Cancer growth suppression using purified azurin from native *Pseudomonas* isolates

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

DOCTOR OF PHILOSOPHY IN MICROBIOLOGY

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Under the guidance of
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This is to certify that this thesis entitled **"Cancer growth suppression using purified azurin from native *Pseudomonas* isolates."** is a bonafide research work done by **Mrs. Sereena M C.,** under my supervision and guidance in the Department of Life Sciences, University of Calicut, for the award of the degree of Doctor of Philosophy in Microbiology, under the faculty of Science of the University of Calicut. I also certify that the same has not been submitted for any other degree diploma or associateship in any other University.

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I also hereby certify that the corrections/suggestions from the adjudicators have been incorporated in the revised thesis. Content of the CD submitted and the hardcopy of the thesis is one and the same.

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(Reg. No. 149/PO/Rc/S/1999/CPCSEA)

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CERTIFICATE

The Institutional Animal Ethics Committee of Amala Cancer Research Centre (No.149/PO/Rc/S/1999/CPCSEA) unanimously approved the animal experiments of the Ph.D. Thesis work of Sereena M.C., Research Scholar, Microbiology, Department of Life Sciences, University of Calicut entitled **"Cancer growth suppression using purified azurin from native *Pseudomonas* isolates"** (Approval No.ACRC/IAEC/2017[5]) carried out under the guidance of Dr. Ramadasan Kuttan.

Dr. Jose Padikkala, Ph.D.

Secretary, IAEC

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Institutional Animal Ethical Committee
Reg.No. 149/1999/CPCSEA
Amala Cancer Research Centre

DECLARATION

I, **Sereena M C**., hereby declare that this thesis entitled **"Cancer growth suppression using purified azurin from native *Pseudomonas* isolates"** is being submitted to the University of Calicut, in partial fulfillment of the requirement of the degree of Doctor of Philosophy in Microbiology under the faculty of Science. This thesis is the result of my work carried out in the department of Life Sciences under the guidance and supervision of Dr. Denoj Sebastian, Assistant Professor in Microbiology, Department of Life Sciences, University of Calicut. This thesis or any part thereof has not been submitted for any other degree, diploma or any other similar title of any university.

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University of Calicut **Sereena M C**
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Sereena M C

Dedicated to.....

All those who supported me in this endeavor

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LIST OF ACRONYMS USED

AGE	:	Agarose gel electrophoresis
BSA	:	Bovine serum albumin
BLAST	:	Basic Local Alignment Search Tool
CTAB	:	Cetyl trimethylammonium bromide
DAPI	:	4',6-diamidino-2-phenylindole is a fluorescent stain
DLA	:	Daltons Lymphoma Ascites
DNA	:	Deoxyribonucleic acid
DMEM	:	Dulbecco's Modified Eagle Medium
EDTA	:	Ethylenediaminetetraacetic acid
EYFP	:	Enhanced Yellow Fluorescent Protein
ECFP	:	Enhanced Cyan Fluorescent Protein
Etbr	:	Ethidium bromide
FBS	:	Fetal Bovine Serum
FDA	:	Food and Drug administration
FRET	:	Fluorescence Resonance Energy Transfer
FTIR	:	Fourier-transform infrared spectroscopy
FSC	:	Forward Scatter
HIV	:	Human Immunodeficiency Virus
HCT 15	:	Human Colorectal Carcinoma
IMViC	:	IMViC: Indole, Methyl red, Voges-Proskauer, Citrate
IPTG	:	Isopropyl β-D-1-thiogalactopyranoside
LB	:	Luria Bertani
LC-MS/MS	:	Liquid Chromatography with tandem mass spectrometry.
MALDI-TOF	:	Matrix Assisted Laser Desorption/Ionization Time of Flight

MEGA	:	Molecular Evolutionary Genetics Analysis
MCF7	:	Michigan Cancer Foundation
MCS	:	Multiple cloning sites
mM	:	Millimolar
MTT	:	3- (4,5-Dimethylthiazol-2-Yl)-2,5Diphenyltetrazolium Bromide
NCBI	:	National Center for Biotechnology Information
Ni NTA	:	Nickel-nitrilotriacetic acid
PAGE	:	Polyacrylamide gel electrophoresis
PBS	:	Phosphate-Buffered Saline
PCR	:	Polymerase chain reaction
RD	:	Restriction Digestion
rDNA	:	Ribosomal DNA
RNA	:	Ribonucleic acid
rpm	:	Rotation per minute
SDS	:	Sodium Dodecyl Sulfate
SW480	:	Spectral Karyotyping of the Human Colon Cancer Cellline
SSC	:	Side Scatter
UV	:	Ultra Violet

Chapter 1
General introduction

The word cancer explains an extensive series of malignant tumors which affect almost every tissues and organs of the body (Mansoori *et al*.,2007). Cancer is the second leading cause of mortality and morbidity worldwide (Ovesna & Horvathova-Kozics, 2005). The reason for this disease is the lack of balance between proliferation and apoptosis of cells (Kianoosh, G, & Mehdi, 2010). According to WHO, cancer is responsible for an estimated 9.6 million deaths in 2018, and approximately 70 % of the deaths caused by cancer occur in low- and middle-income countries. The major reasons that leads to cancer death can be listed as high body mass index, low fruit and vegetable intake, lack of physical activity, tobacco use, and alcohol use. Tobacco use is the most critical risk aspect for cancer in men and is responsible for approximately 22 % of cancer deaths (Walser *et al*., 2008). Cancer arises from the development of a pre-cancerous lesion to a malignant tumor in a multistage process. The progression of cancer is due to the interaction between a person's genetic factors and three categories of external agents, including:

- Physical carcinogens (ultraviolet and ionizing radiation).
- Chemical carcinogens (asbestos, components of tobacco smoke, aflatoxin and arsenic).
- Biological carcinogens (viruses, bacteria, or parasites) (Blagosklonny, 2005).

Viruses associated with cancer include human papillomavirus (genital carcinomas), hepatitis B virus (liver carcinoma), Epstein-Barr virus (Burkitt's lymphoma and nasopharyngeal carcinoma), human T-cell leukemia virus (T-cell lymphoma) and a herpes virus called KSHV (Kaposi's sarcoma and some B cell lymphomas). The transformation of virally induced tumors can be divided into two: acutely transforming and slowly transforming. Acute transforming viruses, induce a rapid tumor growth, since they carry an oncogene called viral-oncogene. Slowly transforming viruses, induces a slow tumor growth. It does not contain any viral oncogene and its genome part is integrated into the host genome. Hepatitis virus and human papillomavirus (HPV), are responsible for up to 25 % of the cancer cases in low- and middle-income countries (zur Hausen, 1991).

