromosome length and centromeric position, recorded among its different accessions. This might have been produced as a result of differences in the degree of chromatin condensation and/or chromosomal changes such as translocations and pericentric inversions **(**Al-Turki et al., 2000; Hamoud et al., 1994). The small size of the chromosomes, together with their unclear centromeres, has hampered a detailed karyotype analysis **(**Al-Turki et al., 2000**;** Song et al., 2001). In contradiction to the above result, the present study recorded 2n = 32 chromosomes for *A. viridis*, which was further clarified from the gametic number (n = 16) observed in the meiotic cells of *A. viridis*.

From the results of karyomorphometric and asymmetric features of different *Amaranthus* species, it was clear that all species showed heterogeneity in their karyotype within and between species. On this basis, it may be inferred that the karyotype differentiation between the species, observed in the present study, may have occurred through minor alternations caused by chromosome repatterning or changes in individual gene or gene complexes which are the major sources of all genetic variability (Das & Borah, 2015). All together various karyomorphometric and asymmetry indices used in the present study were quite significant in identifying and

characterizing eight different karyotypes in the genus *Amaranthus* from a comparative study.

MEIOTIC CHROMOSOME STUDIES

Cytogenetic analysis of the eight species of *Amaranthus* comprising somatic chromosome numbers of 2n = 32, 34 and 64 showed a normal meiosis forming mainly bivalents in the meiotic cells. The gametic numbers observed in the present study was n = 16, n = 17 and n = 32, which was already reported for the genus. Species showing n = 16 are *A. caudatus*, *A. hybridus* subsp. *hybridus* and *A. viridis*, n = 17 are *A. blitum*, *A. hybridus* subsp. *cruentus*, *A. spinosus* and *A. tricolor* and n = 32 was found in *A. dubius*. Similar observations are made by Sheidai and Mohammadzadeh (2008) in a cytogenetic study of 12 populations of 10 *Amaranthus* species and varieties. The species studied showed a normal meiosis forming bivalents in metaphase of meiosis I. According to the study a post pachytene diffuse stage occurred in all the species possibly as a means of adaptation to adverse environmental conditions. In their study, ANOVA test revealed significant differences in relative cytogenetic characteristics including chiasma frequency and distribution as well as chromosome pairing among the species, indicating their genomic differences.

According to Ugborogho and Oyelana (1992), *Amaranthus* species have three ploidy levels of chromosome complements with haploid number of n = 16 for the two varieties of *A. hybridus*, n = 17 for the two varieties of *A. spinosus* and *A. viridis* and n = 32 for *A. dubius*. As per their observations all the species are characterized by aberrant meiosis except *A. dubius*, which showed high degree of meiotic abnormalities such as multivalent and stickiness of chromosomes leading to clumping, overlapping and unequal segregation of chromosomes at anaphase I. In addition, the pollen grains were found to be highly fertile with over 85% pollen stainablity except for *A.*

dubius and *A. hybridus* that showed 65.8% and 72% respectively. But in the present study severe abnormalities were not observed rather than univalent formation in the meiotic cells of *Amaranthus* species studied.

Behera and Patnaik (1974) studied the meiosis in 8 species of *Amaranthus* and observed two basic numbers x = 16 and 17. Species confirming to x = 16 are *A. hybridus*, *A. caudatus*, *A. giganteus* and *A. caturtus*, of which the latter two were found to be tetraploids characterised by n = 32. Most *Amaranthus* species have chromosome numbers of n = 16 or n = 17, but *A. dubius* is unusual for having n = 32 (Grant, 1959 b). Some grain *Amaranthus* are paleoallotetraploids, as indicated by observations of pairing in their hybrids (Greizerstein & Poggio, 1992; Pal et al., 1982).

The relationship between the two basic chromosome numbers (n = 16 and n = 17) in the grain amaranth species was determined by Pal et al. (1982) through an interspecific dibasic cross involving white seeded cultivated *A. hypochondriacus* (n = 16) and black seeded wild *A. hybridus* (n = 17). The study suggested that the origin of n = 17 from n = 16 through primary trisomy.

The occurrence of univalent chromosomes was also found in some species of *Amaranthus* in the present study. Cytological studies of meiotic chromosomes of *A. caudatus* (Plate 25 b) showed n = 16 with 14 bivalents and 4 univalents. Haploid chromosome number of n = 32 with 28 bivalents and 8 univalents was observed in the pollen mother cells of *A. dubius* (Plate 25 c). Meiotic cells of *A. hybridus* subsp. *cruentus* (Plate 25 e) and *A. tricolor* (Plate 25 g) showed the occurrence of n = 17 chromosomes with 14 bivalents and 6 univalents. Gametic chromosome number of n = 16 with 15 bivalents and 2 univalents was observed in the meiotic cells of *A. hybridus* subsp. *hybridus* (Plate 25 d). Independent of the causes of origin of univalent chromosomes, their presence in the meiotic cells will generally favour the

increased chromosome frequency in early migration at metaphase I or delayed chromosomes in the anaphases. In both cases, they can originate micronuclei in telophase I or meiosis II (Pagliarini, 2000). Singh (2003) attributed the presence of univalent and trivalent chromosomes to the reduction in the recombination rate of the homologous chromosomes, and consequently, to an abnormal segregation in the progeny resulting in aneuploids, which in turn will lead to fertility reduction. Chromosome alterations at diakinesis, principally the presence of univalent chromosomes, can be one of the causes of male sterility (Amma et al., 1990, Nirmala & Kaul, 1994). Defani-Scoarize et al. (1995) associated the occurrence of male sterility in maize to the action of meiotic abnormalities of the univalent chromosome type, followed by irregular chromosome segregations (Tomé et al., 2007).

Altogether the haploid chromosome complements observed from the meiotic study helped to confirm the somatic chromosome counts of the eight *Amaranthus* species studied.

3. MICROMORPHOLOGICAL CHARACTERIZATION

Taxonomic delimitation and application of names in vegetable, grain and weedy *Amaranthus* species are still very tentative. Several new taxa at subspecies level, variety level and even at species level have been introduced. Along with morphology, some molecular parameters like isozyme, ITS restriction site variation, AFLP based marker *etc*. have been applied to solve the taxonomic problems (Das, 2016 b). The micromorphological studies have attracted the attention of plant morphologists and systematists to resolve taxonomic conflicts in different groups of plants (Sonibare et al., 2014). Micromorphological studies are sources of useful information relevant to the taxonomic description of species and are also useful in the hands of medicinal plant scientists in comparing and identifying species (Ibrahim et al., 2015). Because of the great genetic and morphological diversity within *Amaranthus*

species, identification based on a single parameter/method alone will not be accurate. The presence of flowers is required to identify most species, and even then, identification to the species level is sometimes difficult. In addition, interspecific hybridization between *Amaranthus* species causes further difficulties in identification, as these hybrids can exhibit characteristics of both parents (Horak et al., 1994). The 'Morelos' accessions are a good example illustrating the taxonomic ambiguity of *Amaranthus* taxa at the basic morphologic level (Costea et al., 2006). Previous studies revealed that micromorphological characters of pollen grains, seed capsule and seed coat can provide significant diagnostic features over various controversies that exist in different *Amaranthus* species (Das, 2016 b; Talebi et al., 2016; Parveen et al., 2015; Das, 2012 b; Franssen et al., 2001; Costea et al., 2006).

Despite pollen micromorphological studies, little attention has been given to the microfeatures of seed coat and seed capsule in different *Amaranthus* species. In the present study micromorphological characters of seed coat, pollen grains and seed capsule were considered for a comparative micromorphological analysis of eight *Amaranthus* species collected which was yet to be documented. The observed micro features can be used to discriminate the species studied in the genus *Amaranthus*.

MICROMORPHOLOGICAL STUDIES OF POLLEN GRAINS

In modern systematics pollen morphology has been extremely useful in clarifying systematic relationships within plant taxa. Moreover, palynology of the species and subspecies can have taxonomic value as supporting evidence to morphological and phylogenetic traits. The studies on pollen grain morphology are considered as the basic necessity for palynology due to its fundamental value in the recognition and identification of grains found in various conditions (Arora & Modi, 2008). A comparative micromorphological study of *Amaranthus* pollen grains using acetolysis (Plate 27) and SEM

(Table 23; Plates 28, 29) analysis revealed similarities and variations in both qualitative and quantitative parameters investigated. The result showed that all the studied species had poly pantoporate pollen, with many pores distributed on the surface. The morphology of the pollen grain is generally a conserved characteristic, which is an excellent means for identification of most species (Iwanami et al., 1988). Pollen morphology is considered to reflect phylogenetic relationships and is used in systematic studies (Nilsson, 1990; Harley et al., 1991; Crane et al., 1995). Studies addressing pollen viability, production and its morphology are also necessary for conducting breeding programs in *Amaranthus*. Identification of *Amaranthus* species using palynological studies is also necessary for an effective weed management (Mayo et al., 1995). The relationship between monoecious and dioecious *Amaranthus* as well as different ploidy level and interspecific hybrids can be determined using pollen grain features (Sousa et al., 2013; Talebi et al., 2016). Pollen grains contain a plasmalemma-encased cytoplasm surrounded by a thin layer of cellulose called intine. Immediately surrounding the intine is a layer of exine. The exine surface often develops various forms of sculpturing and ornamentation, including various types and numbers of apertures (Franssen et al., 2001).

Some studies (Pandey & Misra, 2009; Zhigila et al., 2014 a) showed that pollen morphological characteristics play a major role in solving taxonomic problems. Palynological characteristics have enabled to reposition several disputed genera and interpret problems related to the origin and evolution of many taxa (Nair, 1980) and to undertake a classification of angiosperms (Cronquist, 1981). Quantitative and qualitative analysis of pollen assemblages, on the other hand, provides the primary basis for interpretation of plant community response to climatic and anthropogenic environmental change (Kneller & Peteet, 1999; Watts, 1979; Davis, 1969; Willard et al., 2003, 2004). Zhigila et al. (2014 a) suggested that such analyses rely on

accurate identification of pollens for quantitative and qualitative comparison. Hence pollen spore morphology helps in delimitation of plant taxa. A survey of pollen morphology in Amaranthaceae revealed a remarkable diversity and many clearly distinguishable pollen types (Borsch, 1998). It also led to the recognition of metareticulate pollen (Borsch & Barthlott, 1998) and suggested the utility of pollen characters for reconstructing phylogenetic relationships in the family, with many pollen types. Previous studies (Eliasson, 1988; Costea, 1998 a; Costea 1998 b; Costea, et al., 2004) have confirmed that in *Amaranthus* taxa, flowers lack nectar glands and pollen grains are small (diameter 18 to 28 μm), usually with 30 to 45 pores uniformly distributed on their surface. In addition, they are tectate with granules and spinules. Roulston and Buchmann (2000) reported that *Amaranthus* pollens contain starch up to 7.5%, which protects them against desiccation.

Pollen from different plant species can usually be distinguished based on diagnostic traits such as pollen grain size, exine sculpturing and number and size of the apertures (pores or furrows) *etc*. using SEM (Sonibare et al., 2014). Studies on pollen viability and morphology are of high importance in relation to genetic breeding programmes, aimed at attaining potentially promising selections (Oliveira et al., 2001). A study by Franssen et al. (2001) examined pollen morphological variations among *Amaranthus* species and interspecific hybrids. Ten weedy *Amaranthus* species, a cultivated grain species and several putative hybrids resulting from interspecific mating between common waterhemp and palmer amaranth were used for the study. Mature pollen was collected, viewed, and photographed with a SEM. The results showed that the pollen grains were spherical shaped with poly pantoporate, or golf ball-like, aperture arrangement. Differences were observed between the monoecious and dioecious *Amaranthus* species. Pollen grains of the dioecious species had a greater number of apertures on the visible surface. One exception to these trends was the dioecious species,

palmer amaranth (*A. palmeri*), whose pollen was similar to that of the monoecious species spiny amaranth (*A. spinosus*). Pollen grain diameters did not differ between the monoecious and dioecious plants. Pollen of the hybrids was similar in size to the maternal parent but had an aperture number that was intermediate between parents. The diameters of the pollen grains for the 10 weedy species and the cultivated species ranged from 17.8 to 22 µm, with an average of 19.7 µm. The overall number of visible apertures on the *Amaranthus* pollen grains ranged from 18.1 to 60.3. Aperture number was greater in the pollen of dioecious species than that of monoecious species, with the exception of palmer amaranth.

Based on the P/E ratio, the pollen shape observed in the present study was defined as prolate-spheroidal with a circular polar view. The equatorial diameter of pollen grains for the eight monoecious *Amaranthus* species that belong to grain (*A. caudatus* and *A. hybridus* subsp. *cruentus*), weed (*A. hybridus* subsp. *hybridus*, *A. spinosus* and *A. viridis*) and vegetable (*A. blitum*, *A. dubius* and *A. tricolor*) category ranges from 12.60 to 20.42 µm with a mean value of 17.85 µm. The polar axis length for the eight *Amaranthus* species ranges from 13.87 to 20.67 µm with a mean of 18.58 µm. The largest pollen grain was observed for *A. dubius* with a mean diameter of 20.42 µm, whereas the smallest for *A. blitum* with a mean diameter of 12.60 µm. All the studied species had poly pantoporate pollen, with many pores distributed on the surface. The number of pores observed in the visible aperture was greater in the pollen of *A. dubius* (32), and lesser in *A. blitum* (12); the largest and the smallest pollen grains observed among the species studied respectively. The present observation highlights the positive relationship between the size of pollen grain and aperture number. Comparing the aperture numbers of dioecious species according to Franssen et al. (2001), lower numbers was observed for the monoecious species in the present study. This is in conformity with the report of Franssen et al. (2001), as aperture number was

greater in the pollen of dioecious species than that of monoecious species. In the present study, the aperture membrane ornamentation observed was scabrate for all the species, while its aggregation differed between species. Dense aggregations were observed in *A. dubius* (Plate 28 c1 & c2) and *A. spinosus* (Plate 29 b1& b2), while sparse aggregation was seen in all the other species, especially in *A. viridis* (Plate 29 d1 & d2).

Pollen characters are one of the most frequently used characters in taxonomic studies. The presence of different structures and ornamentation (echinae, spinule, baculum, gemma, *etc*.) on the exine surface is considered to be advanced, while uninterrupted exine is considered to be a primitive characteristic (Takhtajan, 1980; Walker, 1974 a, 1974 b). In addition, pollen grains with a low number of pores are considered to be more primitive than those with a high number of pores (Van Campo, 1966). These pollen characteristics have been used in taxonomic studies of many families and genera (Yıldız, 2001; Huysmans et al., 2003; Yıldız et al., 2010). Based on this the eight species of *Amaranthus* in the present study are advanced in the sense that the aperture of all the species are porate with scabrate/granulate membrane ornamentation, which resulted in interrupted exine. In addition to that, out of the two apertures seen in general *viz*., pores and fissures (colpi), the former are more advanced. As far as the number of pores is concerned, the most developed taxa among the species studied was *A. dubius* as it showed highest number of pores (32) on the visible aperture whereas the least developed species was *A. blitum*. The highest number of aperture in the pollen grains was reported in the dioecious species (Franssen et al., 2001) than monoecious species, which indicates their advanced status.

According to Franssen et al. (2001) the differences in pollen morphology between the monoecious and dioecious species may be related to their mode of pollination and can be explained using the aerodynamics of a

sphere. The addition of apertures on the surface creates a boundary layer of turbulent air surrounding the sphere. This boundary layer reduces the friction between the sphere and the air as the sphere travels through air, thus increasing the potential distance travelled (Jorgensen, 1993). The monoecious *Amaranthus* species typically are self-pollinated plants. When both male and female flowers are on the same plant, the pollen has a short distance to travel, in contrast to the dioecious species, which must disseminate pollen to neighbouring plants. This study revealed that dioecious species pollen has evolved more surface apertures, which may facilitate dispersal over greater distances. Based on the observation it was suggested that, traits that offer a selective advantage (i.e., herbicide resistance) may spread more rapidly in dioecious than in monoecious *Amaranthus* species (Franssen et al., 2001).

The pollen micromorphological study also revealed the close relatedness of a monoecious [spiny amaranth (*A. spinosus*)] and a dioecious [palmer amaranth (*A. palmeri*)] weedy *Amaranthus* species (Franssen et al., 2001). In their study, the pollen of palmer amaranth, did not have the same morphology as pollen of the other dioecious species. Its pollen was similar to that of spiny amaranth in diameter, aperture number and aperture density. Examination of the genetic sequence of the ITS region of selected *Amaranthus* species by Kirkpatrick (1995) demonstrated a high degree of homology between palmer amaranth and spiny amaranth. The present molecular study also agrees with this report that the grouping of *A. palmeri* and *A. spinosus* of the present study, in a single cluster with a high bootstrap support of 87% was observed in the phylograms of ITS region of rDNA. The same observation was also made by Wassom and Tranel (2005), while studying the genetic relationship among 8 weedy amaranth species. According to Murray (1940), although spiny amaranth is typically monoecious, its flowers are unlike those of the other monoecious amaranth species and are spatially separated. According to Franssen et al. (2001) the

relation between these two species was closer than the relation between spiny amaranth and the other monoecious species examined. These two species frequently share a common morphological characteristic - a chevron or v-mark on the leaves. These relationships and the pollen similarities reported suggest that these two species share a relatively recent ancestor (Franssen et al., 2001). According to Mosyakin and Robertson (1996) spiny amaranth might be in a transition stage towards developing dioecious flowering condition, indicating a possible genetic relationship with the dioecious palmer amaranth.

A study was done by Costea et al. (2006) to unravel the taxonomic ambiguity at the basic morphological level in the 'Morelos' accessions of *Amaranthus* from Mexico based on AFLP and micromorphology of seed, fruit and pollen grains. The study resulted in the placement of 'Morelos' accessions in the *A. cruentus* species clade and was clearly separated from *A. caudatus* species clade. The pollen of the 'Morelos' plants has the characteristics of *A. cruentus* (Costea et al., 2001). Pollen grains are pantoporate, apolar, 22-25 µm, with 35-45 sunken pores of 1.9-2.1 µm diameter, uniformly distributed and are having the apertural membrane granulated; the tectum has spinules and not granules as in *A. caudatus* (Costea et al., 2001). In the present study *A. hybridus* subsp. *cruentus* showed poly pantoporate pollen with 22 sunken pores in the visible aperture of 0.96-1.20 µm in diameter with scabrate surface sculpturing. The species showed an interpore distance of 2.65-3.06 µm. Poly pantoporate pollen with 20 shallow pores of 1.14-1.71 µm in diameter, with scabrate aperture membrane ornamentation was observed for the pollen grains of *A. caudatus*.