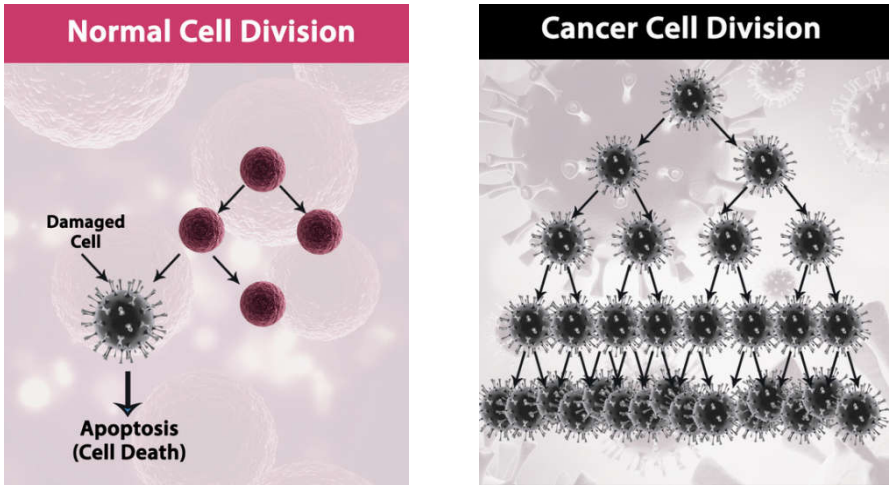


Figure 1.1 :Division of normal and cancer cells.

Many morphological and functional characteristics occur in cancer cells. Morphological changes include large nucleus having an

irregular size and shape; the nucleoli are prominent and the cytoplasm is scarce and intensely colored or pale. Cancer cells show decreased cell adhesion, and production of new enzymes, grow and divide at an abnormally rapid rate, are poorly differentiated, and have abnormal membranes, cytoskeletal proteins. The structure and division of normal cell and cancer cells are entirely different (Figure 1.1&1.2). The abnormality in cells can be progressive with a slow transition from normal cells to benign tumors to malignant tumors (Hanahan & Weinberg, 2000).

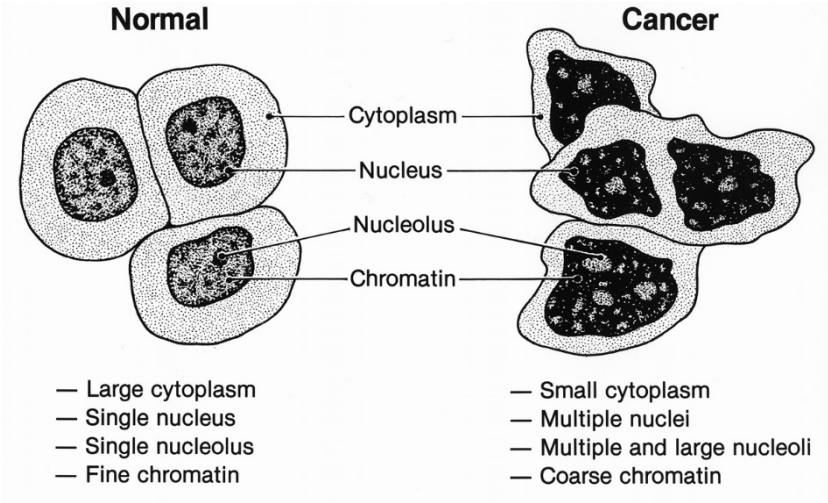


Figure1.2: Structure of normal and cancer cell (Baba, 2007)

Tumors that stay in one spot and have limited growth are generally considered to be benign. Malignant tumors are those in which a cancerous cell manages to move throughout the body using the blood or lymphatic systems, destroying healthy tissue in a process called the invasion. Cancer cell manages to divide and grow to produce

new blood vessels to feed itself in a process called angiogenesis. Angiogenesis is the physiological process through which new blood vessels form from pre-existing vessels (Birbrair *et al.,* 2015). Primary or secondary tumors require utilization of new blood vessels to grow to a large mass. Without a blood supply, a tumor can grow into a mass of about 10^6 cells, roughly a sphere that is 2 mm in diameter. Hence, the division of cells on the outside of the tumor mass is balanced by the death of cells in the center due to an insufficient supply of nutrients. So tumors force the formation of new blood vessels that invade the tumor and nourish it, which constitute a process called angiogenesis (Gallup & Talledo, 1987). The genetic changes allow the cancer cells to break away from the primary cancer, travel through lymph and bloodstream and spread to the other tissues. Hence, it can invade to distant parts of the body. This process is called metastasis and this type of cancer is called metastatic cancer. Metastatic cancer can grow and interfere with the digestive, nervous, and circulatory systems, and they can release hormones that alter body function for many types of cancer. It is also called stage IV (four) cancer (C. A. Klein, 2008).

There are over 100 different types of cancer, and each is classified by the type of the cell that is initial. These types include:

- **Carcinoma:** Cancers that developed from epithelial cells of skin or the tissues that covers the internal organs such as lungs, colon and pancreas.
- **Sarcoma:** Cancers that mainly affect the connective tissues, such as fat, muscle, blood vessels and nerves.

- **Lymphoma and leukemia:** These type of cancer usually begins in the bone marrow. It mainly affects the formation of blood cells.
- **Germ cell tumor:** Germ cell tumors that are originated from reproductive cells, mainly effect testicle or the ovary.
- **Blastoma:** Cancers are derived from precursor cells, more commonly seen in children than adults.
- **Mesothelioma:** These are rare type of cancers, derived from the thin lining of body organs such as lungs, stomach and heart.

Many different types of cancer screening tests exist, depending on the site of the cancer origin. Some of these tests include mammograms, colonoscopies, computed tomography (CT) scans, sigmoidoscopy, and high-sensitivity fecal occult blood tests (FOBTs), lab tests, Pap test, and human papillomavirus (HPV) testing, etc.(National Cancer Institute, 2018).