A pollen morphology study of *A. spinosus, A. dubius* and their hybrids has been carried out by Srivastava et al. (1977). Three pollen types have been observed, namely type A: micrograins, type B: grains with smaller pores and

type C: grains with larger pores. Type B is characteristic of *A. spinosus*, type C of *A. dubius*, and the micrograins of the hybrids. Pollen size range and frequency of the various morphotypes serve to throw light on the biosystematics of the plants studied. Zhigila et al. (2014 b) studied palynological characteristics in five species (*A. spinosus, A. viridis, A. hybridus, A. australis* and *A. tricolor*) of the genus *Amaranthus*. Their results indicated that pollen characteristics may be used to delimit the species in the genus *Amaranthus*.

Palynological traits of seven Iranian *Amaranthus* taxa, including *A. albus, A. blitoides, A. blitum* subsp. *blitum, A. blitum* subsp. *emarginatus, A. caudatus, A. retroflexus* and *A. tricolor* were studied by Talebi et al. (2016) in order to solve taxonomical problems and to aid identification of the taxa. Twelve quantitative and qualitative characteristics were investigated. Results showed that all of the studied taxa had poly pantporate pollen, with many pores distributed on the surface of pollen. Pollen polar shapes were stable in the studied taxa and were circular. In addition, equatorial views in all the taxa revealed that they were elliptic-truncate with the exception of *A. tricolor* that was elliptic-acuminate. Surface sculpturing was seen as scabrate, while its aggregation differed between samples. Dense aggregations were recorded in *A. retroflexus*, while sparse aggregation occurred in *A. blitum* subsp. *blitum, A. albus* and *A. blitum* subsp. *emarginatus*. The present study also observed similar result as above such as poly pantporate pollen with circular polar view and scabrate surface sculpturing for the eight species investigated, but the pollen grains are prolate spheroidal in shape (Plates 27-29).

According to the study of Talebi et al. (2016) quantitative traits varied between taxa. The polar axis length (20.5 μm) and equatorial diameter (21.0 μm) of pollen grain was shortest in *A. blitum* subsp. *blitum*. However largest values for polar (25.4 μm) and equatorial (26.0 μm) length were observed in

A. tricolor and *A. albus*. Pore diameter varied between the studied taxa. The largest pores were found in *A. blitum* subsp. *blitum* (2.45 μm), and smallest in *A. retroflexus* (0.81 μm). The present study also revealed that the polar axis length and equatorial diameter was found to be shortest in *A. blitum*, but highest in *A. dubius*. The largest pores were found in *A. hybridus* subsp. *hybridus* and smallest in *A. spinosus*, this observation was found to be varying from the previous results. The statistical analysis in the previous study showed that some quantitative morphological features were more valuable in identification of the studied taxa, for example, P/E ratio was a distinct characteristic in identification of *A. blitum* subsp. *emarginatus* from the other taxa. Pore diameter, equatorial and also polar axis length were the main characteristics used for recognition of *A. blitum* subsp. *blitum* from the other taxa. The studied taxa were separated from each other using multivariate analysis (Talebi et al., 2016).

Quantitative and qualitative palynological features in the present investigation varied between the species studied and were useful in identification of different *Amaranthus* species. For example, based on the pollen size, *A. dubius* and *A. blitum* can be distinguished from the other species. Degree of sculpturing aggregates and number of pores in the visible aperture was useful in identification of *A. dubius* from the other taxa. Various palynological investigations in different species of the genus *Amaranthus* confirmed the importance of pollen traits for distinguishing taxa.

MICROMORPHOLOGICAL STUDIES OF SEED CAPSULE

Despite pollen micromorphological studies, previous reports on the microfeatures of seed capsule in different *Amaranthus* species is lacking. The micromorphological features of seed capsule of different *Amaranthus* species observed in the present study showed slight variations in size and capsule ornamentations. The capsule wall of *A. blitum* (Plate 30 a1-a3) and *A. viridis*

(Plate 31 d1-d3) was found to be indehiscent and rupture irregularly, whereas the capsule wall of all the other species of *Amaranthus* was found to be dehiscent with a prominent line of dehiscence. The capsule ornamentation in *A. blitum* was uniform throughout the wall and showed entirely different pattern from other species. The capsule wall of *A. viridis* is distinct from other species as it showed very strong rugose structure throughout. Capsule wall ornamentation of other species showed rugose or wrinkled pattern of wall ornamentation and was uniform throughout the capsule of *A. caudatus* (Plate 30 b1-b3), *A. dubius* (Plate 30 c1-c3) and *A. hybridus* subsp. *hybridus* (Plate 30 d1-d3), whereas *A. hybridus* subsp. *cruentus* (Plate 31 a1-a3), *A. spinosus* (Plate 31 b1-b3) and *A. tricolor* (Plate 31 c1-c3) showed strong rugose pattern of ornamentation above the line of dehiscence than the lower region of the capsule. Slight variations are also observed in the capsule size of different *Amaranthus* species. The longest capsule was observed for *A. tricolor* with a mean length of 3.98 mm, whereas the shortest capsule was for *A. blitum* with a mean length of 2.19 mm. The capsule width observed ranges from 0.98 to 1.66 mm in the species studied.

To ascertain the taxonomic identity of the 'Morelos' accessions and their hypothesized species affiliation to *Amaranthus caudatus* or *A. cruentus*, Costea et al. (2006) conducted a comparative analysis of phylogenetic relationships among these taxa/accessions using AFLP and micromorphology methods. The micromorphology of fruits, seeds and pollen was studied on the accessions collected. The basic morphology of the 'Morelos' plants shows characteristics that are somewhat intermediate between *A. cruentus* and *A. caudatus*. Based on AFLP data, all the controversial 'Morelos' accessions can be consistently placed into a single *A. cruentus* species clade, which is clearly separated from the *A. caudatus* species clade. The AFLP-based phylogenetic relationship of 'Morelos' and delimitation of *A. cruentus* and *A. caudatus* are further supported by micromorphology, showing that the combination of these

techniques can provide more reliable data for germplasm identification than each method used alone. The perianth micromorphology using SEM analysis revealed that *Amaranthus caudatus* has overlapping, broadly spatulate, and commonly recurved tepals whereas *A. cruentus* has non-overlapping, obovate and erect tepals, which was more similar to 'Morelos' accessions.

According to the study of Costea et al. (2006) the morphology of the fruit apex towards the stigma region is very important to distinguish *A. cruentus* from both *A. caudatus* and *A. hypochondriacus*. Typically, *A. cruentus* has the fruit abruptly narrowed into a thin rostrum continued with the erect stigmas. In *A. caudatus* and *A. hypochondriacus* the fruit is gradually narrowed apically, and a rostrum is absent. The pattern of pericarp surface in the 'Morelos' plants agrees with the range of variation described in *A. cruentus* such as, 1. cells are irregularly shaped, 1–3 times longer than wide, and anticlinal walls are irregularly waved [Type A] and 2. cells have short lateral ramifications, 4–6 times longer than wide and anticlinal walls are X or S-waved [Type B] (Costea et al., 2001). The pericarp surface of *A. caudatus* is less variable, belonging only to the type A category. The capsule of *A. hybridus* subsp. *cruentus* in the present study also showed narrowed fruit apex with erect stigma but the capsule of *A. caudatus* is narrowed apically with curved stigma.

Micromorphology (Costea et al., 2001) and AFLP (Xu & Sun, 2001) have been successfully used to separate the species of *A. hybridus* complex also, the phylogenetic group in which the cultivated grain amaranth species have evolved.

Quantitative and qualitative features of the seed capsule in the present investigation varied between the studied species and were useful in identification of different *Amaranthus* species. For example, based on the capsule size, *A. tricolor* (largest) and *A. blitum* (smallest) can be distinguished

from the other species. On basis of capsule rupturing *A. blitum* and *A. viridis* (indehiscent) can be identified from other species. On behalf of capsule ornamentation, *A. viridis* and *A. blitum* was distinct from other species. The pattern of capsule wall ornamentation was useful in identification of *A. hybridus* subsp. *cruentus*, *A. spinosus* and *A. tricolor* from the other species. The seed capsule microfeatures investigated in different species of the genus *Amaranthus* confirmed its significance for distinguishing species (Plate 30 & 31).

MICROMORPHOLOGICAL STUDIES OF SEED COAT

The seed coat (testa) is the outer covering of every mature seed. It interacts with the internal structures of the seed and the external environment. Seed coats exhibit complex and highly diverse morphology and anatomy, providing valuable taxonomic characters (Barthlott, 1981, 1990). The micromorphological characteristics of the seed coats may provide valuable information for identification of interspecific and intraspecific variations among species (Sonibare et al., 2014; Parveen et al., 2015). The ultrastructural importance of seed surface, as a reliable approach for resolving taxonomic problems is well accepted (Heywood, 1971; Buth & Ara, 1983; Brochmann, 1992; Koul et al., 2000). The seeds of *Amaranthus* are small in size and not possible to differentiate easily with naked eyes (Parveen et al., 2015). A little work has been focused on comparative seed micromorphological features of different *Amaranthus* species. Hence in the present study, micromorphological typology of seed surfaces was investigated in eight species of *Amaranthus* using SEM.

Many species of *Amaranthus*, especially the grain amaranths, showed great variability in morphological features like plant height, branching, colour and abnormalities in inflorescence in response to many environmental factors like nutritional elements, water availability, light condition, injury *etc*. But

these variabilities are of little taxonomic significance as it is transient. In contrast, seed surface features are quite stable as they are controlled by multiple alleles and additional loci (Kulakow & Jain, 1990). The seed morphological studies are often done to distinguish the taxonomy of genus and species (Kuh et al., 2017). Seed surface micro-morphological features like reticulation of ridges and furrows on spermoderms, shape of spermoderm cavities or elevated areas created by ridges and furrows, respectively, were of significance in delimiting species, or even accessions (Das, 2012 b; Parveen et al., 2015).

Seed surface micromorphological features observed in the present study by SEM analysis revealed variations among different *Amaranthus* species studied. Variations are observed in the seed shape, size, weight, colour and spermoderm/testa ornamentations (Table 25; Plates 32-34). The seed length was found to be higher than width in all the species except *A. viridis* which showed higher seed width (0.85 mm) than seed length (0.82 mm).

According to the study by Costea et al. (2006) the seeds of the 'Morelos' accessions are almost similar in morphology with the seeds of typical *A. cruentus*. They differ from the *A. caudatus* seeds through their less sculptured surface of the testa in the central region of the seeds and the absence of the pink tint shown by the embryo (a characteristic sometimes present in *A. caudatus*). The present study agrees with these observations that the seeds of *A. hybridus* subsp. *cruentus* in the present study were elliptic-lenticular in shape with glossy surface and reddish brown coloured. Seeds showed well defined reticulation forming rectangular and polygonal elevated areas mainly in the flange region and ill defined ornamentation in the central region of the spermoderm (Plate 32 e; 34 a1, a2). The observations of the present study showed that seeds of *A. caudatus* were elongated lenticular in

shape with glossy surface and brownish black coloured. Epidermal cells show ornamentation having prominent reticulation forming irregularly shaped areas on the spermoderm (Plate 32 b; 33 b1, b2). The pinkish tint given by the embryo in *A. caudatus* reported previously (Costea et al., 2006) was not observed in the present study.

Variations in seed micromorphological features even between varieties and accessions of a single species were reported previously (Das, 2016 b; Das, 2012 b; Costea et al., 2006; Parveen et al., 2015). In a study by Das (2012 b) on taxonomical observation on the grain amaranths and new varieties of *A. cruentus*, used morphological and biochemical tools and revealed a close affinity between the two grain amaranth *viz*., *A. hypochondriacus* and *A. caudatus*, and a distinct divergence of *A. cruentus* from the others. Nine species of *Amaranthus* comprising grain and weed members with their respective morphotypes or accessions were included in the study. Accessions of the grain amaranth such as *A. cruentus* showed a prominent variability, especially in seed surface features, which strongly proposed a segregation of the population into two groups; one having rusty brown seeds with reticulate spermoderm and another group having greyish white seeds with rugulate spermoderm. The seeds of *A. hypochondriacus* are creamy white with a reticulate spermoderm. All accessions of *A. caudatus* have pink-coloured seeds with ill-defined to prominent reticulation forming polygonal or hexagonal cavities on the spermoderm. The result was well supported by cluster analysis, showing a clear delimitation of brown and greyish white seeded accession of *A. cruentus*. The morphological and biochemical parameters in the study strongly favour the introduction of new infraspecific categories in *A. cruentus*. In contrast to the above result, the present study showed reddish brown seeds for *A. hybridus* subsp. *cruentus* and brownish black seeds for *A. caudatus*. The spemoderm was found to be

reticulate in *A. hybridus* subsp. *cruentus* rather than rugulate as observed by Das (2012 b).

A recent study by Parveen et al. (2015) was undertaken to unravel the intraspecies variation in the seed coat micromorphology of eight accessions of *Amaranthus hybridus* using SEM. The study was aimed to explore the seed characters which are useful as a tool for identifying crop species and variations among the accessions studied. According to the study the micromorphological characteristics of the seed coats may provide valuable information for the identification of seeds. Considerable diversity in seed size, shape, colour and seed coat micromorphology was observed. Five different patterns of testa ornamentations *viz*., reticulate, polygonal, scalariform, undulating lumps and spindle shaped was observed for the eight accessions of *A. hybridus*. The phenogram exhibits the relationship among the 8 accessions on the basis of overall similarities of seed characters that were found to be useful in better taxonomic resolution of *A. hybridus.* According to the study, seed coat colour was different in different accessions *viz*., four accessions were white, two accessions were black, one dark brown and the other one was blackish red. The seeds of *A. hybridus* subsp. *hybridus* in the present study were subglobose in shape with glossy surface and were black coloured. According to Browning et al. (1997) the colour of the seeds is very important in distinction of the taxonomic status of *Draba* and *Bolboschoenus maritimus.* The seeds of *A. hybridus* subsp. *hybridus* in the present study showed cross linked undulated thread like epidermal cells ornamentation forming polygonal meshes throughout the spermoderm (Plate 32 d; 33 d1, d2), which is similar to the ornamentation of the *A. hybridus* accession IC 120617 that was reported earlier by Parveen et al. (2015).

The seeds of eight *Amaranthus* species studied in the present investigation showed similarities and variations in spermoderm/testa

ornamentations, which were found to be significant for species identification. The seeds of *A. blitum* showed prominent reticulation with polygonal and rectangular elevated areas on the spermoderm (Plate 32 a; 33 a1, a2). Seeds showed prominent reticulation forming hexagonal and polygonal areas on the spermoderm of *A. dubius*. Prominent reticulation forming hexagonal and polygonal cavities throughout the spermoderm (Plate 32 f; 34 b1, b2) was observed in *A. spinosus*, whereas prominent reticulation forming hexagonal and polygonal areas throughout the spermoderm was detected for *A. tricolor*. Seed surface ornamentation of *A. viridis* was entirely different from other species. Here the reticulate pattern of spermoderm ornamentation is absent; instead the entire spermoderm is rugulate forming an irregular muriform arrangement (Plate 32 h; 34 d1, d2). Among the eight species of *Amaranthus* studied, all of the species except *A. hybridus* subsp. *hybridus* and *A. viridis* showed reticulate pattern of testa ornamentations with slight differences in between. Considering mean seed length, width and length-width ratio, the largest seed was for *A. caudatus* (1.47×1.01 mm) and the smallest seed for *A. dubius* (0.82×0.79 mm). Based on the mean weight of 1000 seeds, highest seed weight was for *A. tricolor* (0.82 g) and lowest for *A. blitum* (0.14 g).

The seed micromorphological features of *A. tricolor*, *A. blitum*, *A. caudatus*, *A. hypochondriacus*, *A. hybridus*, *A. retroflexus* and two varieties of *A. cruentus viz*., *A. cruentus* L. var. *cruentus* and *A. cruentus* L. var. *albus* was studied previously by Das (2016 b). The seeds of *A. caudatus* and *A. hypochondriacus*, the two grain amaranth species showed well defined folded flange region and rugulate spermoderm. Seed surface features delimited the two varieties in *A. cruentus* such as *A. cruentus* L. var. *cruentus* with reticulate spermoderm whereas *A. cruentus* L. var. *albus* with regulate spermoderm.

According to Das (2012 b), the weedy amaranths all have small black seeds with undifferentiated flange. The present study agrees with the statement that all the weedy amaranths *viz*., *A. hybridus* subsp. *hybridus*, *A. spinosus* and *A. viridis* together with vegetable amaranths *viz*., *A. dubius* and *A. tricolor* showed small black coloured seeds, with undifferentiated pleurogram/flange. Pleurogram is completely absent in *A. viridis*. According to Das (2012 b) the grain amaranths have discoid grains with well-differentiated folded flange and seed coat colour other than black. The grain amaranths in the present study *viz*., *A. caudatus* and *A. hybridus* subsp. *cruentus* also showed well defined flange region without any folding, with brownish black and reddish brown seeds respectively rather than black. From the result it was clear that seed colour can be used as a promising character for the identification of grain and weed amaranth respectively. Weed amaranths showed some uniformity of features like absence of a well-differentiated flange region, reticulation of furrows creating hexagonal, rectangular or polygonal elevated areas on their spermoderms. Seeds of grain amaranths are quite different from weed amaranths in having a well-differentiated folded flange region, ill-defined or well defined reticulation of ridges over spermoderms (Das, 2012 b).

Quantitative and qualitative features of the seed coat in the present investigation varied between the studied species and were useful in identification of different *Amaranthus* species. For example, based on the seed size, *A. caudatus* (largest) and *A. dubius* (smallest) can be distinguished from the other species. On the basis of seed colour, weed and vegetable amaranth (black) can be distinguished from grain amaranth (brownish black/reddish brown) species. On behalf of spermoderm/testa surface and ornamentation, seeds of *A. viridis* are distinct from other species as it showed rough seed surface with rugulate pattern throughout. The seed surface ornamentation was also useful in identification of *A. hybridus* subsp. *hybridus*

from the other species. Based on the presence of well defined pleuroderm, grain amaranth species can be distinguished from other species. The spemoderm/testa microfeatures investigated in different *Amaranthus* species confirmed its significance for distinguishing species.