1.1 Types of cancer treatment

Different types of cancer treatments are practiced nowadays such as surgery, chemotherapy, radiation therapy, hormonal therapy and monoclonal antibody therapy. The criteria for choosing the type of treatment depends upon the site and stage of tumor and also the health condition of patient (National Cancer Institute, 2018). About two in five people will experience cancer at some point in their lifetime

(Sausville & Longo, 2005). Some of the cancer treatments such as radiotherapy or chemotherapy cause major side effects. The discovery of novel anticancer compounds with lesser side effects are very essential in cancer therapy. The major cause of cancer growth progression is due to the lack of apoptosis. So, induction of apoptosis directly leads to the tumor regression (Woynarowska & Woynarowski, 2002).

1.2 Bacterial therapy of cancer

Live or attenuated pathogenic bacteria or their products were used in the treatment of cancer (A. M. Chakrabarty, 2003). The use of bacteria in the regression of certain forms of cancer has been recognized. In recent times, the microbe based therapy of cancer has attracted considerable interest and new microbial compounds having specific anticancer activity without side effects are assessed for their ability to act as a new anticancer agent (Schulz, 2005). The knowledge of using bacteria in cancer therapy (Figure 1.3) is considered as a new idea, but it originates from ancient times. Researchers have been looking to engineer microorganism to produce medicinally important compounds and to improve their activity via protein engineering so that cancer treatments only become more economical and available (Punj, Das Gupta, & Chakrabarty, 2003).

In ancient times 2600 BC, an Egyptian physician Imhotep reported the relation between infections and certain swellings (most likely cancerous tissue) (Garrison-Morton, 1937). Later, some evidence proved that tumor retardation was observed in cancer patients

suffering from gas gangrene, caused by *Clostridia* (Felgner, Kocijancic, Frahm, & Weiss, 2016). But clinically proved by W. Busch in 1863, they purposefully infected a female cancer patient with *Streptococcus pyogenes*. The lady became infected and tumor growth decreased but she died because of that infection. However, a lack of balance between the therapeutic benefit and infection control occurred (Cheyne, 1886; Hoption Cann, van Netten, & van Netten, 2003). The American physician William Coley (1862–1936) (pioneer of cancer immunotherapy) discovered a mixture containing heat-inactivated *Streptococcus pyogenes* and *Serratia marcescens*, known as "Coley's toxin". He injected this toxin to primary tumor mass and kept increasing the dose throughout therapy to more than 1000 patients (Coley, 1898, 1910). Tumor regression was observed in many cases. Some patients showed complete clearance of the primary tumor mass and a disease-free state was obtained (McCarthy, 2006). This condition arises by increased production of Tumor Necrosis Factor-α (TNF-α) secretion in the body of the patient (Karpiński & Szkaradkiewicz, 2013). A significant disadvantage of bacterial therapy has been the undesired infections caused by the bacteria. So, the discovery of novel approaches for anticancer therapies include the use of purified products from microbial origins must be needed (A. M. Fialho, Stevens, Das Gupta, & Chakrabarty, 2007).

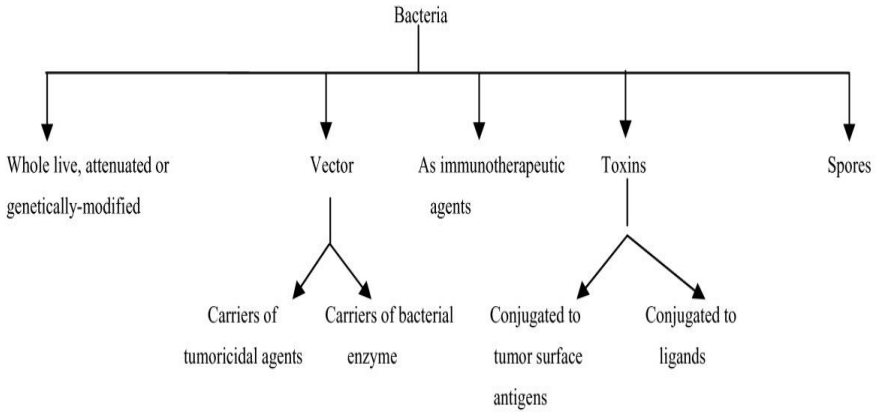


Figure 1.3:Schematic overview of the role of bacteria in cancer therapy (Patyar *et al.,* 2010).

Several microorganisms have been used for the cancer regression (Elahian *et al.,* 2013). *Myxobacterium Sorangium cellulosum* shown antitumor activity to different type of human tumors by the production of epothilone A, epothilone B and desoxyepothilone B (a chemically modified synthetic form of EpoB). The most sticking out example would be the *Mycobacterium bovis* BCG. This vaccine strain is used for the treatment of bladder cancer (A. M. Chakrabarty, 2003). *Listeria monocytogenes* and *Salmonella enterica serovar Typhimurium* that target the antigen-presenting cells and induces an innate immune response (Shahabi, Maciag, Rivera, & Wallecha, 2010). *Shigella* and *Clostridia* species are used for the delivery of therapeutic agents to tumor cells (Ramasamy, Nattarayan, Jayaraj, Arulanandh, & Jaiswal, 2012). A protozoan parasite, *Toxoplasma gondii*, caused tumor regression by blocking angiogenesis (Suzuki, Muto, & Kobayashi, 1986). *Serratia Marcescens* that produces an endotoxin that promoted tumor regression (A. M. Chakrabarty, 2003).

1.3 Azurin: a novel anticancer agent

Azurin, a blue cuprous protein primarily discovered in the 1950s by Verhoeven & Takeda (Verhoeven, 1956). According to Horio *et al.,* (1958), this protein has a brilliant blue colour and named as *Pseudomonas* blue protein. Its physical and chemical properties were studied by Horio *et al.,* (Horio, 1958). Azurin is mainly produced from few Gram-negative bacteria such as *Pseudomonas, Bordetella*, *Nesseria, Methylomonas* and *Alcaligenes*. It participate in the transfer of single electrons between enzymes associated with the cytochrome chain. Azurin (also mentioned as "Paz") is a 128-amino-acid periplasmic protein that also has antimicrobial activity (Hashimoto, Ochiai, Hong, Murata, & Chakrabarty, 2015). These are small proteins (14 KDa), located in the periplasmic space of bacteria where they function as electron carriers (Wood, 1978).

The presence of a single copper atom imparts an intense blue color to the protein, and has a fluorescence emission band centered at 308 nm. It participates in denitrification processes in bacteria (De Rienzo, Gabdoulline, Menziani, & Wade, 2000). Southerland and Wilkinson describe the preparation, purification and some properties of azurin (Horio, 1958; Sutherland & Wilkinson, 1963).

The potentiality of azurin as an anticancer agent is due to its special entry in to cancer cells, and no adverse side effects were observed in anticancer studies (Nguyen & Nguyen, 2016). Water solubility of azurin helps its tissue penetration and clearance from the blood stream (van de Kamp et al., 1990). Azurin is a small protein it

can be hypothesized and its expression may occur in different vectors, including some human cell types (Bernardes, Chakrabarty, & Fialho, 2013). All these reasons make azurin as a novel molecule to be used in cancer therapy.

Azurin's cytotoxicity is only for specific cancer cells and does not affect healthy cells, and it has fewer toxic side effects. So it will show a harmless treatment for patients and prevent them from risky anomalies. A second most vital feature of azurin is that tremendous number of therapeutic substance can link to azurin molecule, So it can acts as a cargo protein (Mahfouz, Hashimoto, Das Gupta, & Chakrabarty, 2007). Azurin has antimalarial and anti-HIV activity, so that it can acts as a prospective therapeutic agents in the treatment of malaria and HIV-1 infections and the toxoplasmosis-causing parasite *Toxoplasma gondii* (Chaudhari et al., 2006). Azurin is a multi-faceted anticancer agent that acts through mainly three pathways; induction of apoptosis through intracellular stabilization of p53 protein, that is azurin p53 complex formation enhancing its intracellular level, which leads to apoptosis through caspase-mediated mitochondrial cytochrome C release pathways. Second one inhibition of cell cycle progression through intracellular binding to Eph receptor tyrosine kinase. Finally, prevention of angiogenesis through inhibition of VEGFA (Vascular endothelium growth factor A) (Apiyo & Wittung-Stafshede, 2005; Chaudhari et al., 2007; Miyashita & Reed, 1995; Pasquale, 2010).

1.4 Structure of azurin

Azurin's structure contains four loops and three distinct binding regions so it can bind to multiple sites on a ligand (Figure 1.4). This protein contains two charged clusters and a prominent neutral aromatic-rich hydrophobic patch on Phe114 and it is structurally similar with variable domains of immunoglobulins. Because of all this arrangement, azurin has scaffold properties (A. M. Chakrabarty, Bernardes, & Fialho, 2014; M. Fialho, Bernardes, & M Chakrabarty, 2016). Azurin belong to the type I copper protein family. It has an intense absorption band in the visible spectra (~max = 595-630 nm, e _~ 5000 M -1 cm-1), and a narrow hyper- fine splitting in the electron paramagnetic resonance spectra (All ~ 0.006cm -1) (Adman, Canters, Hill, & Kitchen, 1982). The copper ion is coordinated by the Sγ of a cysteine and the N δs of two histidines. Azurin, that can be repeatedly oxidized and reduced lose its color in reduced form and regain it on reoxidation.

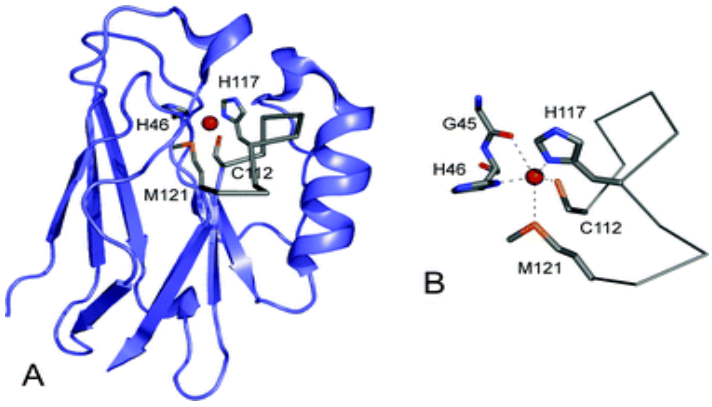


Figure 1.4: **A**-The structure of the azurin from *Pseudomonas aeruginosa* prepared using Pymol, **B**-Ligand containing loops (Dennison, 2008).

1.5 Azurin derived peptides

Azurin-derived peptides includes **p28,**Leu50-Asp77 (LSTAADMQGVVTDGMASGLDKDYLKPDD) **p18,** Leu50-Gly67 (LSTAADMQGVVTDGMASG **p18b,**Val60-Asp77 (VTDGMASGL DKDYLKPDD) and **p12** Gly66-Asp77 (SGLDKDY LKPDD) (B. N. Taylor et al., 2009).

Figure1.5: Azurin derived peptides (p28 and p18).

P28, an important peptide fragment from azurin also used as an anticancer drug. Experiments on p28 held by CDG Therapeutics, and found that it can used as a new anticancer drug (IND 77.754) approved by the FDA. In first-human, phase I clinical trial of p28 (NSC745104), different factors such as safety, tolerability, pharmacokinetics and preliminary activity of p28 in patients with $p53^{+}$ metastatic solid tumors were investigated. Nobody shows toxicity symptoms, hence p28 can be used as a good therapeutic drug (Warso *et al.,* 2013). After that certain studies have also shown that p28 is safe and well accepted in children with progressive CNS malignancies (Bernardes, Chakrabarty, *et al.,* 2013). *In vivo* studies of p28 on male

mice shows good result without any toxicity symptoms (Jia et al., 2011). P28 (50-77) of azurin is also known as amphipathic peptide because it contains both hydrophobic domain (50-66aa) and hydrophilic domain (67-77aa) (Bernardes, Chakrabarty, et al., 2013).

P18 causes the cell death of most K562 cells by disrupting plasma membrane with enhancing depolarizing plasma membrane potential and membrane permeability (Tang *et al.,* 2010). Azurin p18 and p28 have high affinity towards cancer cells compared to other anticancer cationic peptides (Kirkham & Parton, 2005).