4. PHYTOCHEMICAL CHARACTERIZATION

Plant-derived drugs have become of great interest owing to their harmless applications over allopathic medicines. Use of herbal medicine for the treatment of diseases and infections is as old as mankind. Plants are endowed with various phytochemical molecules such as vitamins, terpenoids, phenolics, lignins, tannins, flavonoids, quinones, coumarins, alkaloids, amines, betalains *etc*. According to the World Health Organization, the use of traditional medicine has proven to be efficacious and safe. The ingestion of natural antioxidants has been associated with reduced risks and in recent years, there has been a worldwide trend towards the use of the natural phytochemicals present in plants. In order to promote the use of herbal medicines and the determination of their potentials, the scientific studies of medicinal plants should be more intensified especially those used as folk medicines and as traditional medicine. To ensure reproducible quality of herbal products, proper control of starting material is important. The first step towards ensuring quality of starting material is authentication. Several modern and conventional methods are there, for the standardization of selected medicinal plants of potential therapeutic significance. Such studies are important in the path of acceptability of herbal drugs in the present scenario of lacking regulatory laws to control quality of herbal drugs (Mishra et al., 2011). Recently chemotaxonomic studies through the phytochemical characterization in order to detect primary (carbohydrates, amino acids, proteins, chlorophyll *etc*.) and secondary metabolites (phenol, flavonoid, terpenoid, alkaloid, *etc*.) have gained significant attention.

Many Amaranthaceae species have been used as medicinal plants by native people in the tropical and subtropical countries as well as temperate climate for their numerous activities and are confirmed in the contemporary biological tests. Among these, *Amaranthus* is a food plant with health and medicinal benefit. Plants have provided a source of inspiration of novel drug compounds, as plant derived medicines have made large contributions to human health and well-being (Abukakar et al., 2008). The reported pharmacological studies revealed that species of the genus *Amaranthus* exhibited various bioactivities, which are mentioned in Table 3.

The phytochemical constituents of plants can be detected by various techniques. Qualitative screening can be done to get an overall idea regarding the major classes of compounds and quantitative techniques like GC/MS, HR-LC/MS, HPLC, HPTLC *etc*. can be used to find out the specific bioactive components present. In the present study, the phytochemical constituents present in eight *Amaranthus* species were screened by using both qualitative and quantitative methodologies. The classes of compounds present were identified by preliminary screening and quantification was done for major classes. The volatile and non-volatile components present in the methanolic plant extract was screened by using GC/MS and HR-LC/MS techniques. A comparative phytochemical analysis was also done among species to find out the chemical affinity among different *Amaranthus* species.

QUALITATIVE PHYTOCHEMICAL SCREENING

Traditional medicine system has gained global importance. Hence, a thorough knowledge of their organic constituents and trace element contents is essential for formulating safe and effective medications (Salahuddin et al., 1998; Choudhari, 1988; Cohen et al., 1991). The plant kingdom represents a treasure trove of structurally diverse bioactive molecules, which are referred to as secondary metabolites and are biosynthesized by plants (Iyengar, 1995).

The most important bioactive constituents of the plants are terpenoids, alkaloids, tannins, flavonoids and phenolic compounds and have several biological properties. The beneficial physiological and therapeutic effects of plant materials typically result from the combinations of these secondary products. It is clearly known that they have roles in the protection of human health, when their dietary intake is significant. It is believed that the phytochemicals may be effective in combating or preventing disease due to their antioxidant effect. In recent years, phytochemicals previously with unknown pharmacological activities have been extensively investigated as a source of medicinal agents which would further be valuable in discovering the actual value of folkloric remedies (Zheng & Wang, 2001; Mojab et al., 2003; Cai et al., 2003; Krishnaraju et al., 2005).

Qualitative phytochemical screening in the present study led to the disclosure of the presence of primary and secondary metabolites such as carbohydrate, protein, phenols, flavonoids, terpenoids, alkaloids, tannins, saponins, steroids, carotenoids and cardiac glycosides in the species of *Amaranthus* studied (Table 26). Quinones are absent in all the species except *A. viridis*. Anthraquinones are observed in *A. spinosus* alone, whereas *A. blitum* was devoid of saponins and phlobatannins. Phlobatannin was absent in *A. tricolor*. Former studies have also reported the presence of these major classes of compounds in different *Amaranthus* species (Table 2). These phytochemicals form the basis of the biological activities attributed to the extracts (Table 3). *Amaranthus* is an important traditional herb widely used in folk medicine for centuries. Current scientific opinion advocates consumption of whole plant instead of isolated compounds, the way nature prepared it with full compliments of naturally occurring synergistic phytonutrients to attain holistic wellbeing. Polyphenol rich *Amaranthus* based diet can provide significant protection against many chronic disease conditions (Peter & Gandhi, 2017). Phenolic compounds have strong free radical-scavenging

activity (Proteggente et al., 2003) and hence they are potent antioxidants (Amin et al., 2006; Oboh, 2005; Pacifico et al., 2008; Saxena et al., 2007). An important effect of flavonoids is the scavenging of oxygen-derived free radicals. *In vitro* experimental systems also showed that flavonoids possess anti-inflammatory, antiallergic, antiviral, anticarcinogenic and antiatherosclerotic effects (Middleton, 1998). The terpenoids have shown to possess antioxidant (Grassmann, 2005; Pacifico et al., 2008), antimicrobial (Griffin et al., 1999; Singh & Singh, 2003) and anti-inflammatory activities (Neukirch et al., 2005). *Amaranthus* species are rich source of triterpenes (saponin), having reported biological efficacies *viz*., antioxidant, anticancer (Ronco & Stéfan, 2013; Garg et al., 2010), anti-hypercholesterolemic (Bhilwade et al., 2010), cardioprotectant activities (Farvin et al., 2010) *etc*. Tannins have reported antimicrobial activities (Kaur & Arora, 2009). The antimicrobial potential of tannins, phlobatannins, alkaloids, coumarins, cardiac glycosides, terpenes, phenylpropanes, organic acids, flavonoids, isoflavonoids and saponins were reported previously (Souza et al., 2005; Matasyoh et al., 2009). The carotenoids in *Amaranthus* (canthaxanthin) having anti-tumor potential was previously reported by Dlamini et al. (2010). Saponins and tannins are the two anti-nutritional components observed in the *Amaranthus* species studied. Saponin is a triterpene found in all the species except *A. blitum* in the qualitative screening, but trace amounts were observed during the quantitative estimation. There are previous reports on the occurrence of saponins in *Amaranthus* species (Rastrelli et al., 1995).

QUANTITATIVE ESTIMATION OF MAJOR PHYTOCOMPONENTS

Quantitative estimation of major phytocomponents such as phenols, flavonoids, terpenoids, alkaloids, tannins, carotenoids and saponin was performed in all the species and the results are summarized in Figures 8 to 11. The analysis revealed the occurrence of phenols and terpenoids in appreciable

amount, whereas flavonoids, alkaloids, tannins, saponins and carotenoids in lesser amounts. This gives an estimate of major secondary metabolites in the plant extract. Earlier phytochemical analysis of the aerial parts of *Amaranthus* species has established the presence of active constituents like alkaloids, flavonoids, glycosides, phenolic acids, steroids, saponins, amino acids, vitamins, minerals, terpenoids, lipids, betaine, catechuic tannins and carotenoids (Nana et al., 2012; Sharma et al., 2012; Kumar et al., 2011; Clemente & Desai, 2011; Akubugwo et al., 2007).

In the present study the total phenolic content in the extracts of *Amaranthus* were estimated using the Folin-Ciocalteu reagent and was expressed as gallic acid equivalents using the standard equation from the linear calibration curve ($y = 0.0068x - 0.007$, $R^2 = 0.997$; Fig. 3). Highest phenolic content (mg GAE/g DW) was observed in *A. hybridus* subsp. *hybridus* (248.78 ± 1.14) and the least was in *A. caudatus* (117.48 ± 1.12) (Fig. 8 a). Polyphenols are a class of phytochemicals that contribute to the total antioxidant capacity of dark green leafy vegetables (Duthie et al., 2000). They have aromatic rings and achieve their antioxidant activities mainly through the donation of hydrogens (Puoane et al., 2002). A study by Van der Walt et al. (2009) reported that *A. hybridus* extract was enriched with phenolic compounds and the total phenolic concentrations reported were similar to those reported in commercial spinach (Pandjaitan et al., 2005). Similar observation was also made from the present study that, among the eight *Amaranthus* species investigated, highest phenolic content was found in *A. hybridus* subsp. *hybridus*. In recent years, plant-derived bioactive substances especially anticancer ones have gained considerable attention (Oueslati et al., 2012). Moreover, many plants containing phenolic compounds have been found to possess anticancer activity (Cai et al., 2004; Owen et al., 2000) and based on this fact, plants rich in phenolic content have

been considered as interesting source of anticancer potential (Huang et al., 2009; Vuorela et al., 2005).

The vegetable *Amaranthus* has been reported to have high concentration of antioxidant components such as vitamin C, vitamin E, carotenoids, polyphenols and other phytochemicals (Hunter & Fletcher, 2002). Green leafy vegetables are generally consumed in the cooked form apart from the salads. Therefore there is a need to assess the changes that occur in the antioxidant activity on cooking. Losses of antioxidant components from vegetables during cooking were already reported before (Chu et al., 2000; Yadav & Sehgal, 1995). The effect of blanching on the phenolic content and antioxidant activity of four *Amaranthus* species was investigated by Amin et al. (2006) and found that antioxidant activities and phenolic contents of all the species were in the order of raw > blanched 10 min > blanched 15 min. Blanching up to 15 min may lead to losses of antioxidant activity and phenolic content. According to the study the decreased antioxidant activity may be due to the losses or degradation of certain types of phenolic compounds responsible for the free radical-scavenging activity during blanching. This result supports Papetti et al. (2002) who reported that the radical-scavenging activity would decrease if the vegetables were exposed to heat. According to Joubert (1990), blanching causes solubilization of phenolic compound and hence leads to loss of total phenolic compounds from the final product. Oboh (2005) studied the effect of blanching on Nigerian green leafy vegetables and concluded that the blanching of vegetables reduces their antioxidant properties drastically.

In the present study the total flavonoid content of the plant extracts was determined using the aluminium chloride colorimetric method. The flavonoid content was measured and expressed in terms of quercetin equivalent employing the standard equation obtained from the linear calibration curve (y

= 0.010x – 0.006, R^2 = 0.997; Fig. 4). Highest flavonoid content was found in *A. dubius* (21.88 ± 0.10) and the least was in *A. blitum* (9.54 ± 0.18) (Fig. 8 b). Flavonoids isolated from a wide range of vascular plants, with over 8000 individual compounds known. Many studies have suggested that flavonoids exhibit biological activities including anti-allergenic, antiviral, anti-inflammatory and vasodilating actions. However, most interest has been devoted to the antioxidant activity, which is due to their ability to reduce free radical formation and to scavenge free radicals. The capacity of flavonoids to act as antioxidants *in vitro* has been the subject of several studies in the past years, and important structure-activity relationships of the antioxidant activity have been established (Pietta, 2000).

In the present study, the total terpenoid content in the extracts was expressed in terms of linalool equivalent employing the standard equation obtained from the linear calibration curve (y = 0.004x + 0.003, R^2 = 0.999; Fig. 5). The terpenoid content was estimated to be highest in *A. blitum* (168.48 ± 1.21 mg linalool/g DW) and lowest in *A. hybridus* subsp. *hybridus* (49.66 ± 1.06) (Fig. 9 a). Terpenes are the largest and the oldest group of natural products that are biosynthetically constructed from isoprene (2-methylbutadiene) units (Ruzicka, 1953; Gershenzon & Kreis, 1999). Bioactivities of terpenoids includes antioxidant, anticancer (Grassman, 2005; Pacifico et al., 2008; Ronco & Stéfan, 2013; Garg et al., 2010), antimicrobial (Griffin et al., 1999; Singh & Singh, 2003), anti-inflammatory (Neukirch et al., 2005), anti-hypercholesterolemic (Bhilwade et al., 2010), cardioprotectant (Farvin et al., 2010) *etc*.

In the present study the total alkaloid content in the extracts was estimated and was expressed in percentage. Among the species studied highest alkaloid content was observed in *A. spinosus* (34.60 ± 2.41) and lowest in *A. blitum* (8.18 ± 1.53) (Fig. 9 b). Alkaloids are plant derived

pharmacologically active, basic compounds from amino acids that contain one or more heterocyclic nitrogen atoms. Plant derived alkaloids are suggested to be natural antioxidants associated with prevention of various diseases and pathological conditions including malaria, diabetics, cancer, cardiac dysfunction *etc*. and also used as local anesthetics and pain relievers (Yahara et al., 2005). Newly extracted alkaloids from medicinal plants are used as therapeutic agents (Sugisawa et al., 2004). Many alkaloids are used as anti-arrhythmic, anticholinergic, anti-tumor, vasodilating, antihypertensive, anesthetic, antiprotozoal, antidiabetic, antihyperlipidemic and antioxidant agents (Saravanan et al., 2013). Alkaloids are known to control development in living system and have protective role in animals (Sasikumar et al., 2015). The protective effect of isolated alkaloids from *A. viridis* leaves against H_2O_2 induced oxidative damage in RBC was reported by Sasikumar et al. (2015).

In the present study the tannin content in the extracts was estimated using vanillin reagent and was expressed as mg tannic acid/g DW from the standard curve equation ($y = 0.078x + 0.003$, $R^2 = 0.999$; Fig. 6). The highest content was observed in *A. spinosus* (9.35 ± 0.25) and lowest in *A. caudatus* (4.06 ± 0.44) (Fig. 10 a). Tannins are poly-phenolic compounds and are widespread in nature which is proven as antioxidants, astringent (Okwu, 2004) and possesses chemo-protective potential. They are complex secondary metabolites having various medicinal properties and have a large influence on the nutritive value of humans and animals foodstuff (Saxena et al., 2013). Tannins are often considered as anti-nutrients, but were found in acceptable amounts in all the species studied.

In the present study the total carotenoid content in the extracts was estimated and expressed in terms of *β*-carotene equivalent employing the standard equation obtained from the linear calibration curve ($y = 0.271x + 0.045$, $R^2 = 0.997$; Fig. 7). The highest carotenoid content was observed in *A.*

blitum (0.65 ± 0.00) and lowest in *A. hybridus* subsp. *cruentus* (0.16 ± 0.01) (Fig. 10 b). Carotenoids comprise a large group of natural pigments widely distributed in the plant and animal kingdoms. They are yellow-orange pigments, insoluble in water but soluble in organic solvents. They are present as pigments in many vegetables and fruits and are associated with chlorophyll in higher plants, playing important role during photosynthesis. The most widespread and important carotene is *β*-carotene which is found abundantly in some plants. The essential role of *β*-carotene as a dietary source of vitamin A has been known for many years (Britton, 1995). Among the provitamin A carotenoids in food namely beta-carotene, alpha-carotene, gamma-carotene and beta-cryptoxanthin, beta-carotene is the one that is most efficiently converted to retinol (Olson et al., 2000). Vitamin A is essential for a variety of biological processes, many of which are related to growth cellular differentiation and interactions of cells with each other or with extracellular matrix (Roberts & Sporn, 1984; De Luca, 1991). Vitamin A is important for normal vision, gene expression, growth and immune function by its maintenance of epithelial cell functions (Lukaski, 2004).

Nature produces a variety of compounds adequate for food colouring, such as the water-soluble anthocyanins, betalains and carminic acid as well as the oil soluble carotenoids and chlorophylls. The health-benefit properties of natural pigments have been focused by many workers, especially those of carotenoids and anthocyanins, whose antioxidant properties have been extensively studied. Another pigment betalain was also reported in *Amaranthus* species (Nana et al., 2012) and some studies have indicated their potential as antioxidant pigments (Kanner et al., 2001). A study by Dlamini et al. (2010) concluded that *A. cruentus* is potentially a good dietary source of the pro-vitamin A carotenoid (*β*-carotene). Carotenoid content was highest in leaves, followed by seeds, stem and roots. The major carotenoid identified in the leaves was canthaxanthin, followed by *β*-carotene and lutein. The level of

β-carotene (28.5 mg/100 g) in *A. cruentus* was seven times higher than in tomatoes and thus it can be considered as a vitamin A source.

In the present study, the total saponin content present in different *Amaranthus* species was estimated and results were expressed in percentage. Highest saponin content was observed in *A. spinosus* (27.76 ± 0.08) and lowest in *A. blitum* (1.66 ± 0.00) (Fig. 11). Saponins were isolated from numerous Amaranthaceae plants and have been proved to be the major effective constituents in some species (Mroczek et al., 2012). Zhang et al. (2014) reported the antifungal and antibacterial potential of isolated saponin from tea seeds. Four new saponins from the seeds of *A. cruentus* were previously isolated by Junkuszew et al. (1998). Oleszek et al. (1999) determined the toxicity of four saponins present in the *A. cruentus* seeds against hamsters and found that low contents of saponins in amaranth seeds and their relatively low toxicity guarantee that amaranth derived products are safe. In the present study the saponin content observed was comparatively lower especially in vegetable amaranth species, which improves their food value. Saponins can also inhibit the growth of cancer cells, boost immune system and energy, lower cholesterol, act as natural anti-inflammatory, antibiotic, anti-oxidant, and can reduce the uptake of certain nutrients including glucose and cholesterol at the gut through intralumenal physicochemical interaction (Aberoumand, 2012; Ray-Sahelian, 2012; De Silva et al., 2013) and have haemolytic activity (Khalil & Eladawy, 1994).

Natural crude extracts from plants have been used in traditional medicine to treat various ailments, *Amaranthus* species is one of them; though its complete therapeutic uses are still unexplored. Scientific interest in *Amaranthus* and its health promoting benefits has increased significantly in the recent past with various reviews presenting nutraceutical properties (Jose & Ana, 2011), its composition, antioxidant properties, applications and

processing (Venskutonis & Kraujalis, 2013). *Amaranthus* extracts have been used in ancient Indian, Nepalese, Chinese and Thai medicine to treat several conditions including urinary infections, gynaecological conditions, diarrhoea, pain, respiratory disorders, diabetes and also as diuretic (Baral et al., 2011; Agra et al., 2008; Kirtikar & Basu, 2001). In India, root extract of *A. spinosus* is given as a vermicide among the Santhali and Paharia tribes of eastern Bihar, while an aqueous decoction of the plant is used for chronic diarrhoea in southern Orissa (Hussain et al., 2009). Some tribes apply *A. spinosus* to induce abortion. The juice of *A. spinosus* is used by tribals of Kerala to prevent swelling around of stomach while leaves are boiled without salt and consumed for 2–3 days to cure jaundice (Hema et al., 2006). Along with medicinal use *Amaranthus* species are valuable food sources of nutrients with high quality proteins, vitamins, minerals *etc* (Silva-Sánchez et al., 2008). In addition to being consumed as a staple food, both the grain and vegetable *Amaranthus* have also been commercially exploited for natural dyes, pharmaceuticals, fodder, ornamental plants *etc*. The grains of *Amaranthus* have become one of the most favoured new foods in recent years, because they are gluten-free, and contain a balanced essential amino acid profile.