1.6 Entry mechanism of azurin on human cells

Caveolae-mediated endocytosis pathway of plasma membrane helps the entrance of azurin to human cells (B. N. Taylor *et al.,* 2009). A short peptide derived from azurin called p28 (50-77 amino acids) or Protein Entry Domain (PED) is responsible for the entrance of the entire protein into cells. It can form an extended amphipathic α-helix with both hydrophobic amino acids (50-66) and hydrophilic amino acids (67-77) due to its negative charge. PED was further reduced to form p18 amino-acids (50-67), it helps the translocation of azurin to cancer cells (B. N. Taylor *et al.,* 2009; Yamada *et al.,* 2005). Azurin enters to cancer cell and forms a complex with p53 (21 kDa; 393 amino acids), with the aid of p28. This complex formation stabilize p53 and protect from proteosomal degradation. Stabilization of p53 with azurin leads to the increased intracellular level of p53. So cell death occurs due to induction of apoptosis through caspase activation (Shetty, Shao, & Weng, 2008). P53 is also known as the guardian of

the genome because of its innumerous role in cellular processes such as transcription, DNA repair, genomic stability, and cell cycle control. It is also able to induce cellular death by apoptosis, sometimes, it may be inactivated by oncogenes and mutations (Apiyo & Wittung-Stafshede, 2005; Martin *et al.,* 2002). Most important feature of azurin is that it shows a preferred attraction to the cancer cells than the normal ones. Hence, the application of this bacterial protein on cancer therapy will bring a new way to fight this disease (Yamada et al., 2005).

1.7 Objectives of current study

Azurin is a novel molecule for cancer treatment (Yamada, Goto, Punj, Zaborina, Kimbara, et al., 2002) and researchers termed azurin as a drug for the future. Its potentiality can be increased through genetic engineering and it is also used in combination with other cytotoxic agents in cancer research. The study is focused on screening of azurin producing bacterial strains from Malabar region of Kerala, India. The purity and yield of azurin enhanced using recombinant DNA technology. It also looks into the characterization and anticancer study of azurin (Sutherland, 1966).

So far in the Indian scenario, the reports for an anti-cancer activity of bacterial azurin are very limited. Presently azurin is available commercially with very high price which counteracts its direct application in research and development. This study provides an economical source for the production of purified azurin that can be used in cancer research. In the light of these facts present study was undertaken with the following research objectives:

Objectives

- Isolation and identification of azurin gene harboring *Pseudomonas aeruginosa* isolates from Malabar region, Kerala.
- Cloning, expression, purification and characterization of azurin.
- Study of growth suppression by azurin on various cancer cell lines.

Chapter 2

Isolation and identification of azurin gene harboring *Pseudomonas* isolates from Malabar region, Kerala.

2.1 Introduction

Proteins and other products from bacteria are becoming increasingly relevant in anticancer treatment due to their high specificity and minimal side effects. This study was conducted on bacteria having anticancer products isolated from the Malabar region of Kerala. Every year, ten million people are diagnosed with cancer, and more than half of these patients die from this disease (Bernardes, Seruca, Chakrabarty, & Fialho, 2010). In the 1890s, William Coly has detected the presence of a pathogen in cancer patients, and found that it causes the regression of tumor (A. M. Chakrabarty, 2003). Since then, a large number of bacterial species such as *Salmonella, Clostridia, Bifido bacterium* and others have been used for tumor regression studies with varying efficiency (Lee, Wu, Chen, & Shiau, 2009; Rhee et al., 2000; Theys & Lambin, 2015). Large effort has been spent over the years in developing attenuated bacterial strains for the treatment of cancer (Jain & Forbes, 2001; Kirn, 2000).

Azurin is a secondary metabolite from *Pseudomonas aeruginosa* that function as donors in terminal electron transfer. Microorganisms mainly use azurin as an electron transport protein during electron transport cycle. It transports an electron between cyt c-551 and cyt oxidase in their respiration process (Kakutani, Watanabe, Arima, & Beppu, 1981). Azurin has a unique property to penetrate the cell membranes of cancer cells without effecting other healthy cells. This novel protein is reported to have antiviral and antiparasitic activity along with anticancer activity (A. M. Chakrabarty, 2016).

Several environmental samples were collected from the Malabar region and screened for the presence of *Pseudomonas Species*. For that purpose, bacterial cultures were subjected to different tests for identification and classification. In the 20th century, a number of bacterial identification and classification methods were initiated. In 1980, Willcox *et al.,* introduced numerical taxonomy methods for the bacterial classification (Willcox, Lapage, & Holmes, 1980). Later on, various molecular biology techniques like DNA base composition, DNA-DNA hybridization (Wilson, 1995) and 16S rRNA sequencing was developed for identification process (Rossello-Mora & Amann, 2001). Recently, even though improved technologies such as fatty acid profiling, MALDI-TOF, metabolic finger profiling using BIOLOG, ribotyping etc.(Phumudzo, Ronald, Khayalethu, & Fhatuwani, 2013) have been introduced. 16S rRNA sequencing is still the most widely and routinely used for identification and classification of bacteria in most microbiological laboratories (Stackebrandt & Goebel, 1994).

In this study, we explore the antitumor activity of *Pseudomonas* strains from environmental samples. We intend to evaluate *in vitro* therapeutic efficacy of these strains in DLA cell lines by trypan blue exclusion assay and the highest activity showing strain was selected for further studies. Screening of the selected strain for the presence of azurin genes was done using PCR with specific primers. Partially purified protein was used for characterization studies such as FTIR and MALDI-TOF. Functional groups were studied by FTIR, and compared with standard azurin, also helps to confirm the presence of azurin.

2.2 Objectives of the study

- Isolation and identification of *Pseudomonas* isolates from Malabar region, Kerala.
- Cytotoxicity assay of isolated *Pseudomonas* strains by trypan blue exclusion assay.
- Azurin gene detection in the isolates using PCR with specific primers.
- Characterization of partially purified azurin protein by FTIR and other advanced methods.

2.3 Review of Literature

The drastic increase in the incidence of cancer demands the development of effective approaches for cancer treatment with high specificity and minimal side effects. Cancer treatment based on microbial products attracted more attention among the various methods. There are some bacteria which used in cancer treatment are listed as below: *Mycobacterium bovis BCG, Streptococcus pyogenes OK-432,Clostridium novyi* and *Salmonella enteric Serovar Typhimurium* (Lukasiewicz & Fol, 2018). In this study, we mainly concentrate on *Pseudomonas aeruginosa.* It was first isolated by Gessard from green pus (Gessard, 1984). It i*s* an aerobic Gram-negative bacterium and they are motile, non-spore forming rods that are oxidase positive and lactose non fermenters. It produces two water-soluble pigments, pyocyanin and pyoverdin, give its blue-green color

and the presence of polar flagella and pili gives motility (Haynes, 1951). It is an inhabitant of terrestrial, aquatic, animal surfaces and an important causative agent of nosocomial infections (Wiehlmann *et al.,* 2007). *Pseudomonas aeruginosa* also produces a variety of toxins, such as phytotoxic factor, pigments, hydrocyanic acid, proteolytic enzymes, phospholipase, enterotoxin, exotoxin, and slime. Exotoxins plays a major role in the pathogenicity of this organism, they can produce leukopenia, acidosis, circulatory collapse, necrosis of the liver, pulmonary edema, hemorrhage, and tubular necrosis of kidneys (Liu, 1974). Azurin act as a virulence factor of *Pseudomonas aeruginosa,* and gives the ability to escape the host defense system. Major amounts of this protein could be isolated from *Pseudomonas aeruginosa* grown under oxygen limitation denitrifying conditions (Parr, Barber, Greenwood, & Brunori, 1977; Wood, 1978).

Southerland and Wilkinson described the preparation, purification and some properties of a blue protein present in *Bordetella species*. They suggested the name of azurin to this protein (Sutherland & Wilkinson, 1963). It can be repeatedly oxidized and reduced, lose its color in reduced form and regain it on reoxidation. Purification studies of azurin was done by zone-electrophoresis on starch, and crystallized from ammonium sulfate solution. This crystal contains approximately one copper atom and have a redox-potential of +300mV. The unique nature of azurin such as intense blue color, a high reduction potential and a small parallel hyperfine coupling in the electron spin resonance spectrum is due to the presence of a single copper atom (Solomon, Baldwin, & Lowery, 1992).

Bonander *et al.,* (1997) reported that the large stability of azurin is due to the coordination of copper ion in five positions to the polypeptide chain. This protein consists of an α-helix and two β-sheets that form a β-barrel motif (Leckner, Bonander, Wittung-Stafshede, Malmstrom, & Karlsson, 1997). It displays an intense charge-transfer absorption band at 625 due to the bond formed between Cu (II) and Cys-112 (Gilardi, Mei, Rosato, Canters, & Finazzi-Agro, 1994). Azurin consists of a cupredoxin fold, which consists of a Greek-key folding motif, is formed by eight β-strands arranged in two β-sheets opposing each other in a β-sandwich (Adman, 1991). Three-dimensional studies of azurin structure was done primarily by Adman *et al.,* (Adman, Stenkamp, Sieker, & Jensen, 1978). Azurin consists of, carbonyl oxygen (O=C) at a distance of ~ 0.21 nm from the copper ion (Walter et al., 1996).

Azurin contains a single tryptophan residue at position 48 masked in the hydrophobic core of the protein (Kim et al., 1993). This buried tryptophan gives rise to a strong fluorescence signal at 308 nm in the folded (apo) state (Gilardi et al., 1994). The stability of azurin protein is due to the presence of a disulfide bond, metal and the tyrosine corner (Bonander, Leckner, Guo, Karlsson, & Sjolin, 2000). A variant of azurin has been engineered (called purple Cu_A Az) where the blue-copper site is replaced by the purple Cu_A center (Hay, Richards, & Lu, 1996).

The isolation of azurin from environmental samples are very few. In a previous study done by Sankar *et al.,* reported the isolation

of azurin from four different MTCC strains (*Pseudomonas aeruginosa* MTCC 1934, 741, 2453 and 1942) and concluded that *Pseudomonas aeruginosa* MTCC 2453 shown enhanced production of azurin than other strains. They also studied about the purification of azurin from these different strains using ammonium sulphate precipitation and dialysis. Biophysical analysis of these extracted azurin were done using FTIR (Sankar, Siddik, Abhijit, & Mahitosh, 2011). Osman *et al.,* (2013) reported the isolation of 95 *Pseudomonas aeruginosa* strains from clinical samples. They were identified the whole samples using manual biochemical tests and confirmed by the Microscan Walk away 90 systems. After primary screening of *Pseudomonas* isolates azurin gene was detected in 11 isolates using specific *azu* gene primers by PCR and amplifying 545bp corresponding gene product (Osman, Douaa, & Younis, 2013). In a recent study from Egypt, they extracted azurin from 5 *Pseudomonas aeruginosa* samples isolated from infected animals and gene detection was done by *azu* specific primers using PCR (Sohier M. Syame, 2018).

Azurin mainly produced from certain Gram negative bacteria *Pseudomonas, Bordetella* , *Alcaligenes, Nesseria and Methylomonas* (Joseph, Anthony, & Bateson, 1994). The three species (*Pseudomonas, Bordetella* and *Alcaligenes*) showed azurin production in a maximum level at medium containing copper above 5 µg/ml. *Pseudomonas aeruginosa* produced similar amount of azurin on both aerobical and anaerobical conditions (Sutherland, 1966). A recent study done by Mohamed *et al.,* reported about the metal variant of azurin, modified by replacement of copper at the active site by cobalt and nickel metals. Functional groups of these two types were analysed using FTIR (Ali,

Mohamed, & Sedek, 2017). In a purification study of azurin, characterization of purified sample was done using SDS PAGE, FTIR and MALDI-TOF (Sankar Ramachandran, Singh, & Mandal, 2012).

Mass spectrometric analysis of two types of azurin variants from *Alcaligenes xylosoxidans* GIFU1051, were analysed using MALDI-TOF (Fukuo *et al.,* 1998). The secondary structure of azurin from *Pseudomonas aeruginosa* MTCC 2453 was determined by Mandel in 2010 by circular dichorism (CD) and SDS –PAGE (Mandal, 2010). A lipid-modified variant of azurin named Laz, was isolated from human pathogens *Neisseria gonorrhoeae* and *Neisseria meningitidis*, are hypersensitive to hydrogen peroxide and copper (Wu *et al.,* 2005). Laz is very similar to azurin in certain properties such as copper ion redox activity, exhibition of intense blue colour on oxidation, absorption of visible light around 626 nm and the presence of copper chelating agent (Deeudom, Huston, & Moir, 2015). Two distinctive variants of azurin were extracted from the obligate *Methylotroph methylomonas J* (Inoue *et al.,* 1999). Dinarieva *et al.,* (2012) reported the characterization of azurin from *Methylobacillus flagellatus* KT. The molecular mass of purified azurin was determined by SDS–PAGE and MALDI-TOF (Dinarieva, Trashin, Kahnt, Karyakin, & Netrusov, 2012).