The medicinal plants contain several phytochemicals, which possess strong antioxidant activities. These antioxidant phytochemicals may be flavonoids (flavones, isoflavones, flavonones, anthocyanins, catechins, isocatechins, quercetin), terpenoids, polyphenols (ellagic acid, gallic acid, tannins), alkaloids, saponins, vitamins (A, C, E, K), carotenoids, minerals (selenium, copper, manganese, zinc, chromium, iodine), enzymes (superoxide dismutase, catalase, glutathion peroxidase), polysaccharides, saponins, lignins, xanthones, pigments *etc*. The antioxidants may cure different diseases by protecting the cells from damage caused by free radicals, the highly reactive oxygen compounds (Madhuri, 2008; Pandey & Madhuri, 2010; Govind, 2010; Gupta & Sharma, 2006; Heber, 2004;

Kathiresan et al., 2006; Kaur & Kapoor, 2002; Ray & Hussan, 2002). The present phytochemical analysis also detected classes of compounds having antioxidant activity.

PHYTOCHEMICAL ANALYSIS FOR THE IDENTIFICATION OF VOLATILE AND NON-VOLATILE COMPONENTS

Extraction is the main step for the recovery and isolation of bioactive phytochemicals from plant materials, before component analysis (Karimi & Jaafar, 2011). There are several ways for extracting compounds from fresh vegetable foods; one of them is by using solvents such as water, ethanol, methanol, ethyl acetate, hexane *etc*. (Singh et al., 2002). Conventional solvents like methanol and hexane are recognized for providing high extraction yields. Different techniques can be used for extraction; some of them are magnetic stirring, ultrasound homogenization, soxhlet extraction, maceration and supercritical fluid extraction (Gorinstein et al., 2007; Ozsoy et al., 2009; Barba de la Rosa et al., 2009; Repo-Carrasco-Valencia et al., 2010). According to López-Mejía et al. (2014) soxhlet extraction method gave best results for extracting antioxidant compounds, including phenolics, from amaranth leaves and seeds and found that leaf extracts had more antioxidant capacities than seed extracts. The present study used soxhlet extraction method for obtaining methanolic extract, which was subjected to screen volatile and non-volatile fractions present in different *Amaranthus* species by GC/MS (Table 27; Figs 12-19; 20 (i) - 20 (xxii) and HR-LC/MS (Table 28; Figs 21-28; 29 (i) –29 (xii).

Gas chromatography-mass spectrometry (GC/MS) analysis

In this modern period, the gas chromatography and mass spectrometry (GC/MS) studies have been more progressively useful for the investigation of most of the medicinal plants because this technique has established to be a

precious method for the chemical analysis of non polar components and volatile essential oil, fatty acids and lipids (Khare, 2007). GC/MS is the excellent practice to determine the bioactive components of alcohols, alkaloids, acids, esters, long chain hydrocarbons, steroids, amino acids and nitro compounds (Muthulakshmi et al., 2012). Association of chromatographic and spectroscopic methods is essential in analytical chemistry since it offers high sensitivity and selectivity, also has immense value in modern natural product analysis, which may further facilitate an insight of the medicinal applications of the traditional plant. GC/MS method can serve as an interesting tool for testing the amount of some active principles of herbs. It combines two analytical techniques to a single method of analyzing mixtures of chemical compounds. Gas chromatography separates the components of the mixture and mass spectrometry analyzes each of the components separately (Vuorela et al., 2005). Generally its application is oriented towards the specific detection and potential identification of compounds based on the molecular mass in a complex mixture. The combination of a principle separation technique (GC) with the best identification technique (MS) made GC/MS an ideal tool for qualitative and quantitative analysis for volatile and semi volatile compounds (Karthishwaran et al., 2012). Therefore, an attempt was made to screen the bioactive compounds present in different *Amaranthus* species, so as to evaluate the bioactive potential and to characterize them by GC/MS analysis.

The phytochemical profile of the methanolic extracts of eight species of *Amaranthus* determined using GC/MS analysis revealed a total of 171 compounds (Table 27) that belonged to various classes like phenols, flavonoids, terpenoids, alkaloids, fatty acids, ketones, esters, sterols, vitamins *etc.* Among the eight species studied highest number of compounds was found in *A. spinosus* and lowest in *A. blitum*. Among the 28 compounds identified from the methanolic extract of *A. blitum*, the major component

detected was α-tocopherol (vitamin E) with highest area percentage of 19.52. Other major components obtained were palmitic acid ester (13.28%), 25,26-Dihydroelasterol (11.63%), tabersonine (9.09%), 3-Methyl-1-phenyl-2-azafluorenone (8.82%) and 1(22),7(16)-diepoxy- tricyclo[20.8.0.0(7,16)] triacontane (8.53%). The methanolic extract of *A. caudatus* revealed a total of 36 compounds, of which compounds with highest area percentage was represented by palmitic acid ester (25.25%), 7,22-Ergostadienol (16.34%), phytol (14.31%) and 1(22),7(16)-diepoxy-tricyclo[20.8.0.0(7,16)]triacontane (12.26%). The methanolic extract of *A. dubius* revealed the presence of 36 compounds. The prominent compounds were E,E,Z-1,3,12-Nonadecatriene-5,14-diol (27.34%), 25,26-Dihydroelasterol (14.38%), 4-Isopropyl-1 ,6-dimethyldecahydronaphthalene (11.93%), 1,5,5-Trimethyl-6-methylene-cyclohexene (11.44%) and 1(22),7(16)-diepoxy-tricyclo[20.8.0.0(7,16)] triacontane (6.50%). Forty five compounds were identified from the methanolic extract of *A. hybridus* subsp. *hybridus.* The major compounds detected were phytol (14.39%), palmitic acid ester (12.53%), 1(22),7(16)-diepoxy-tricyclo[20.8.0.0(7,16)]triacontane (12.09%) and ergosta-7,22-dien-3-ol, (3.beta.,5.alpha.,22E) (9.94%). The plant *A. hybridus* subsp. *cruentus* revealed the presence of 30 compounds, the prominent compound obtained was a triglyceride, trilinolein (23.43%). Fatty acids and their esters are other major components obtained which included methyl palmitate (8.62%), linoleic acid methyl ester (0.79%), palmitic acid ester (9.99%), palmitic acid (3.26%) and docosahexaenoic acid, 1,2,3-propanetriyl ester (1.15%). The methanolic extract of *A. spinosus* revealed a total of 53 compounds, of which compounds with highest area percentage was represented by 1,5,5-Trimethyl-6-methylene-cyclohexene (15.92%), 3,5,24-Trimethyltetracontane (9.63%), chondrillasterol (8.27%), 1(22),7(16)-diepoxy- tricyclo[20.8.0.0(7,16)] triacontane (6.69%) and bis(2-Methylpropyl) ester 1,2-benzenedicarboxylic acid (6.21%). GC/MS analysis of the methanolic extract of *A. tricolor*

revealed the presence of 29 compounds. The prominent compounds obtained were dasycarpidan-1-methanol, acetate (ester) (23.29%), Z,Z-3,15-Octadecadien-1-ol acetate (16.11%), tetracontane-1,40-diol (10.98%), phytol (6.98%) and S-[2-[N,N-Dimethylamino]N,N-dimethylcarbamoyl thiocarbohydroximate (5.29%). The extract of *A. viridis* also showed the presence of potent compounds with palmitic acid ester (27.77%) being the major one. A total of 44 compounds were detected from this extract. The compound tabersonine, a terpenoid indol alkaloid was also occupied a major portion of the extract with 13.54%. The amount of hydrocarbon 1(22),7(16)-diepoxy tricyclo[20.8.0.0(7,16)]triacontane, diterpene alcohol phytol, triterpenoid squalene and sterol chondrillasterol were also noteworthy with 9.17, 7.0, 5.43 and 4.48% respectively.

GC/MS analysis of eight species of *Amaranthus* revealed an array of bioactive compounds. Fatty acids, terpenes, phenolic compounds and ketones were the predominant classes observed. Minor amounts of flavonoid were observed in all the extracts. Phytol (diterpene alcohol) and palmitic acid (fatty acid) are the common compounds found in all the species studied. Unique compounds observed in *A. blitum* are carvacrol (monoterpenoid), 1,2-Dihydro-1,1,6-trimethylnaphthalene, P-propiolic acid, 3-(1-hydroxy-2-isopropyl-5-methylcyclohexyl) and 1-Benzoylamino-5-piperidinyl-1-phenylpentane (organic compound), maytansine, 3-Methyl-1-phenyl-2-azafluorenone and 1-(1H-[1,2,4]Triazole-3-carbonyl)-piperidine (alkaloid), 2-Butanone, 1-(2,3,6-trimethylphenyl) (ketone), n-acetyl-l-phenylalanine methyl ester (amino acid derivative), propanoic acid (carboxylic acid) and linoleic acid methyl ester (fatty acid methyl ester). Unique compounds in *A. caudatus* are α-cubebene and (E)-*β*-farnesene (sesquiterpenoid), 1,7-Dimethyl-4-(1-methylethyl)-Spiro[4.5]dec-6-en-8-one and 9-(3,3-Dimethyloxiran-2-yl)-2,7-dimethylnona-2,6-dien-1-ol (organic compound), 15-Ethenyl-15-methyl 1-oxacyclopentadecan-2-one, 9,10-

Dimethyltricyclo[4.2.1.1(2,5)]decane-9,10-diol, 1-Hexacosene (hydrocarbon), Estra-1,3,5(10)-trien-17.beta.-ol (fatty acid), 6,9,12,15-Docosatetraenoic acid, methyl ester (fatty acid methyl ester), isophytol (diterpene alcohol), 1,10-Cycloeicosanedione (ketone), 2-Hexyl-1-decanol (alcohol), 6,10,14,18,22-Tetracosapentaen-2-ol,3bromo-2,6,10,15,19,23-hexamethyl-,(all-E)- (terpenoid) and 7,22-Ergostadienol (sterol). Unique compounds observed in *A. dubius* are bornyl chloride, 1-Naphthalenol, decahydro-1,4a-dimethyl-7-(1-methylethylidene), alpha-trans-bergamotenol, cis–Z- alpha-Bisabolene epoxide and calamenene (sesquiterpenoid), Tricyclo[6.3.0.0(1,5)]undecan-4-one, 5,9-dimethyl-, 4-Isopropyl-1,6-dimethyldecahydronaphthalene, 5-Methyl-Z-5-docosene and 1,54-Dibromotetrapentacontane (hydrocarbon), Benzene,1-methyl-3-[(1 methylethylidene)cyclopropyl]- (organic compound), (Z)-9,17-Octadecadienal (aldehyde) and tetracosanoic acid methyl ester (ester). Unique bioactive compounds found in *A. hybridus* subsp. *hybridus* are oblonginine (steroidal alkaloid), methyl salicylate (organic ester), eugenol (phenol), thujopsene (sesquiterpenoid), lignoceric acid methyl ester, 9-Octadecenoic acid, (2-phenyl-1,3-dioxolan-4-yl)methyl ester, cis- and hexacosanoic acid, methyl ester (fatty acid methyl ester). Unique bioactive compounds found in *A. hybridus* subsp. *cruentus* are mequinol, beta-ionol and o-eugenol (phenol), linoleic acid methyl ester, methyl palmitate and docosahexaenoic acid, 1,2,3-propanetriyl ester (fatty acid methyl ester) and ingol 12-acetate (diterpenoid). Unique bioactive compounds found in *A. spinosus* are methyl isolaurate (fatty acid methyl ester), 1,1,6-Trimethyl-1,2,3,4-tetrahydronaphthalene and limonen-6-ol, pivalate (Terpenoid), 1,3,8-p-Menthatriene, 1-chlorohexadecane (alkene), (*E*)-2-Methoxy-5-(1-propenyl) phenol (phenol), 3-tert-butyl-4-hydroxyanisole (ether), α-cuparenol (sesquiterpenoid phenol), benzyl benzoate (aromatic hydrocarbon), oleic acid (fatty acid), myristic acid ester, oleic acid ester (fatty acid ester), 1-Hentetracontanol (alcohol), 2,3,16-trihydroxy-5-pregnan-17(R)-20-yl acetate,

Pregna-3, 5-dien-20-one and 9,19-Cyclolanost-24-en-3- ol,acetate (sterol). Unique bioactive compounds found in *A. tricolor* are 4-Hydroxy-.beta.-ionone (ketone), trans-isoeugenol (phenylpropene), palmitic acid methyl ester (fatty acid methyl ester), tetracontane-1,40-diol (hydrocarbon) and nonanoic acid, 9-(3-hexenylidenecyclopro- (fatty acid derivative). Unique bioactive compounds found in *A. viridis* are 4-ketoisophorone (ketone), desulfogluconasturtiin and exo-2-Hydroxycineole (Organic compound), anethofuran (monoterpene), 9-Oximino-2,7-diethoxyfluorene (hydrocarbon), indolizine (heterocyclic aromatic compound), tetrahydro-22-desoxy-tomatillidine and conessine (steroidal alkaloid), 2,3-dibromo-8-phenyl-*p*-menthane (monoterpene hydrocarbon), cadinene (sesquiterpene), 7-Dehydrocholesteryl isocaproate (sterol), N-Ethyl-desoxy-veratramine (alkaloid) and methyl isopalmitate (fatty acid ester).

GC/MS analysis revealed an array of both saturated and unsaturated fatty acids in the plant extracts like palmitic acid, oleic acid, arachidic acid, lauric acid and esters of fatty acids such as lauric acid, linoleic acid, palmitic acid, myristic acid, pentadecanoic acid, oleic acid, linolenic acid, tricosanoic acid, lignoceric acid, docosahexaenoic acid, hexacosanoic acid *etc*. From the present investigation it was found that all the species of *Amaranthus* are excellent sources of fatty acids. The present study revealed essential fatty acids such as linoleic acid, linolenic acid and docosahexanoic acid. The fatty acids and their esters found in different *Amaranthus* species are as follows; *A. blitum* (palmitic acid, palmitic acid ester, linoleic acid methyl ester, linolenic acid methyl ester, tricosanoic acid methyl ester and 14-methylheptadecanoic acid methyl ester), *A. caudatus* (palmitic acid ester, lauric acid, pentadecanoic acid methyl ester, 14-methylheptadecanoic acid methyl ester, linolenic acid methyl ester and tricosanoic acid methyl ester), *A. dubius* (palmitic acid, palmitic acid ester, arachidic acid, linolenic acid methyl ester, pentadecanoic acid methyl ester and tricosanoic acid methyl ester), *A. hybridus* subsp.

hybridus (palmitic acid, palmitic acid ester, lauric acid, pentadecanoic acid methyl ester, linolenic acid methyl ester, lignoceric acid methyl ester and hexacosanoic acid methyl ester), *A. hybridus* subsp. *cruentus* (palmitic acid, palmitic acid ester, linoleic acid methyl ester), *A. spinosus* (oleic acid, oleic acid ester, myristic acid ester, methyl isolaurate, palmitic acid ester and pentadecanoic acid methyl ester), *A. tricolor* (palmitic acid, lauric acid, pentadecanoic acid methyl ester and linolenic acid methyl ester), *A. viridis* (lauric acid, arachidic acid, palmitic acid ester, linolenic acid methyl ester and 14-methylheptadecanoic acid methyl ester). A study by Fernando and Bean (1984) reported fatty acids and sterols from *Amaranthus tricolor* by GC/MS analysis. The fatty acids observed were linoleic acid, linolenic acid, palmitic acid, lignoceric acid and arachidic acid. Five sterols were identified and spinasterol was found in highest amounts. Several studies have been reported on the fatty acid composition in different *Amaranthus* species and their nutraceutical significance (Qureshi et al., 1996; Jahaniaval et al., 2000; Berganza et al., 2003; Martirosyan et al., 2007).

Compound having nutraceutical potential obtained in the present study are essential fatty acids (linoleic acid, linolenic acid, oleic acid and docosahexanoic acid), α-tocopherol (vitamin E) in *A. blitum* and *A. spinosus*. Among the studied species, significant amount of vitamin E was found in *A. blitum* (19.52%), hence the present study suggests that the aerial part of *A. blitum* may be used as a vitamin E source. Some phytosterol compounds, amino acid derivatives and squalene are also found in the extract. Certain anti-nutritional compounds such as alkaloids, tannins, saponin *etc*. were also observed in the extracts of *Amaranthus* in trace amounts.

A comparative phytochemical analysis was done between species in the present study to find out the chemical affinity among different *Amaranthus* species. Based on the compounds from GC/MS analysis, highest

chemical similarity found beween *A. hybridus* subsp. *hybridus* and *A. viridis* was that, out of the total 89 compounds from both the species, 21 compounds are similar.

The GC/MS analysis revealed an array of bioactive compounds and belongs to various classes such as fatty acids, terpenes, phenolic compounds, flavonoids, alkaloids, ketones, esters, sterols, vitamins *etc.* Guaiacol, mequinol, 2-Methoxy-4-vinylphenol, syringol, beta-ionol, and *o*-eugenol are the major phenolic compounds identified from the extracts. The phenolic compounds are one of the largest and most ubiquitous groups of plant metabolites. A number of studies have focused on these phytocompounds having many biological properties such as antiapoptosis, anti-ageing, anticarcinogen, anti-inflammation, anti-artherosclerosis, cardiovascular protection and improvement of the endothelial functions, as well as inhibition of angiogenesis and cell proliferation (Han et al., 2007). Phenolic compounds have been extensively used in disinfections and as bacteriocides (Okwu, 2001). Mequinol is a common active ingredient in drugs used for skin depigmentation and have anti-inflammatory (Vennila & Udayakumar, 2016) and anticancer (Uddin et al., 2015) activities. Mequinol has been reported from many plant species such as *Nerium oleander* (Hase et al., 2017), *Croton bonplandianum* (Vennila & Udayakumar, 2016) *etc*. In the present study mequinol was detected in the extract of *A. hybridus* subsp. *cruentus* alone. Syringol is present in all the species except *A. blitum*, *A. caudatus* and *A. dubius* in the present study. According to the study of Intisar et al. (2013), syringol is an anticancer phenolic compound. *o*-eugenol is a volatile phenolic compound and is the major component of the essential oil extracted from clove buds (*Syzygium aromaticum*), holy basil leaf (*Ocimum sanctum*) and cinnamon leaf (*Cinnamomum zeylanicum*) but the compound was also identified in less aromatic crops such as *Raphanus sativus* (Anandjiwala et al., 2006). In the present study *o*-eugenol was found in *A. hybridus* subsp.

cruentus alone. Daniel et al. (2009) reported the anti-inflammatory and antinociceptive activities of eugenol. It also possesses antimicrobial activity (Amiri et al., 2008).