Azurin shows structural similarity with variable domains of an immunoglobulin. Antiparasitic activity of azurin is due to structural similarity with surface antigen (SAG1) of the parasite *Toxoplasma gondii*. Antimalarial activity is due to the structural similarity with surface protein 1 (MSP1) of *Plasmodium falciparum* merozoite. An anti-HIV activity of azurin is due to the structural similarity with

glycoprotein gp120, which binds the host receptor CD4, or the host proteins DC-SIGN and ICAM-3. These activities of azurin make it a promiscuous drug for future that can hit many target diseases in a better and more effective way with fewer side effects. Azurin elicit less resistance than drugs that target a single step of a disease (Chaudhari et al., 2006).

2.4 Materials and methods

2.4.1 Isolation of samples and identification

Environmental samples were collected from different locations of Malabar region. Soil and water samples were collected aseptically, serially diluted and 0.1ml of the appropriately diluted samples were spread on the surface of Kings B agar (E. O. King, Ward, & Raney, 1954). Greenish mucoid colonies were selected, and further identification of the isolates were carried out by their biochemical characteristics. The isolates morphologically examined for size, shape, color, pigment. All purified isolates were stained with Gram stain and examined microscopically.

Media composition: Kings B Agar

Proteose peptone	: 20.00 g
Dipotassium hydrogen phosphate	: 1.50 g
Magnesium sulphate.heptahydrate	: 1.50 g
Agar	: 20.00 g
Distilled water	: 1 L
Final pH (at 25°C)	: 7.2±0.2

The plates were incubated at 37 ºC for 24 h and observed for the presence of organisms. These colonies were picked and purified by repeated subculturing on nutrient agar and were stored as glycerol stocks at– 20 ^{o}C. Also, working cultures were maintained in nutrient agar slants by subculturing at monthly intervals.

2.4.2 Phenotypic characterization

2.4.2.1 Morphology

Morphology of the isolate was studied by Grams staining. A clean glass slide was taken and a thin smear of the culture was prepared under sterile conditions. The slide was allowed to air dry and then heat fixed. The slide was flooded with the primary stain - crystal violet and allowed to stand for 1 min. Excess dye was removed by rinsing with tap water. Gram's iodine mordant solution was added and the slide was left for 1 min and then rinsed with water. The decolorizing reagent - ethanol was added slowly to the slide until alcohol runs clear, showing a bluish tinge. The slide was washed with water and then stained with the counter stain safranin for 45 s. Finally, the slide was washed with water and allowed to dry. It was then observed under oil immersion under bright field microscope. Gram-positive bacteria retained the crystal violet stain and appeared blue or purple whereas Gram-negative bacteria took up the counterstain and seemed to be red or pink in color (Gerhardt, 1981).

2.4.2.2 Hanging drop

Motility test was done using the hanging drop method. Vaseline was applied to the corners of a clean coverslip using a toothpick. A loopful of 24-h old broth culture was placed at the centre of the cover slip. A depression cavity slide was put onto the cover slip with the concavity facing downwards, such that the drop protrudes into the centre of the concavity of the slide. The slide was gently pressed against the cover slip to seal and immediately inverted. A drop of immersion oil was put on the cover slip just above the hanging drop and the edge of the hanging drop was observed under the oil-immersion objective of the microscope (Collins, Lyne, & Grange, 1995).

2.4.3 Biochemical tests

2.4.3.1 Indole test

Indole test is used to determine the degradation of the amino acid tryptophan to its metabolic products namely indole, pyruvic acid and ammonia. Test organism was inoculated into tryptone broth and incubated overnight at 37 ^{o}C. When Kovac's Reagent was added a cherry red coloration in the top layer indicated a positive result (Isenberg & Sundheim, 1958).

2.4.3.2 Methyl red-Voges-Proskauer test (MR-VP)

Test organisms was inoculated in to MR-VP broth and incubated overnight at 37 ^{o}C. After incubation, the p^{H} indicator, methyl

red was added to the broth. The development of red coloration indicated a positive result (Cowan, 1953). For Vogues-Proskauer test, Barritt's reagent A (0.6 ml) and B (0.2 ml) was added and shaken for 30 s; the positive result was indicated by the development of pink color in 2-5 min, which turns to crimson color in 30 min. For maximum aeration tube was shaken in intervals (Mac Faddin, 1976).

2.4.3.3 Starch hydrolysis (Priest, 1977)

Spot inoculation of the test organisms was done on starch agar plates, incubated at 37 ^{o}C for 24 h. After the growth appeared, the plates were covered with Grams iodine and examined for the clear zone around the growth.

Media composition: Starch agar

Beef extract	: 3.0 g
Soluble starch	: 10.0 g
Agar	: 12.0 g
Distilled water	: 1 L

2.4.3.4 Gelatin hydrolysis (Clarke, 1953)

Nutrient gelatin tube was inoculated with test organisms, incubated at room temperature.

Media composition: **Gelatin Medium**

Gelatin	: 30.0 g
Casein enzymichydrolysate	: 10.0 g

Sodium chloride	: 10.0 g
Agar	: 15.0 g
Distilled water	: 1 L
Final pH at 25°C	: 7.2±0.2

2.4.3.5 Oxidase test (Farmer et al., 1981)

Using a sterile needle, the test organisms were transferred on to the surface of oxidase (Himedia, Bangalore) disc and the development of purple color was observed which indicated a positive result.

2.4.3.6 Catalase test (W. I. Taylor & Achanzar, 1972)

Using a sterile loop, a 24-h old culture of test organisms were transferred on to the surface of a slide, added two drops of 3 % hydrogen peroxide and observed for foaming. The effervescence indicated a positive test.

2.4.3.7 Citrate utilization test (Koser, 1923)

Simmons citrate agar slants were inoculated with the test organisms and incubated at 37 ^{o}C for 24 h. The presence of blue colouration on slants indicated a positive result.

Media composition: **Simmons citrate agar**

Ammonium Dihydrogen Phosphate	: 1.0 g
Dipotassium Phosphate	: 1.0 g
Sodium Chloride	: 5.0 g

Sodium Citrate	: 2.0 g
Magnesium Sulfate	: 0.2 g
Bromothymol	: 0.08 g
Agar	: 15.0 g
Final pH	: 6.9 ± 0.2 at 25 °C
Distilled water	: 1 L

2.4.3.8 Nitrate reduction test (Cheesbrough, 2005)

Nitrate broth was inoculated with test organisms and after incubation, reagent A and B were added. The development of cherry red color is the positive result.