Minor occurrence of flavonoid was observed in the GC/MS analysis of plant extract. Sinensetin is the only flavonoid found in the extract of *A. spinosus* and *A. viridis* in trace amounds. It has been previously reported in many plant species such as *Orthosiphon stamineus*, *Cordyceps militaris*, *Citrus etc*. and are reported to have bioactivities such as anticancer (Dong et al., 2011), antioxidant (Akowuah et al., 2004), anti-diabetic (Mohamed et al., 2012) *etc*.

Terpenoids are the other major class of compounds detected in GC/MS analysis. Terpene compounds such as anethofuran, carvacrol, 1,1,6-Trimethyl-1,2,3,4-tetrahydronaphthalene, 2,3-dibromo-8-phenyl-*p*-menthane, limonen-6-ol, pivalate, ingol 12-acetate, isophytol, phytol, squalene, oleanolic acid and sesquiterpenoids such as α-cubebene, calamenene, thujopsene, cadinene, (E)-*β*-farnesene, alpha-trans-bergamotenol and α-cuparenol were identified from the plant extracts. *Anethum graveolens* is the natural source of the monoterpene anethofuran, which was detected in *A. viridis* alone in the present study. According to Zheng et al. (1992), three monoterpenes such as anethofuran, carvone and limonene are potential cancer chemopreventive agents. Carvacrol is a monoterpenoid, a major component of essential oil bearing aromatic plants such as *Origanum*, *Ocimum etc*. It is a potent natural antimicrobial and bio-herbicidal compound (Lambert et al., 2001; Nostro et al., 2007; Azirak & Karaman, 2008) found in *A. blitum* alone in the present study. Limonen-6-ol, pivalate has been reported as a major component in the essential oils of many plants species such as *Nigella sativa*, *Artemisia*, *Tagetes, Origanum etc*. and are reported to have many bioactivities. Ingol 12-acetate is a derivative of ingol, initially reported as a new macrocyclic

diterpene alcohol from *Euphorbia ingens* by Opferkuch and Hecker (1973) and then from the latex of other *Euphorbia* species (*E. kamerunica*, *E. pekinensis*, *E. royleana etc*.) and also from *Zingiber officinale* and are reported to possess medicinal properties. Phytol is a common compound observed in all the species of *Amaranthus* in significant quantity. Among the eight species, highest phytol content was found in *A. hybridus* subsp. *hybridus* (14.39%) and *A. caudatus* (14.31%) and least in *A. dubius* (1.59%) and *A. spinosus* (1.34%). Phytol is a diterpene alcohol having reported anti-microbial activity against *Mycobacterium tuberculosis* with a minimum inhibitory concentration of 2 μg/ml (Rajab et al., 1998) and *Staphylococcus aureus* (Inoue et al., 2005). It has also shown to possess antispasmodic activity (Pongprayoon et al., 1992). Steinberg et al. (1966) reported the toxicity of dietary phytol in animals. Feeding of phytol in large doses (2-5% by weight in the diet) led to accumulation of phytanic acid in the mouse, rat, rabbit, and chinchilla, the degree of accumulation depending upon the level of dietary intake. Comparatively low phytol content found in the vegetable species analyzed in the present study may enhance their nutritional potential.

Squalene is an important triterpenoid which acts as a biosynthetic precursor to all steroids in plants and animals. Because of its significant dietary benefits, biocompatibility, inertness and other advantageous properties, squalene is extensively used as an excipient in pharmaceutical formulations for disease management and therapy. This compound is now widely used as an important ingredient in skin cosmetics and as a lubricant for precision instruments (Budin et al., 1996; Sun et al., 1995). Squalene has also been reported to have important beneficial effects on health, such as decreasing the risk for various cancers (Rao et al., 1998) and reducing serum cholesterol levels, enhances the immune response to various associated antigens, exhibits chemopreventive activity (Reddy & Couvreur, 2009) *etc*. In the present study squalene was identified from the methanolic extract of all

the amaranth species except *A. caudatus* and *A. hybridus* subsp. *cruentus* in significant proportions. Application of squalene in nutraceutical and pharmaceutical fields has been increasing progressively, so the demand for this substance is expected to increase continuously. Shark liver oil is the primary source of natural squalene. Plant resources were considered as another potential source of squalene and have been widely prospected. Squalene contents in some commercially important oils, such as olive, rice bran, corn, peanut, rapeseed, sunflower and cottonseed oils, are in the range of 0.01-0.4% (Becker, 1994), which is not high enough for them to be considered as viable resources. Recently, more attention has been focused on squalene from *Amaranthus* grain. Oils from *Amaranthus* grain have been reported to contain larger amounts of squalene (2.4-8.0%) than other common vegetable oils (Bruni et al., 2001; León-Camacho et al., 2001). Squalene content in *A. cruentus* has been reported to be 0.43% of the total seed weight (Becker, 1994). Earlier reports reveal that squalene was present at 0.73% in the seed of *A. hypochondriacus* and at 1.32% in the seed of *Amaranthus pumilus* (Marcone, 2000). The present study observed significant squalene content in the aerial parts of eight *Amaranthus* species except *A. caudatus* and *A. hybridus* subsp. *cruentus*. The amount of squalene ranges from 2.74% (*A. blitum*) to 5.43% (*A. viridis*).

Oleanolic acid is a ubiquitous triterpenoid in plant kingdom having many reported therapeutic properties such as antihyperlipidemic, antioxidant, diuretic/saluretic, hypoglycemic (Somova et al., 2003), anti-HIV (Kashiwada, 1998), anticancer (Li et al., 2002), anti-inflammatory (Singh et al., 1992) *etc*. In the present study oleanolic acid was found in *A. hybridus* subsp. *hybridus*, *A. hybridus* subsp. *cruentus* and *A. spinosus*. Calamenene is a sesquiterpenoid observed in the extract of *A. dubius* alone in the present study. It has been isolated from many plant species and have reported

bioactivities such as antioxidant, antimicrobial, antiproliferative (Azevedo et al., 2013; Dai et al., 2012) *etc*.

Alkaloids are a diverse group of low molecular weight, nitrogen containing compounds found in about 20% of plant species. The potent biological activity of some alkaloids has also led to their exploitation as pharmaceuticals, stimulants, narcotics and poisons (Facchini, 2001). Alkaloids are the other major class of compounds observed in the present study by GC/MS analysis. The major ones include oblonginine, tetrahydro-22-desoxy-tomatillidine, conessine, conhydrine, 2-cyclohexylpiperidine, maytansine, N-Ethyl-desoxy-veratramine and tabersonine. Oblonginine, tetrahydro-22-desoxy-tomatillidine and conessine are steroidal alkaloids. Oblonginine has been previously isolated from *Veratrum oblongum* and reported in *Solanum* and *Allium*. Conessine has been previously reported in *Holarrhena antidysenterica* and it possesses a wide range of activities against four insect species *viz*., *Aedes aegypti*, *Dysdercus koenigii*, *Spodoptera litura* and *Pieris brassicae* **(**Thappa et al., 1989). The present study showed trace amount of maytansine in the extract of *A. blitum*. Maytansine is a toxic natural alkaloid having anti-mitotic and anti-tumor activity (Remillard et al., 1975).

Eugenol (*A. hybridus* subsp. *hybridus*), cis-isoeugenol (*A. hybridus* subsp. *hybridus* and *A. viridis*) and trans-isoeugenol (*A. tricolor*) are the phenolic compounds identified in the present study and were previously reported in many plant species especially in *Ocimum*. Plants synthesize the volatile phenolic compounds *viz*., eugenol and isoeugenol to serve in defense against herbivores and pathogens (Koeduka et al., 2008). Eugenol have many reported bioactivities such as antimicrobial, anthelmintic, cytotoxic, antioxidant, antipyretic (Asha et al., 2001; Ali et al., 2005; Ogata et al., 2000) *etc*. Beta-Ionone is a ketone compound observed in *A. caudatus* and *A.*

spinosus in the present study and earlier studies report that it possess chemopreventive, antiproliferative and antioxidant potential (Asokkumar et al., 2012).

Trilinolein is a triglyceride identified from the methanolic extract of *A. blitum* and *A. hybridus* subsp. *cruentus* in the present study. Trilinolein is the major compound in the extract of *A. hybridus* subsp. *cruentus* with highest peak area percentage of 23.42%. It was previously isolated and purified from a commonly used traditional Chinese medicine *Panax notoginseng* and have antioxidant and cardio protective potential (Chan & Tomlinson, 2000; Chan et al., 2002).

α-tocopherol (vitamin E) is the major component identified from *A. blitum* with the highest peak area percentage of 19.52. It was also found in *A. spinosus* (1.53%). α-tocopherol was previously reported in *Amaranthus* species, especially in grain amaranth (Lehmann et al., 1994; Ozsoy et al., 2009; Akubugwo et al., 2007). Potential antioxidant (Patra et al., 2001) and antiatherogenic activity (Carr et al., 2000) of α-tocopherol was reported previously. Vitamin E is essential for the formation and normal functioning of red blood cells and muscles (Lukaski, 2004). The present result suggests that *A. blitum* may be included in the daily diet by considering it as a vitamin E source.

Sterols such as 7,22-Ergostadienol, Chondrillasterol, 25,26-Dihydroelasterol *etc*. was also found in the different amaranth species studied. A study by Xu et al. (1986) analyzed nineteen species and varieties of Amaranthaceae for sterol composition using GC/MS and HPLC and found that spinasterol and 7-stigmastenol were the dominant sterols in all species. Minor sterols identified in one or more species included cholesterol, campesterol, stigmasterol and sitosterol as well as 7,22-ergostadienol, 7,24(28)-ergostadienol, 7-ergostenol, 7,25-stigmastadienol and 7,24(28)-

stigmastadienol. Chondrillasterol is reported previously in *A. palmeri* (Dailey et al., 1997) and is considered as an allelochemical. The phytochemical compound, 25,26-Dihydroelasterol is also reported in many plant species such as *Momordica charantia*, *Cucurbita pepo*, *Cayaponia africana etc*. after GC/MS analysis.

High-resolution liquid chromatography mass spectrometry (HR-LC/MS)

HR-LC/MS analysis of all the eight species of *Amaranthus* was done to detect the presence of phytoconstituents which were not revealed through GC/MS. Total of 96 non- volatile compounds were identified from all the species [Table 28; Figs 21-29 (i) - 29 (xii)], which includes phenols, flavonoids, alkaloids, fatty acid derivatives, amino acids and their derivatives, peptides, vitamins, glycosides, sesquiterpenoids, tetranortriterpenoids, phospholipids, ketones *etc*. HR-LC/MS is one of the powerful analytical techniques used for the quantitative and qualitative estimation of non-volatile fractions present in the plant extract. This technique detects trace amount of compounds from the complex biological mixture and provides high levels of sensitivity and selectivity (Lu et al., 2008). The methanolic extract of *A. blitum* revealed 23 compounds and *A. caudatus* showed 27 compounds. Twenty one compounds are obtained from the extract of *A. dubius*, *A. tricolor* and *A. viridis*. Thirty eight compounds are obtained from the extract of *A. hybridus* subsp. *hybridus* whereas 25 and 17 compounds are obtained from *A. hybridus* subsp. *cruentus* and *A. spinosus* respectively. Most of the compounds obtained are of proven biological and nutraceutical potential. The LC/MS profile revealed variations in chemical constituents, with swietenine, a tetranortriterpenoid being the only common compound. The extract of *A. blitum* showed a single unique compound, which was N-Carboxyethyl-gamma-aminobutyric acid (non-protein amino acid derivative). Alkaloids such as ecgonine and ajmaline, peptides like Thr-Phe, Lys-Gln-Leu, Thr-Arg-

Phe, Lys-His-Cys, Arg-Pro-Gly and Arg-Phe-Gln, aromatic carboxylic acid like benzoic acid and a ketone compound such as 3,7-Epoxycaryophyllan-6-one were the unique compounds found in *A. caudatus*. The unique compounds in the extract of *A. dubius* are betaine and hydroxylysine (Amino acid derivative), Ser-Lys-Lys and Arg-Pro-Pro (peptides) and nervonic acid (fatty acid). Among the eight species studied *A. hybridus* subsp. *hybridus* scored highest number of compounds (38), of which 28 compounds are unique to the species, the major ones include omega-hydroxy myristic acid (fatty acid derivative), dimethylglycine and tyramine (amino acid derivatives), 1-Aminocyclopropane-1-carboxylic acid (α-amino acid), normetanephrine (catecholamine), 2-methoxy-4-vinylphenol (phenol), beta-Carboline and ambelline (alkaloids), dihydromyricetin and rutin (flavonoids), dihydrocaryophyllen-5-one (sesquiterpenoid), 4-heptanone (ketone), 12-hydroxy-10-octadecynoic acid (fatty acid), embelin (glycoside), peruvoside (cardiac glycoside), calotropin (Cardenolide) and peptides such as Pro-Ile, Thr-Arg-Tyr, Ile-Asp-Asp *etc*. Unique compounds found in *A. hybridus* subsp. *cruentus* include peptides like Gly-Ala-Val and Phe-Arg-Thr and an alkaloid compound such as norharman. Peptides like Ala-Phe-Pro and Phe-Val-Leu are the two unique compounds found in the extract of *A. spinosus*. A sesquiterpenoid, artemisinin and peptides like Ala-Ala-Leu and Thr-Pro-Asp are the unique compounds found in *A. tricolor*. Peptides such as Phe-Ala-Pro, Ala-Pro-Asp and 6-Deoxotyphasterol (Brassinosteroid precursor) are the unique compounds found in *A. viridis*. Based on the compounds obtained from HR-LC/MS analysis highest chemical affinities was found between *A. blitum* and *A. hybridus* subsp. *cruentus*, as out of the 48 compounds from both the species, 13 compounds are similar. HR-LC/MS analysis revealed many compounds to prove the nutritional potential of *Amaranthus* species such as amino acid derivatives, vitamins, peptides, amino acids, steroids, lipids *etc*. In addition several bioactive compounds which were not detected in GC/MS

analysis such as beta-carboline, cuscohygrine, embelin, pantothenic acid, ambelline, betaine, carnitine, dihydromyricetin, rutin, artemisinin, punctaporin B, 2,6-dihydrofissinolide, peruvoside, phytosphingosine, dihydrosphingosine, swietenine and many bioactive peptides *etc*. were also observed.

Many diseases prevalent in the world nowadays present an important relation with the diet. A healthy diet combined with the intake of functional foods may help minimize or even prevent certain diseases (Jew et al., 2009). In recent years, there has been much interest in studying old crops such as quinoa, amaranth and chia, which were widely used by people during the past. HR-LC/MS analysis revealed the occurrence of many bioactive peptides in the present study (Table 28). The recognition of the role of proteins as physiologically active components is actually increasing. Many foods that contain physiologically active proteins, native or processed, are rich sources of bioactive peptides. Today the most important sources of bioactive peptides are milk proteins (Korhonen & Pihlanto, 2006) and plant proteins (soy, wheat, corn, rice, barley, wheat, sunflowers *etc*.) (Wang & Gonzalez de Mejia, 2005). The functional components can be diverse molecules, among them are bioactive peptides which generally present short sequences and are encrypted in food proteins (Quian et al., 2008; Udenigwe & Aluko, 2012). Over the last decade there have been several reports which describe different biological activities performed by the bioactive peptides. Among the bioactive food components, some proteins and peptides from different sources are under clinical or preclinical studies to be used as alternative therapies to conventional cancer treatments (Bhutia & Maiti, 2008; Perez Espitia et al., 2012; Udenigwe & Aluko, 2012). Peptides and proteins exert their action through different mechanisms covering all stages of cancer development including initiation, promotion and progression (Bhutia & Maiti, 2008; Gonzalez de Mejia & Dia, 2010). There are some well known examples of

peptides and proteins derived from plants with a recognized antitumor activity such as lunasin and Bowman Birk inhibitor from soybean (Hernandez-Ledesma et al., 2009; Kobayashi, 2013). The potential associated to amaranth proteins has been reported by Mendonça et al. (2009). Silva-Sánchez et al. (2008) have found a lunasin-like peptide in the glutelin fraction from *A. hypochondriacus* with potential antitumor properties. From the point of view of human health, amaranth contains, either in leaves or seeds, numerous health- and nutritional-beneficial compounds. Besides, and due to the absence of prolamins in the protein composition, amaranth is a functional food alternative for celiac patients. In addition, a renewed interest in amaranth consumption is due to the presence of encrypted peptides with several biological activities such as antihypertensive, anticarcinogenic, antidiabetic (Silva-Sánchez et al., 2008; Velarde-Salcedo et al., 2013) *etc*.

Bioactivities of herbal formulations are believed to be due to its wide range of phytoconstituents alone, but recently studies showed the role of bioactive peptides in the treatment of various ailments. In addition to numerous preventive and therapeutic drug regimens, there has been increased focus on identifying dietary compounds that may contribute a healthy life in recent years. Food-derived bioactive peptides represent one such source of health-enhancing components. A recent comprehensive review by Montoya-Rodríguez et al. (2015) identified bioactive peptide sequences from *A. hypochondriacus* seed proteins and studied their role in preventing chronic diseases especially cardiovascular disease, cancer and diabetes. The plant, *A. hypochondriacus* is a pseudocereal with higher protein concentration than most cereal grains but there is limited information about the bioactivity of peptides from amaranth proteins. Enzymatic hydrolysis and food processing could produce biopeptides from amaranth proteins. Amaranth proteins, reported in UniProt database, were evaluated for potential bioactive peptide using BIOPEP database and revealed that 15 major proteins are present in

amaranth seeds. All proteins showed high occurrence frequencies of angiotensin-converting enzyme-inhibitor peptides as well as of DPP-IV inhibitor. Other proteins showed antioxidative and glucose uptake-stimulating activity and also found antithrombotic and anticancer sequences. Their results support the concept that amaranth grain could be part of a "healthy" diet and thereby prevent chronic human diseases. This also emphasizes the opportunity of the health benefits from these bioactive peptides to be gained from regular food consumption, rather than a formulated drug therapy (Cavazos & Gonzalez de Mejia, 2013).

A catecholamine such as normetanephrine was observed in the methanolic extract of *A. hybridus* subsp. *hybridus* in the present study. Three catecholamines such as dopamine, norepinephrine and normetanephrine were identified by GC/MS in *Solanum tuberosum* previously (Szopa et al., 2001). Catecholamines are biologically active substances with various cellular regulatory functions. Norepinephrine and epinephrine are neurotransmitters that are closely related with the aetiology of different physiological and neurodegenerative diseases of the central nervous system. This indicates the significance of determining the levels of catecholamines and their metabolites (normetaepinephrine and metanephrine) in tissue, serum and urine samples, which can serve as biochemical markers for a number of diseases (Pheochromocytoma, Parkinson's disease, Alzheimer's *etc*.) (Mateva & Mitev, 2008).

Both GC/MS and HR-LC/MS analysis revealed a phenolic compound such as 2-methoxy-4-vinylphenol, which was found to have anti-inflammatory effect via the suppression of NF-κB and MAPK activation, and acetylation of histone H3 (Jeong et al., 2011). It has also found to possess antimicrobial, antioxidant and analgesic activity (Vadivel & Gopalakrishnan, 2011). Alkaloids such as beta-carboline, ambelline, alpha-erythroidine,

ajmaline, cuscohygrine, rescinnamine *etc*. were also found in the extract. Beta-carboline was previously detected from the seeds of *Peganum harmala.* It has various types of pharmacological activities such as antimicrobial, antifungal, antitumor, cytotoxic, antiplasmodial, antioxidant, antimutagenic, antigenotoxic and hallucinogenic properties (Patel et al., 2012; Nenaah, 2010). Cuscohygrine was previously extracted from *Atropa belladonna* (Van Haga, 1954) and *Withania somnifera* and it's cytotoxic and genotoxic effects on bone marrow of mice were evaluated (Sharma et al., 2011; Said et al., 2013).

Betaine is an important compound identified from the extract of *A. dubius*. It is distributed widely in animals, plants and microorganisms, and rich dietary sources include seafood, especially marine invertebrates (~1%), wheat germ or bran (~1%) and spinach (~0.7%). The principal physiologic role of betaine is as an osmolyte and methyl donor (transmethylation). As an osmolyte, betaine protects cells, proteins and enzymes from environmental stress. Betaine has been shown to protect internal organs, improve vascular risk factors and enhance performance. Databases of betaine content in food are being developed for correlation with population health studies. Evidences show that betaine is an important nutrient for the prevention of chronic disease. It is an important human nutrient obtained from the diet from a variety of foods. It is rapidly absorbed and utilized as an osmolyte and source of methyl groups and thereby helps to maintain liver, heart and kidney health. Betaine can reduce the elevated serum homocysteine concentrations associated with mild or severe hyperhomocystinuria via the methionine cycle and may play a role in epigenetics and athletic performance (Craig, 2004). According to Ji and Kaplowitz (2003), betaine decreases hyperhomocysteinemia, endoplasmic reticulum stress and liver injury in alcohol-fed mice. Blunden et al. (1999) examined the presence of betaines

from the family Amaranthaceae and isolated two important betaines such as glycine betaine and trigonelline.

Carnitine is a quaternary ammonium amino acid found in the extract of *A. caudatus*, *A. dubius*, *A. hybridus* subsp. *cruentus*, *A. tricolor* and *A. viridis*. According to Bourdin et al. (2007) carnitine is associated with fatty acid metabolism in plants. According to Rospond and Chłopicka (2013), L-carnitine plays a vital function in the metabolism of lipids and it carries long-chain fatty acids into mitochondria for beta-oxidation. An increase of the amount of L-carnitine in the human body may lead to reduction and inhibition of production of fatty tissue. Despite the fact that human body can synthesise L-carnitine, about 80% of this chemical compound is delivered by food. Animal by-products and milk are the major source of L-carnitine. Vegetables and fruits are products which contain less than 5 mg of L-carnitine per 100 g of dry matter. Carnitine can provide protection against lipid peroxidation hence is an antioxidant. Carnitine was also known for their hepatoprotective potential in mice (Yapar et al., 2007).

Dihydromyricetin and rutin are the two important flavonoids obtained from the extract of *A. hybridus* subsp. *hybridus*. Dihydromyricetin was previously reported from the leaves of *Ampelopsis grossedentata* (Du et al., 2002) and having anti-alcohol intoxication efficacy (Shen et al., 2012). It has numerous pharmacological activities, such as antioxidant (Liu et al., 2009; Zhang et al., 2003), anti-inflammatory (Chen et al., 2015; Hou et al., 2015), relieving cough, antimicrobial activity, anti-hypertension, hepatoprotective effect and anticarcinogenic effect (Ye et al., 2008; Kundaković, et al., 2008; Zhang et al., 2012; Qi et al., 2012). Recently, plenty of data supported that dihydromyricetin could inhibit the growth and metastasis of prostate cancer *in vitro* and *in vivo* (Ni et al., 2012). The existing data confirmed that dihydromyricetin has a strong inhibitory activity against breast cancer MCF-7

cells and MDA-MB-231 cells, nasopharyngeal carcinoma HK-1 cells, liver cancer Bel-7402 cells, leukemia HL-60, K-562 cells and lung cancer H1299 cells (Jeon et al., 2008).

Rutin is a well known flavonoid, already established as a therapeutic compound with many reported bioactivities such as anti-inflammatory (Guardia et al., 2001), anti-tumor (Deschner et al., 1991), anti-ulcer, antioxidant (La Casa et al., 2000), hepatoprotective (Janbaz et al., 2002) *etc*. Rutin is used in many countries as vasoprotectants and are ingredients of numerous multivitamin preparations and herbal remedies (Erlund et al., 2000). The rutin content in five amaranth species ranged from 0.08 (seeds) to 24.5 g/kg DW (leaves), among which *A. hybridus* and *A. cruentus* being the best sources of rutin (Venskutonis & Kraujalis, 2013). By using HPLC technique, Paśko et al. (2008) explored the phenol and flavonoid content present in the seed sprouts of *A. cruentus*. The main flavonoid found in the sprouts was rutin. But the present study detects rutin in the extract of *A. hybridus* subsp. *hybridus* alone. A study by Noori et al. (2015) on root, leaf, inflorescence and seed flavonoids of seven *Amaranthus* species revealed the presence of isorhamnetin, kaempferol, quercetin and rutin in the aerial parts of all the taxa studied.

Sesquiterpenoids such as artemisinin, dihydrocaryophyllen-5-one, punctaporin B and tetranortriterpenoids such as swietenine and 2,6-dihydrofissinolide were also obtained from the extract. Artemisinin is a potent antimalarial compound previously isolated from *Artemisia annua*. Dihydrocaryophyllen-5-one and punctaporin B are previously reported from *Vernonia cineria* by LC/MS analysis and are found to be potent antitumor agents (Pichette et al., 2002). *Swietenia macrophylla* seeds are the major source of swietenine in nature. According to Dewanjee et al. (2009) swietenine showed hypoglycemic and hypolipidemic activity in type 2

diabetic rats. Its antidiarrhoeal, anti-tumor, antidiabetic and antimutagenic activities were also have been documented (Maiti et al., 2007; Moghadamtousi et al., 2013). The compound 2,6-dihydrofissinolide was previously reported from the extract of *Khaya senegalensis* and it was found to be having cytotoxic and antimicrobial activities (Kubmarawa et al., 2008; Ayo et al., 2007).

Pantothenic acid (vitamin B_5) was detected in the extract of *A. blitum* and *A. dubius*. It plays an essential role in the synthesis of fatty acids, membrane phospholipids and for the synthesis of amino acids such as leucine, arginine and methionine (Pesofsky-Vig, 1996). Methyl dihydrojasmonate detected in the *Amaranthus* extract is a proven cytotoxic and anticancer agent (Heyfets & Flescher, 2007).

Embelin is identified from the extract of *A. hybridus* subsp. *hybridus*. *Embelia ribes* is the major source of embelin in nature and it possess various bioactivities such as antitumor, anti-inflammatory, analgesic (Chitra et al., 1994), antibacterial (Chitra et al., 2003), wound healing (Swamy et al., 2007), chemopreventive (Sreepriya & Bali, 2005) *etc*.

A brassinosteroid precursor such as 6-deoxotyphasterol is identified from the extract of *A. viridis*, which was previously reported in *Arabidopsis thaliana* (Fujioka et al., 1998) and from the cultured cells of *Catharanthus roseus* (Yong-Hwa et al., 1997). Brassinosteroids are a group of naturally occurring plant steroidal compounds with wide-ranging biological activity that offer the unique possibility of increasing crop yields through both changing plant metabolism and protecting plants from environmental stresses (Krishna, 2003).

Peruvoside identified from the extract of *A. hybridus* subsp. *hybridus* in the present study was previously reported as a new cardiac glycoside from

Thevetia neriifolia (Arora et al., 1967). The immediate haemodynamic effects of peruvoside obtained from *Thevetia neriifolia* were studied in six patients with congestive heart failure (Bhatia et al., 1970). The drug was found to have an immediate and powerful positive inotropic and negative chronotropic effect. Oral peruvoside was also found to be effective in the treatment of congestive heart failure when used on a short-term as well as long-term basis. Thus peruvoside is a useful cardiac glycoside used for the management of congestive heart failure in man as a quick-acting intravenous preparation and oral administration was also found to be equally effective. The cardiac glycoside has been used for over two centuries as stimulant in cases of cardiac failure and diseases (Trease & Evans, 1978; Olayinka et al., 1992).

Two sphingolipids such as phytosphingosine and dihydrosphingosine identified from the *Amaranthus* extracts in the present study are the major sphingolipids found in plants and yeast (Imamura et al., 2007). Sphingolipids are a class of lipids derived from the aliphatic amino alcohol sphingosine, which are ubiquitous constituents of eukaryotic cells that also occur in a few prokaryotes. They are known to function as membrane structural components, as bioactive molecules involved in signal transduction and cell regulation, and in cell-cell interactions but the biological roles of sphingolipids and its derivatives remain unclear (Lynch & Dunn, 2004). Fatty acids and their derivatives such as docosanedioic acid, nervonic acid, 4-methyl-decanoic acid, omega-hydroxy myristic acid and 12-hydroxy-10-octadecynoic acid were found in the extracts studied. Nervonic acid was previously detected in plants such as *Malania oleifera*, *Macaranga adenantha* and *Thlaspi arvense* (Ma et al., 2004). A study by Henry et al. (2002) revealed that there was an increase in antioxidant activity with increasing chain length from octanoic acid to myristic acid (C-8 – C-14) and a decrease thereafter. According to their study, all unsaturated fatty acids tested at 60 µg/ml showed good

antioxidant activity except for undecylenic acid, cis-5-dodecenoic acid and nervonic acid.

The present phytochemical investigation of eight *Amaranthus* species revealed plenty of bioactive compounds which proves their pharmacological and nutraceutical potential. Qualitative and quantitative phytochemical screening gave an overall idea about the major classes of compounds present and their abundance in different *Amaranthus* species. GC/MS and HR-LC/MS analysis specifically detected that the bioactive compounds exist in different species. The analysis revealed compounds having nutritional significance such as plenty of fatty acids, vitamins, phytosterols, amino acids, lipids, squalene *etc*. Whereas insignificant amounts of anti-nutrients such as tannins, saponins, alkaloids *etc*. were also detected. In addition, phytochemical investigation using GC/MS and HR-LC/MS analysis precisely detected several bioactive compounds of potential therapeutic significance, preferably anticancer, anti-inflammatory, antioxidant, antimicrobial, anti-ulcer, antidiabetic, hepatoprotective, analgesic and chemopreventive activities. All together the present phytochemical characterization highlights the phytochemical profile of eight *Amaranthus* species studied, together with their pharmacological and nutraceutical efficacy brought about by the bioactive components detected.

PROXIMATE COMPOSITION ANALYSIS

The challenge for agricultural practices to increase food production to obtain food security still persists even after 40 years of the Green Revolution (Hobbs, 2007). The first millennium development goal is to reduce hunger and poverty by 2015 (Dixon et al., 2006). The demand for food is increasing, not only to meet food security for growing populations, but also to provide more nutritious food, rich in good quality proteins and nutraceutical compounds (Barba de la Rosa et al., 2009). According to the recent estimate

of Food and Agricultural Organization (FAO), 12% of the population suffering from chronic undernourishment across the world, indicates towards finding diet-based ways of combating such nutritional disorders (Nehal et al., 2016). One such remedy can be the exploration of underutilized foods like indigenous leafy vegetables which are the inexpensive source of nutrition and can be used to eradicate micronutrient malnutrition and degenerative diseases (Gupta et al., 2005). One such vegetable is *Amaranthus*, which is a valuable nutritious foodstuff with high production ability and adaptability to thrive extreme agronomic conditions. The edible parts are the seeds, leaves and tender stems. They are non-grass, broad-leaved plants that produces small seeds on a sorghum-like head. The grain is a pseudocereal consumed in various parts of the world due to its higher dietary benefits. Grain amaranths constitute three principal species such as *Amaranthus cruentus*, *Amaranthus hypochondriacus* and *Amaranthus caudatus* (Shukla et al., 2003) and vegetable amaranth represents species such as *A. blitum*, *A. dubius* and *A. tricolor*. Grain amaranth has higher protein than other cereal grains and has significantly higher lysine and methionine content (Bressani, 1989; Lehman, 1989). It is a gluten-free pseudocereal having significant levels of squalene, an important precursor for all steroids (He et al., 2002), also containing various valuable constituents and from these points of view it is attractive as a raw material for foods with health and medicinal benefits, including the prevention and treatment of some diseases and disorders. The nutritional analysis of amaranth grain was extensively studied rather than leaves. A comparative analysis of the nutritional profile of different amaranth species was scarce, which was attempted in the present study. Previous reports on various functional attributes exhibited by *Amaranthus* species are consolidated in Figure 1.

Proximate composition analysis of different *Amaranthus* species was investigated using standard analytical methods in order to assess their

nutritional potential. The percentage content of protein, lipid, carbohydrate, ash and dietary fibre was determined and represented in Fig. 30. The results showed that moisture, protein, ash (mineral) and dietary fibre content are present in significant proportions in all the species but carbohydrate and lipid content was found in lower quantities.

The results of proximate composition of different *Amaranthus* species in the present study showed higher moisture content among the eight species (79.60% to 87.70%). Comparatively highest moisture content was observed for *A. spinosus* and lowest for *A. viridis*. Moisture content and dry matter analysis during nutrition reporting is very important because it directly affects its nutritional content, stability and storage (Nehal et al., 2016). The proximate composition of *A. lividus* leaves showed high moisture content (85.01%) in a previous study by Nehal et al. (2016). *A. lividus* is the synonym of *A. blitum*. The present study also showed more or less similar percentage of moisture content of 84.73% in *A. blitum*. The high moisture content may induce a greater activity of water soluble enzymes and co-enzymes involved in metabolic activities of these plants (Iheanacho & Udebuani, 2009). According to Dewole et al. (2013), high moisture content is an index of spoilage whereas the low moisture content of the leaf increases the storage life by hindering the growth of microorganisms.

The use of proteins obtained from vegetables is presented as a prospect response to worldwide food needs (Aiking, 2011). For this reason, further information on the functional behaviour of proteins of vegetable origin is needed so that they can be incorporated to a greater variety of products. Amaranth grains are already known protein sources having significantly higher lysine content than other cereals such as wheat, rice and maize and sulphur amino acids than legumes, which makes it an important and inexpensive source of protein (Segura-Nieto et al., 1994). A comparative

analysis to determine the protein content in the aerial part of eight *Amaranthus* species was done in the present study. The crude protein content was found to be highest in *A. tricolor* (31.8 ± 0.18), followed by *A. blitum* (19.4 ± 0.08), *A. dubius* (18.8 ± 0.21), *A. caudatus* (18 ± 0.18), *A. viridis* (13.5 ± 0.09), *A. hybridus* subsp. *hybridus* (12 ± 0.12), *A. spinosus* (12 ± 0.11) and *A. hybridus* subsp. *cruentus* (6.4 ± 0.12). The highest protein content in the vegetable species such as *A. tricolor*, *A. blitum* and *A. dubius* highlights their nutritional potential. Plant food that provides more than 12% of its calorific value from protein is considered as a good source of protein (Ali, 2009). This suggests that the species such as *A. tricolor*, *A. blitum*, *A. dubius*, *A. caudatus* and *A. viridis* investigated are good sources of protein and could play a significant role in providing cheap and available proteins for rural communities. A previous study by Nehal et al. (2016) reported 17.28% crude protein in *A. lividus*. The Recommended Dietary Allowance (RDA) for protein is in the range of 28-65 g for children, lactating mothers, pregnant women and adults (Adinortey et al., 2012). The protein content detected for *A. tricolor*, *A. blitum*, *A. dubius* and *A. caudatus* in the present study is higher than the protein content reported for some leafy vegetable species such as *Momordica balsamia* (11.29%), *Lesianthera africana* (13.1-14.9%) and *Momordica foecide* (4.6%) (Ogle & Grivetti, 1985; Isong & Idiong, 1997; Hassan & Umar, 2006).

Physicochemical evaluation is an important parameter to identify the drug and to establish its quality and purity. Ash values are used to determine the presence of impurities like inorganic salts, carbonates, phosphates, silicates and silica. The amount of ash remaining after combustion of plant material varies considerably according to the part of the plant, age, treatment *etc*. The constituents of the ash also vary with time and from organ to organ. Ash usually represents the inorganic part and is an index of mineral content preserved in the food materials (Nehal et al., 2016). It contains inorganic

material of the plant because ashing destroys all the organic material present in the sample (Vermani et al., 2010). In the present study, significant amount of ash content was present in all the species in more or less equal proportions, of which highest content was found in *A. hybridus* subsp. *cruentus* (21.31 ± 0.07) and lowest in *A. caudatus* (17.53 ± 0.07). Almost equal percentage of ash content was observed for *A. blitum* (20.57 ± 0.06), *A. dubius* (20 ± 0.20), *A. hybridus* subsp. *hybridus* (20.83 ± 0.04) and *A. viridis* (20.75 ± 0.03). Ash content of *A. spinosus* and *A. tricolor* was 19.87 ± 0.03 and 19.72 ± 0.04 respectively. The results revealed that *Amaranthus* species can be used as a mineral source, since inorganic elements play an important role in physiological process involved in human health. Comparing with the ash value (2-10%) obtained for cereals and tubers (FAO, 1986), the eight species of *Amaranthus* studied can be considered as excellent mineral sources.

Appreciable amount of dietary fibre content was observed in all the *Amaranthus* species studied. The crude fibre content in *A. blitum*, *A. caudatus*, *A. dubius*, *A. hybridus* subsp. *hybridus*, *A. hybridus* subsp. *cruentus*, *A. spinosus*, *A. tricolor* and *A. viridis* was 6.14 ± 0.02, 6.25 ± 0.005, 7.67 ± 0.01, 5.26 ± 0.006, 8.57 ± 0.01, 6.25 ± 0.005, 7.27 ± 0.01 and 6.46 ± 0.006 respectively. Of which *A. hybridus* subsp. *cruentus* recorded highest fibre content (8.57%) whereas *A. hybridus* subsp. *hybridus* (5.26 %) recorded the lowest content. A study by Akubugwo et al. (2007) reported 8.61% of dietary fibre in *A. hybridus* subsp. *hybridus*. A previous study by Nehal et al. (2016) reported 8.35% crude fibre in *A. lividus*. Adequate intake of dietary fibre can lower the serum cholesterol level, risk of coronary heart disease, hypertension, constipation, diabetes, colon and breast cancer (Ishida et al., 2000; Rao et al., 1998) and also helps in digestion and regulation of blood sugar. The crude fibres in the leafy vegetables would be advantageous for their active role in the regulation of intestinal transit, increasing the dietary bulk due to their ability to absorb water (Jenkin et al., 1986). High level of

fibre is known to act as anti-tumorigenic and hypocholestrolaemic agent (Okoro & Achuba, 2012).

Amaranthus species showed negligible crude fat content, which ranges from 2.01 ± 0.05 (*A. tricolor*) to 2.82 ± 0.08 (*A. dubius*). Lipid content observed in other species was 2.61 ± 0.05 (*A. blitum*), 2.42 ± 0.06 (*A. caudatus*), 2.42 ± 0.02 (*A. hybridus* subsp. *hybridus*), 2.22 ± 0.07 (*A. hybridus* subsp. *cruentus*), 2.63 ± 0.09 (*A. spinosus*) and 2.02 ± 0.01 (*A. viridis*). Negligible crude fat content in *A. lividus* (0.69%) reported by Nehal et al. (2016), make the species an ideal component in several diets and makes it more palatable, as dietary fats function to increase palatability by absorbing and retaining flavours. The lower values of lipids in *Amaranthus* confirmed the findings of many authors (Nehal et al., 2016; Odhav et al., 2007; Patricia et al., 2014; Akubugwo et al., 2007) which showed that leafy vegetables are poor sources of lipids (Ejoh et al., 1996). Diet providing 1 – 2% of its calorific energy as fat is said to be sufficient to human beings, as excess fat consumption yields to cardiovascular disorders such as atherosclerosis, cancer and aging (Kris-Etherton et al., 2002). Therefore, the lower fat content observed in the *Amaranthus* species analyzed in the present study highlights its possible role in the diet of obese people and could be recommended as good source of food supplement for patients with cardiac problems or at risk with lipid induced disorders.

The carbohydrate content was also very low in all the species, which ranges from 1.92 ± 0.01 (*A. blitum*) to 7.40 ± 0.005 (*A. viridis*). Carbohydrate content in other species was 7.0 ± 0.02 (*A. caudatus*), 2.61 ± 0.01 (*A. dubius*), 1.98 ± 0.04 (*A. hybridus* subsp. *hybridus*), 2.25 ± 0.01 (*A. hybridus* subsp. *cruentus*), 2.82 ± 0.02 (*A. spinosus*) and 3.8 ± 0.06 (*A. tricolor*). Low carbohydrate content was also observed for *A. lividus* (4.13%) in a previous study by Nehal et al. (2016). According to Mnzava (1997) and Fasuyi (2006),

vegetables are not the major sources of carbohydrate rather contains proteins, vitamins, essential amino acids, minerals *etc*. Hence according to the present study the eight *Amaranthus* species investigated are poor source of carbohydrate. The low calorific value (Kcal/100 g) obtained for different *Amaranthus* species was estimated to be 76.03, 89.16, 78.79, 56.60, 42.22, 61.36, 107.98, 76.26 Kcal/100 g for *A. blitum*, *A. caudatus*, *A. dubius*, *A. hybridus* subsp. *hybridus*, *A. hybridus* subsp. *cruentus*, *A. spinosus*, *A. tricolor* and *A. viridis* respectively. Comparatively high calorific value for *A. tricolor* and low value for *A. hybridus* subsp. *cruentus* was recorded. The low calorific value of African green leafy vegetables including *Amaranthus* was reported by Patricia et al. (2014). Thus, the calorific value is in agreement with general observation that vegetables have low energy values (Lintas, 1992). The low calorific value of any food makes it good for the diet of obese people (Nehal et al., 2016). A previous study by Odhav et al. (2007) reported the nutritional profile of three *Amaranthus* species such as *A. dubius*, *A. hybridus* and *A. spinosus*. The study estimated moisture, protein, fat, fibre, ash, carbohydrate and energy value and was found to be comparable with the present study.

ELEMENTAL ANALYSIS (ICP-AES)

All human beings require a number of complex organic/inorganic compounds in diet to meet the need for their activities. The important constituents of diet are carbohydrates, fats, proteins, vitamins, minerals, fibres and water (Indrayan et al., 2005). Every constituent plays an important role and deficiency of any one constituent may lead to abnormal developments in the body. Plants are the rich source of all the elements essential for human beings. There is a relationship between the element content of the plant and its nutritional status. These micronutrients are essential food nutrients useful for the body as protective agent against diseases, thus necessary for health and growth (Ertan et al., 2002; Falade et al., 2003). Inadequate intake of

micronutrients known as “hidden hunger” contributes to the increasing rates of illness and death from infectious diseases and disability such as mental impairment (Black, 2003). Therefore, indigenous leafy vegetables having medicinal importance may be used as basic strategy for fighting against diseases, poverty, hunger, malnutrition and under nourishment (Barminas et al., 1998).

Traditional vegetables represent inexpensive but high quality nutrition sources for the poor segment of the population, especially where malnutrition is widespread. But decline in the use of indigenous vegetables by many rural communities has resulted in poor diets and increased incidence of nutritional deficiency disorders and diseases in many countries especially in Africa. There is also growing ignorance among young people about the existence of these nutritionally rich food plants (Odhav et al., 2007). Hence knowledge of indigenous plant use needs urgent scientific investigation and documentation before it is irretrievably lost to future generations (Guarino, 1997). Nutrient-rich foods are vital for proper growth both in adults and children. Minerals play important metabolic and physiological roles in the living system (Enechi & Donwodo, 2003; Ujowundu et al., 2010) and they serve as cofactors for many physiological and metabolic functions (Balogun & Olatidoye, 2012). Minerals are essential for proper tissue functioning and a daily requirement for human nutrition (Iniaghe et al., 2009). The recommended dietary allowance (RDA) for some minerals is as follows; calcium - 1000 mg/day, phosphorous - 800 mg/day, copper - 900 mg/day, zinc – 10 mg/day, magnesium – 400 mg/day, manganese - 7 mg/day and iron - 8 mg/day for adults (FND, 2002).

Analysis of mineral elements *viz*., Na, K, Ca, Mg, Fe, Zn, P and Mn in different *Amaranthus* species using ICP-AES in the present study revealed their presence in all the species in varying concentrations (Fig. 31 a, b). The

results showed that all the species of *Amaranthus* contained higher proportions of macro-minerals such as K, Ca, Mg and P and lower proportions of micro-minerals/trace minerals such as Fe, Mn, and Zn. Exceptionally the macro-mineral Na was found in low percentage in all the species studied.

According to Van Duyn and Pivonka (2000) dark-green leafy vegetables are primary sources of minerals and trace elements. A study by Nehal et al. (2016) reported higher proportions of macro-minerals such as K, Ca, Mg, Na and P and lower proportions of micro-minerals such as Fe, Mn, Zn and Cu in *A. lividus* after elemental analysis. A previous study by Odhav et al. (2007) reported the mineral content in three *Amaranthus* species such as *A. dubius*, *A. hybridus* and *A. spinosus* and observed the presence of Ca, P, Na, Mn, Cu, Zn, Mg, and Fe. Among which Ca content was found to be highest followed by Mg, P and then Na. In the present study among the macro and micro-minerals analyzed K content was highest in all the species followed by Ca, Mg, P, Na, Fe, Mn and Zn. Minerals are known as constituents of biological molecules that play important roles in metabolic and physiologic processes, such as co-factors for various metabolic processes (Mayer & Vyklicky, 1989; Brody, 1994). Potassium, calcium and magnesium take part in neuromuscular transmission and, together with other elements like manganese, they are involved in biochemical reactions in the body. Magnesium is a constituent of chlorophyll and it is an important component in connection with ischemic heart disease and calcium metabolism in bones (Ishida, 2000). Iron is a trace element essential for haemoglobin formation, normal functioning of central nervous system and oxidation of carbohydrates, proteins and fats (Adeleye & Otokiti, 1999). Manganese is an antioxidant which is also known to boost the immune system (Talwar, 1989). High potassium could be an advantage for people who take diuretics to control

hypertension and who suffer from excessive excretion of potassium through the body fluids (Siddhuraju et al., 2001).

In the present study Ca is found in highest percentage in all the species, of which *A. hybridus* subsp. *cruentus* showed highest quantity of 18.71% whereas *A. hybridus* subsp. *hybridus* and *A. blitum* showed lower percentages of 10.72% and 10.90% respectively. Ca content observed in other species was 17.77% in *A. dubius*, 16.51% in *A. tricolor*, 14.86% in *A. viridis*, 13.86% in *A. caudatus* and 12.77% in *A. spinosus*. A comparative study by Srivastava (2011) revealed higher Ca content in the dry leaves of *A. spinosus* (4500 mg/100 g dry weight) followed by *A. tricolor*, *A. viridis* and *A. blitum*, while iron content was maximum in *A. viridis* (15 mg) followed by *A. spinosus*, *A. tricolor* and *A. blitum*. Thus according to their study, *Amaranthus* species can be used as a source of biogenic calcium in antacid preparations. Calcium plays a vital role in regulating cellular transmembrane trafficking of elements and molecules (Borek, 2003).

Phosphorus content among the macro-minerals was found to be significant in all the species studied. Among the species, highest P content was observed in *A. tricolor* (2.85%) followed by *A. blitum* (2.60%), *A. caudatus* (2.46%), *A. hybridus* subsp. *cruentus* (2.27%), *A. spinosus* (2.24%), *A. hybridus* subsp. *hybridus* (1.88%), *A. dubius* (1.80%) and *A. viridis* (1.42%) (Fig. 31 a). Phosphorus is an important constituent of nucleic acids and cell membranes, and is directly involved in all energy-producing cellular reactions (Knochel et al., 2006).

Calcium and phosphorus are associated with the growth and maintenance of bones, teeth and muscles (Turan et al., 2003). Ca and P are directly involved in the development and maintenance of the skeletal system and participate in several physiological processes and play an important role in muscle contraction, blood clot formation, nerve impulse transmission, the

maintenance of cell integrity and acid-base equilibrium and activation of several important enzymes (Olagbemide & Ogunnusi, 2015). According to Adeyeye and Aye (2005), the Ca/P ratio higher than 1 might be advantageous for consumption of the studied leaves because diet is considered good if the ratio Ca/P is > 1 and as poor if < 0.5. Based on this all the species of *Amaranthus* investigated in the present study are good for diet since they showed Ca/P ratio greater than one (4.19 for *A. blitum*, 5.61 for *A. caudatus*, 9.82 for *A. dubius*, 5.68 for *A. hybridus* subsp. *hybridus*, 8.21 for *A. hybridus* subsp. *cruentus*, 5.70 for *A. spinosus*, 5.77 for *A. tricolor* and 10.41 for *A. viridis*).

Among the macro-minerals Na was found in lowest percentage in all the species of *Amaranthus* studied and was 0.95% in *A. tricolor*, 0.40% in *A. hybridus* subsp. *cruentus*, 0.39% in *A. dubius*, 0.38% in *A. caudatus*, 0.34% in *A. blitum*, 0.28% in *A. viridis*, 0.235% in *A. hybridus* subsp. *hybridus* and 0.23% in *A. spinosus* (Fig. 31 b). K content was found to be highest among the macro-minerals studied in all the species, which ranges from 18.76% (*A. tricolor*) to 25.19% (*A. hybridus* subsp. *hybridus*). K content in other species was 22.40% in *A. blitum*, 20.55% in *A. caudatus*, 21.68% in *A. dubius*, 21.23% in *A. hybridus* subsp. *cruentus*, 22.53% in *A. spinosus* and 24.66% in *A. viridis*. Sodium and potassium are important intracellular and extracellular cations respectively, which are involved in the regulation of plasma volume, acid-base balance, nerve and muscle contraction (Akpanyung, 2005).

The Na/K ratio in the body is of great concern for prevention of high blood pressure. Na/K ratio less than one is recommended (FND, 2002). Hence, consumption of different *Amaranthus* species in the present study would probably reduce high blood pressure diseases because the Na/K ratio of all the species was found to be less than one (0.01 for *A. blitum*, *A. caudatus*,

A. dubius, *A. hybridus* subsp. *cruentus*, *A. spinosus* and *A. viridis*, 0.009 for *A. hybridus* subsp. *hybridus*, 0.05 for *A. tricolor*).

Significant percentage of Mg was found in all the species studied, of which *A. blitum* showed higher content of 10.62% and *A. hybridus* subsp. *cruentus* showed least amount of 4.35%. Percentage of Mg content observed in other species such as *A. caudatus*, *A. spinosus*, *A. hybridus* subsp. *hybridus*, *A. tricolor*, *A. dubius* and *A. viridis* was 8.14, 7.36, 6.94, 6.84, 5.40 and 5.30% respectively. Mg is an essential cofactor in many enzymatic reactions in intermediary metabolism (Akpanabiater et al., 1998) and is an important component of chlorophyll (Akwaowo et al., 2000). It is known to prevent cardiomyopathy, muscle degeneration, growth retardation, alopecia, dermatitis, immunologic dysfunction, gonadal atrophy, impaired spermatogenesis, congenital malformations and bleeding disorders (Chaturvedi et al., 2004).

Among the trace minerals Fe was found in significantly higher proportions than Mn and Zn. The Fe content ranges from 0.14% (*A. blitum*) to 0.89% (*A. tricolor*). The percentage of Fe content in *A. caudatus*, *A. dubius*, *A. hybridus* subsp. *hybridus*, *A. hybridus* subsp. *cruentus*, *A. spinosus* and *A. viridis* was 0.37, 0.31, 0.191, 0.199, 0.296 and 0.293% respectively. According to Geissler and Powers (2005), Fe plays numerous biochemical roles in the body, including oxygen blinding in haemoglobin and acting as an important catalytic center in many enzymes as the cytochrome oxydase. Thus, the studied *Amaranthus* species could be recommended in diets for reducing anemia which affects more than one billion people worldwide (Trowbridge & Martorell, 2002).

Micro-minerals such as Mn and Zn were also found in all the species, even though in lesser amounts. Compared to Zn, Mn content was higher in all the species except in *A. hybridus* subsp. *cruentus* and *A. viridis*. Among the

species highest Mn content was in *A. hybridus* subsp. *hybridus* (0.085%) and lowest in *A. viridis* (0.034%). Mn content in other species was 0.069% in *A. dubius*, 0.054% in *A. caudatus* and *A. spinosus*, 0.045% in *A. tricolor*, 0.040% in *A. blitum* and 0.038% in *A. hybridus* subsp. *cruentus*. Highest Zn content was found in *A. hybridus* subsp. *cruentus* (0.052%), followed by *A. dubius* (0.038%), *A. caudatus* (0.037%), *A. viridis* (0.035%), *A. hybridus* subsp. *hybridus* (0.031%), *A. tricolor* (0.024%), *A. spinosus* (0.022%) and least amount in *A. blitum* (0.021%) (Fig. 31 b). Zinc is an important requirement in protein synthesis, normal body development and recovery from illnesses and is also involved in normal function of immune system and cell division (Olagbemide & Ogunnusi, 2015). It is a co-factor that hepls in the function of the enzyme carbonic anhydrase which is required for carbon dioxide transport and as part of peptidases needed for protein digestion (Muhammad et al., 2011). Mn plays important role in fat and carbohydrate metabolism, calcium absorption and blood sugar regulation. It is also necessary for normal brain and nerve function and is a component of the antioxidant enzyme superoxide dismutase (SOD), which helps fight free radicals (http[24]). Low Mn content observed in the present study reflects that *Amaranthus* is a poor dietary source of Mn.

Based on the above results *Amaranthus* species studied are found to be a good source of protein, ash (mineral) and dietary fibre but poor source of carbohydrate and lipids, which point out that they are low energy food. Presences of mineral elements such as Na, K, Ca, Mg, Fe, Zn, P and Mn in detectable amounts confirm that these species may be used as mineral sources, which helps to solve malnutrition and diseases that are mainly caused due to those deficiencies.

SUMMARY AND CONCLUSIONS

Alimentary and pharmaceutical dietary research is increasingly focusing on plant-derived nutraceuticals owing to their versatile applications. This is reflected in the increasing interest in potentially exploitable sources of food stuffs such as functional foods that are able to overcome any dietary insufficiencies. *Amaranthus* is an unexploited crop, which shows promising potential as a global resource by supplying nutritious grains as well as leafy vegetables. Recent decades have witnessed a resurgence of interest in *Amaranthus* species as nutraceutical and natural protector against chronic ailments. Hence currently this crop got global attention and is widely cultivated and consumed throughout the world. As an emerging food crop in the world, amaranth is considered as the 'food for future', in the sense that it is the food for the coming dry season. So, it is worthy to bring these plants into the light with respect to their various potentialities which are least explored. Hence a detailed investigation had been carried out on this genus in the present study with the following objectives: (1) species authentication using molecular sequencing studies (2) mitotic and meiotic chromosome characterization (3) micromorphological characterization of pollen grains, seed capsule and seed coat using SEM analysis (4) phytochemical characterization by GC/MS and HR-LC/MS analysis (5) comparative proximate composition and elemental analysis using ICP-AES (Inductively Coupled Plasma Atomic Emission Spectrometer).

The major findings emerged out from the study can be summarized as follows:

1. MOLECULAR CHARACTERIZATION

Molecular phylogenetic characterization of the eight species of *Amaranthus* collected for the present study has been conducted for the precise conformation of their species identity as it is difficult morphologically alone due to the occurrence of many natural interspecific hybrids. The PCR product of the samples was successfully sequenced with a base pair length of 697, 893 and 701 for rbcL, matK and ITS region of rDNA genes respectively. The rbcL, matK and ITS region of rDNA sequence data analysis of the eight *Amaranthus* species using MEGA6 software revealed the information of the conserved sites (C), variable sites (V), parsimony informative sites (Pi) and singleton sites (S) and found that more variable sites occur in the ITS region of rDNA gene sequences. The gene regions of rbcL and matK were found to be highly conserved with few variable regions. Among the 3 gene regions studied the Pi sites were more in ITS region of rDNA gene sequences. The rbcL, matK and ITS sequences obtained from the eight taxa studied and their relative species were compared with the help of GENEIOUS software. Phylogenetic analysis was done by using the sequences generated from rbcL, matK and ITS gene regions of the samples together with related sequences and out-groups downloaded from GenBank. The out-groups selected were genera close to *Amaranthus* (*Celosia*, *Pleuropetalum, Beta* and *Chenopodium*). Tree-based method was used to assess species specific clusters. Maximum Likelihood (ML) method yielded well resolved phylograms with comparable topologies. Best fit model nucleotide substitution was found out after Bayesian Inference (BI) analysis of the sequences and it was based on the lowest BIC value scored. The best fit model nucleotide substitution obtained after Bayesian Inference analysis for each gene region was Kimura 2-Parameter (K2) for rbcL, Tamura 3-Parameter (T92+G) for matK and Kimura 2-Parameter (K2+G) for ITS region of rDNA and were used for the phylogenetic tree construction. The Maximum

Likelihood dendrogram of rbcL, matK and ITS gene regions revealed the clustering of species and their conspecifics at distinct nodes with high support values and revealed the monophyly of the genus *Amaranthus* with a congruent topology for ML and BI analysis. The present phylogenetic study successfully resolved species identity of the collected *Amaranthus* species, since all the collected species and their close allies forms a monophyletic group/clade with a strong bootstrap support of >90%.

2. CYTOGENETICAL CHARACTERIZATION

Cytogenetical studies such as mitotic and meiotic chromosome characterization were made in eight species of *Amaranthus* collected. Mitotic squash experiment using aceto-orcein and DAPI staining technique revealed three somatic chromosome numbers *viz*., 2n = 32, 2n = 34 and 2n = 64 in the genus. Meiotic chromosome studies revealed three gametic numbers *viz*., n = 16, n = 17 and n = 32 in the genus *Amaranthus*. Among the eight species of *Amaranthus* studied *A. caudatus*, *A. hybridus* subsp. *hybridus* and *A. viridis* showed 2n = 32 chromosomes with a gametic number of n = 16. The other species studied *viz*., *A. blitum*, *A. hybridus* subsp. *cruentus*, *A. spinosu*s and *A. tricolor* showed 2n = 34 with a haploid chromosome number of n = 17. One tetraploid species observed was *A. dubius* with 2n = 64 (n = 32). The genus *Amaranthus* is dibasic, showing two basic numbers such as x = 8 and x = 9. Hence the diploid chromosome number 2n = 32 might have originated from secondary basic chromosome number of x_2 = 16, which may be originated from the primary base number x_1 = 8 by protoautoploidy. The chromosome number 2n = 34 might have originated either from the primary base number 8 or 9 through ascending dysploidy/descending dysploidy/amphiploidy. Apart from the few earlier chromosome count reports, detailed karyomorphometric studies were found to be lacking in the genus, hence many parameters such as total length of chromosomes, short arm length, long arm length, arm ratio,

centromeric index, karyotype formula *etc.* were considered in the present study. The chromosomes in all the species were found to be small in size less than 2.5 μm. Based on the chromosome size and centromeric index, chromosomes were classified mainly into metacentric, submetacentric, telocentric *etc*. The karyotypes showed variations in chromosome size and morphology among species which showed a tendency towards asymmetry. Various asymmetry indices *viz*., DI, VC, TF%, As K%, Syi-Rec, A_1-A_2, A and AI analyzed resulted in intermediary values, which depicts asymmetric karotype for all the species and are in a path of evolutionary advancement. A comparative cytogenetic analysis revealed that *A. tricolor* possessed the highest asymmetric karyotype which leads to the conclusion that *A. tricolor* is the most evolved species among the eight *Amaranthus* species investigated.

3. MICROMORPHOLOGICAL CHARACTERIZATION

Micromorphological characterization of pollen grains, seed capsule and seed coat of eight *Amaranthus* species collected was undertaken in the present study for observing possible micro features under SEM. A comparative micromorphological analysis of different *Amaranthus* species, which was yet to be documented, was attempted in the present study. The study revealed significant micromorphological features of pollen grains, seed coat and seed capsule, which was found to be enough to distinguish different species in the genus. Similarities and variations are observed in both qualitative and quatitative traits analyzed among the species.

A comparative micromorphological study of *Amaranthus* pollen grains using acetolysis and SEM analysis resulted that all the studied species had poly pantoporate pollen, with many pores distributed on the surface. The number of pores observed in the visible aperture was greater in the pollen of *A. dubius* (32), and lesser in *A. blitum* (12); the largest and the smallest pollen grains observed among the species studied respectively. The present

observation highlights the positive relationship between the size of pollen grain and aperture number. The aperture membrane ornamentation observed was scabrate for all the species, while its aggregation differed between species. Dense aggregations were observed in *A. dubius* and *A. spinosus*, while sparse aggregation was seen in all the other species, especially in *A. viridis*. Pollen grains are small with a mean size range of 13.87 to 20.67 µm. largest pollen grain was observed for *A. dubius* with a mean diameter of 20.42 µm, whereas the smallest for *A. blitum* with a mean diameter of 12.60 µm. Based on the P/E ratio the pollen shape observed in the present study was defined as prolate-spheroidal for all the species. Based on the palynological features observed, the eight *Amaranthus* species showed evolutionary advancement in the sense that the aperture of all the species are porate with scabrate/granulate membrane ornamentation, which resulted in interrupted exine. As far as the number of pores are concerned, the most developed taxa among the species studied was *A. dubius* as it showed highest number of pores (32) on the visible aperture, whereas the least developed species was *A. blitum*. A comparison of the present result with the previously reported micro features of the dioecious amaranth species revealed their highly advanced micro features over the monoecious species analyzed in the present work. The variations observed among species in the present study can be used as a species identification tool. Based on the pollen size, *A. dubius* and *A. blitum* can be distinguished from the other species. Degree of sculpturing aggregates and number of pores in the visible aperture was useful in identification of *A. dubius* from the other taxa. All together the observed palynological features might be used to discriminate the species studied in the genus *Amaranthus* to some extent.

The micromorphological features of the seed capsule of different *Amaranthus* species showed slight variations in size and capsule ornamentations. The capsule wall of *A. blitum* and *A. viridis* was found to be

indehiscent and rupture irregularly. The capsule ornamentation in *A. blitum* was uniform throughout the wall and showed entirely different pattern from other species. The capsule wall ornamentation of *A. viridis* was distinct from other species as it showed very strong rugose structure throughout. Capsule wall of all the other species of *Amaranthus* was found to be dehiscent with a prominent line of dehiscence and showed rugose or wrinkled pattern of wall ornamentations. The rugose pattern of ornamentation was uniform throughout the capsule of *A. caudatus*, *A. dubius* and *A. hybridus* subsp. *hybridus* whereas *A. hybridus* subsp. *cruentus*, *A. spinosus* and *A. tricolor* showed strong rugose above the line of dehiscence than the lower region of the capsule. Slight variations are also observed in the capsule size of different *Amaranthus* species. The longest capsule was observed for *A. tricolor* with a mean length of 3.98 mm, whereas the shortest capsule was for *A. blitum* with a mean length of 2.19 mm respectively. Quantitative and qualitative features of the seed capsule in the present investigation varied between the studied species and were useful in identification of different *Amaranthus* species. Based on the capsule size, *A. tricolor* (largest) and *A. blitum* (smallest) can be distinguished from the other species. On the basis of capsule rupturing *A. blitum* and *A. viridis* (indehiscent) can be identified from other species. On behalf of capsule ornamentation, *A. viridis* and *A. blitum* was distinct from other species. The pattern of capsule wall ornamentation was useful in identification of *A. hybridus* subsp. *cruentus*, *A. spinosus* and *A. tricolor* from the other species. Hence the seed capsule micro features observed in the present study confirmed its significance to discriminate *Amaranthus* species studied.

Seed surface micromorphological features observed by SEM analysis revealed variations among different *Amaranthus* species studied. Variations are observed in the seed shape, size, weight, colour and spermoderm/testa ornamentations. Various seed shapes observed *viz*., orbicular, elongated-

lenticular, lenticular, subglobose, elliptic-lenticular, and globose indicate that slight variations exist among the species studied. Considering mean seed length, width and length-width ratio, the largest seed was for *A. caudatus* and the smallest seed for *A. dubius*. Based on the mean weight of 1000 seeds, highest seed weight was for *A. tricolor* and lowest for *A. blitum*. The seed length was found to be higher than width in all the species except *A. viridis* which showed higher seed width than seed length. The seeds of eight *Amaranthus* species studied showed similarities and variations in spermoderm/testa ornamentations, which were found to be significant for species identification. The seeds of *A. blitum* showed prominent reticulation with polygonal and rectangular elevated areas on the spermoderm. Seeds showed prominent reticulation forming hexagonal and polygonal areas on the spermoderm of *A. dubius*. Prominent reticulation forming hexagonal and polygonal cavities throughout the spermoderm was observed in *A. spinosus*, whereas prominent reticulation forming hexagonal and polygonal areas throughout the spermoderm was for *A. tricolor*. Seeds showed well defined reticulation forming rectangular and polygonal elevated areas mainly in the flange region and ill defined ornamentation in the central region was found in the spermoderm of *A. hybridus* subsp. *cruentus*, whereas epidermal cells ornamentation having prominent reticulation forming irregularly shaped areas was observed on the spermoderm of *A. caudatus*. Seed surface ornamentation of *A. viridis* was entirely different from other species. Here the reticulate pattern of spermoderm ornamentation is absent; instead the entire spermoderm is rugulate forming an irregular muriform arrangement. A distinct pattern of testa ornamentation observed in *A. hybridus* subsp. *hybridus* was that seeds showed cross linked undulated thread like epidermal cells ornamentation forming polygonal meshes throughout the spermoderm. Among the eight species of *Amaranthus* studied, all of the species except *A. hybridus* subsp. *hybridus* and *A. viridis* showed reticulate pattern of testa

ornamentations with slight differences in between. The present study observed small black coloured seeds with undifferentiated pleurogram/flange for weed amaranths (*A. hybridus* subsp. *hybridus*, *A. spinosus* and *A. viridis*) and vegetable amaranths (*A. dubius* and *A. tricolor*). Pleurogram is completely absent in *A. viridis*. The grain amaranths in the present study *viz*., *A. caudatus* and *A. hybridus* subsp. *cruentus* showed well defined flange region without any folding, with brownish black and reddish brown seeds respectively rather than black. From the result it was clear that seed colour can be used as a promising character for the identification of grain and weed amaranth respectively. Quantitative and qualitative features of the seed coat in the present investigation varied between the studied species and were useful in identification of different *Amaranthus* species. Based on the seed size, *A. caudatus* (largest) and *A. dubius* (smallest) can be distinguished from the other species. On the basis of seed colour, weed and vegetable amaranth (black) can be distinguished from grain amaranth (brownish black or other than black) species. On the basis of spermoderm/testa surface and ornamentation, seeds of *A. viridis* are distinct from other species as it showed rough seed surface with rugulate pattern throughout. The seed surface ornamentation was also useful in identification of *A*. *hybridus* subsp. *hybridus* from the other species. Based on the presence of well defined pleurogram /flange, grain amaranth species can be distinguished from other species. The spemoderm/testa micro features investigated in different *Amaranthus* species confirmed its significance for species identification and might be used to discriminate different taxa under the genus *Amaranthus* to some extent.

4. PHYTOCHEMICAL CHARACTERIZATION

The phytochemical characterization studies included qualitative and quantitative screening of the methanolic extract of eight *Amaranthus* species together with their proximate composition analysis. Qualitative

phytochemical screening led to the disclosure of the presence of primary and secondary metabolites such as carbohydrate, protein, phenols, flavonoids, terpenoids, alkaloids, tannins, saponins, steroids, carotenoids and cardiac glycosides in the *Amaranthus* species studied. Quinones are absent in all the species except *A. viridis*. Anthraquinones are observed in *A. spinosus* alone, whereas *A. blitum* was devoid of saponins and phlobatannins. Phlobatannin was absent in *A. tricolor*. Quantitative analysis revealed the occurrence of phenols and terpenoids in appreciable amounts, whereas flavonoids, alkaloids, saponins, tannins and carotenoids in lesser amounts. Lower proportions of anti-nutrients such as saponins, tannins and alkaloids in the *Amaranthus* species studied may have a positive influence on their nutritional value. GC/MS and HR-LC/MS analysis disclosed the volatile and non-volatile components in the methanolic extract of *Amaranthus* species studied. The phytochemical profile of the methanolic extracts of eight species of *Amaranthus* determined using GC/MS analysis revealed a total of 171 compounds (Table 27) that belonged to various classes like phenols, flavonoids, terpenoids, alkaloids, fatty acids, ketones, esters, sterols, vitamins *etc.* of which phenolic and terpenoid compounds represents the major classes. GC/MS analysis unveiled the presence of 28 compounds in *A. blitum*, 36 compounds in *A. caudatus* and *A. dubius*, 45 compounds in *A. hybridus* subsp. *hybridus*, 30 compounds in *A. hybridus* subsp. *cruentus*, 53 compounds in *A. spinosus*, 29 compounds in *A. tricolor* and 44 compounds in *A. viridis*. The major components detected in *A. blitum* were α-tocopherol (vitamin E), palmitic acid ester, 25,26-Dihydroelasterol and tabersonine and that of *A. caudatus* were palmitic acid ester, 7,22-Ergostadienol, phytol *etc*. The dominant compounds in *A. dubius* were E,E,Z-1,3,12-Nonadecatriene-5,14-diol, 25,26-Dihydroelasterol, 4-Isopropyl-1,6-dimethyldecahydronaphthalene and 1,5,5-Trimethyl-6-methylene-cyclohexene. Phytol, palmitic acid ester and 1(22),7(16)-diepoxy-tricyclo[20.8.0.0(7,16)]triacontane were the predominant

compound observed in *A. hybridus* subsp. *hybridus*. Trilinolein, methyl palmitate, linoleic acid methyl ester, palmitic acid ester and palmitic acid were the dominant compounds in *A. hybridus* subsp. *cruentus*. The major compounds observed in the extract of *A. spinosus* includes 1,5,5-Trimethyl-6-methylene-cyclohexene, 3,5,24-Trimethyltetracontane, chondrillasterol *etc*. The prominent compound obtained from the extract of *A. tricolor* included dasycarpidan-1-methanol, acetate (ester), Z,Z-3,15-Octadecadien-1-ol acetate, tetracontane-1,40-diol *etc*. The extract of *A. viridis* also showed the presence of potent compounds with palmitic acid ester being the major one, followed by tabersonine, squalene, chondrillasterol *etc*. Phytol and palmitic acid are the common compounds found in all the species studied. Compounds having potential biological activity observed in GC/MS analysis include mequinol, syringol, eugenol, sinensetin, anethofuran, carvacrol, phytol, squalene, oleanolic acid, calamenene, trilinolein, α-tocopherol *etc*. Fatty acids, α-tocopherol and squalene are the major compounds observed having nutritional significance.

HR-LC/MS analysis unveiled a total of 96 compounds from all the species, which includes phenols, flavonoids, alkaloids, fatty acid derivatives, amino acids and their derivatives, peptides, vitamins, glycosides, sesquiterpenoids, tetranortriterpenoids, phospholipids, ketones *etc*. The methanolic extract of *A. blitum* revealed 23 compounds, and the extract of *A. caudatus* showed 27 compounds. A total of 21 compounds are obtained from the extract of *A. dubius*, *A. tricolor* and *A. viridis*. Thirty eight compounds are observed in *A. hybridus* subsp. *hybridus*, whereas 25 and 17 compounds are obtained from *A. hybridus* subsp. *cruentus* and *A. spinosus* respectively. The LC/MS profile revealed variations in chemical constituents, with swietenine, a tetranortriterpenoid being the only common compound. In addition several bioactive compounds which were not detected in GC/MS analysis such as beta-carboline, cuscohygrine, embelin, pantothenic acid, ambelline, betaine,

carnitine, dihydromyricetin, rutin, artemisinin, punctaporin B, 2,6-dihydrofissinolide, peruvoside, phytosphingosine, dihydrosphingosine, swietenine and many bioactive peptides were also observed. All together the present phytochemical analysis that unveiled the significant occurrence of bioactive nutraceutical constituents and irrelevant amount of anti-nutritional components in the *Amaranthus* species studied, which highlights their medicinal and food value.

The results of the proximate composition analysis revealed that significant occurrence of moisture, protein, ash (mineral) and dietary fibre in all the species rather than carbohydrate and lipid. Elemental analysis using ICP-AES showed that all the species of *Amaranthus* contained higher proportions of macro-minerals such as K, Ca, Mg and P and lower proportions of micro-minerals/trace minerals such as Fe, Mn, and Zn. Exceptionally the macro-mineral Na was found in low percentage in all the species studied. A comparative proximate composition analysis among the eight species revealed that highest protein content, more calorific value and highest percentage of minerals such as P, Na and Fe were found in *A. tricolor*, which highlights its nutritional significance as an edible leafy vegetable species. Highest proportion of ash, dietary fibre, Zn and Ca content were observed in *A. hybridus* subsp. *cruentus*. The highest percentage of minerals such as K and Mn was found in *A. hybridus* subsp. *hybridus*. The plant, *A. blitum* was found to be the major source of Mg. The highest percentage of moisture, crude fat and carbohydrate content was found in *A. spinosus*, *A. dubius* and *A. viridis* respectively. In a nutritional point of view, the eight *Amaranthus* species may be exploited as a good source for protein, minerals and dietary fibres, that may help to solve malnutrition and diseases that are mainly caused due to their deficiency. All the species studied were found to be poor sources of carbohydrates and crude fat, thus have low energy values. Low fat content and calorie make it an ideal component in several diets

suitable for obese people. The studies mentioned above conclusively indicate that *Amaranthus* species contains appreciable amount of nutrients and can be included in diet to supplement daily nutritional requirements to combat diseases, hence serving as a nutraceutical for fortification of food.

Deliverables

- Molecular studies of the selected *Amaranthus* species using rbcL, matK and ITS region of rDNA sequencing unraveled the species identity and genetic relatedness.
- Detailed mitotic and meiotic chromosome characterization led to the disclosure of the karyological identity and evolutionary status of the eight species of *Amaranthus*.
- Comparative micromorphological studies of pollen, seed capsule and seed coat revealed the common and unique micro features to discriminate the species studied.
- Phytochemical profiling by qualitative and quantitative techniques unveiled a multitude of compounds having nutraceutical significance.
- Proximate composition and elemental analysis revealed the nutraceutical potential of the species studied.

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