Media composition: **Nitrate broth**

Peptone	: 5.0 g
Meat extract	: 3.0 g
Potassium nitrate	: 1.0 g
Final pH	: 7.0 ± 0.2 at 25 °C

Reagent A : 8 g of sulfanilic acid in 1 liter 5N acetic acid

Reagent B: 6 g of N,N-Dimethyl-1-naphthylamine in 1L5N Acetic acid

2.4.3.9 Carbohydrate fermentation test

This test is used to define the capability of bacteria to ferment different sugars. Media containing different carbohydrates namely glucose, sucrose, lactose and mannitol were prepared and inoculated

with the test organisms. Carbohydrate fermentation broth with different sugar substrates were inoculated with the test organisms, incubated overnight at 37 ^{o}C, observed for the presence of acid and or gas.

2.4.4 Extraction of Cellular Protein

Isolated *Pseudomonas* strains were inoculated separately in medium containing peptone (10 g/l), K_2HPO_4 (0.75 g/l), $MgSO_4$ (0.75 g/l) and $CUSO_4$ (0.005 g/l) and incubated at 37 ^{o}C overnight. After incubation, culture medium was centrifuged at 12000 rpm for 15 min using cold centrifuge (REMI C- 24 PLUS). Cell pellets were collected and suspended in 0.02 M Potassium phosphate buffer at pH 7 and kept in the basket containing ice cubes for sonication. Cells were sonicated for 45 s at 100 W using Ultrasonicator (Sonics Vibra cell). After sonication, the suspension was centrifuged at 10000 rpm for 15 min and the supernatant was stored at 4 °C (Parr, Barber, & Greenwood, 1976; Sutherland, 1966).

2.4.4.1 Ammonium sulfate precipitation

The supernatant was saturated to 70 % ammonium sulfate salt at 4 °C for overnight (Parr et al., 1976). After precipitation, the solution was centrifuged at 20000 rpm for 25 min, and the supernatant was discarded. The precipitate was collected and resuspended in 0.02 M Potassium phosphate buffer at pH 7.

2.4.4.2 Dialysis

The precipitate suspended in 0.02M potassium buffer at pH 7, was dialyzed by standard dialysis bag (Himedia LA 395) having 12

KDa MW cut off at 4 °C for 24 h. The solution was kept at 4 °C after dialysis for further purification (Sutherland, 1966).

2.4.4.3 DEAE cellulose treatment

Dialysate was initially treated with DEAE cellulose. 100 ml slurry of DEAE cellulose equilibrated in 0.02 M potassium phosphate buffer at pH 7 were treated with the dialysate and stirred for 25 min at 4 ^{o}C . After that centrifuged at 10,000 rpm for 15 min. The supernatant was collected because azurin does not adsorbed in the DEAE cellulose (Sankar & Mahitosh, 2011; Sankar, Moganavelli, & Mahitosh, 2012; Sutherland, 1966).

2.4.5 Lowry's method (Lowry, Rosebrough, Farr, & Randall, 1951)

BSA solution (1 mg/ ml) was mixed with water and the final volume was made up to 5 ml. From the different dilutions of BSA solution, protein solution was pipetted out to different test tubes and 2 ml of alkaline copper sulfate reagent was added. After 10 min incubation at room temperature, 200 µl Folin Ciocalteau solution was added. After 30 min incubation OD (measure the absorbance) was measured at 660 nm and a standard curve was plotted.

2.4.6 Trypan blue exclusion assay

Total protein from all *Pseudomonas* samples was extracted and partially purified using 70 % ammonium sulfate precipitation followed by dialysis (Sereena & Sebastian, 2016). *In vitro* cytotoxicity of the dialysate was studied using trypan blue exclusion assay on Dalton's

lymphoma ascites cells (Elia *et al.,* 1993; Sereena .M.C, 2018). The tumor cells were extracted from the peritoneal cavity of tumor-bearing mice and washed repeatedly with PBS. Viable cells (10^6 cells/100 µl) were added to tubes containing different concentrations (200 µg/ml, 100 µg/ml, 50 µg/ml, 20 µg/ml and 10 µg/ml) of dialysate. After incubation for 3 h at 37 ºC , the cell suspension was mixed with 100 µl of trypan blue (1%) and loaded on a hemocytometer after 2 min. Then separately counted the number of dead (blue colour) and live cells (Elia et al., 1993). The isolate showing the highest cytotoxic activity was selected and stored as glycerol stock for further studies.

$$Cytotoxicity\,(\%) = \frac{No.of\ dead\ cells}{No.of\ living\ cells + No.of\ dead\ cells} x100$$

2.4.7 DNA extraction

DNA extraction was done using modified Unal *et al.,* method (Unal *et al.,* 1992). A single pure colony was inoculated in 4 ml of LB broth and incubated at 37 ºC for 24 h. Cells were acquired by centrifuging 5 ml of overnight broth culture at 5000 rpm for 5 min. Cells were re-suspended in 5 µl of lysozyme (50 mg/ ml distilled water) and incubated at 37 ºC . After 10 min, 10 µl of Proteinase K (20 mg/ml) solution and 500 µl of 0.1 M Tris HCl (pH 7.5) buffer were added. Cell suspension were then incubated at 37 ^{o}C for an additional 20 min and placed in a boiling water bath for 5 min. After that, centrifuged at 4000 rpm for 5 min. Supernatant was taken into a new microcentrifuge tube and mixed with an equal volume of isopropanol. For pelleting DNA, this mixture was centrifuged at 12000 rpm for 10

min. The pellet was then desalted with 200 µl of 70 % cold ethanol and tubes were inverted to dry. The pellet was then re-suspended in 100 µl TE buffer [10 mM Tris HCl (pH 7.5) and 1 mM EDTA (pH 8.0)] and stored at -20 ^{o}C until further analysis.

2.4.8 Analysis of DNA Purity and Quality (Sambrook, Fritsch, & Maniatis, 1989)

The DNA stock samples were quantified using UV spectrophotometer at 260 nm and 280 nm using the convention that one absorbance unit at 260 nm wavelength equals 50 µg DNA per ml. The Ultraviolet (UV) absorbance was checked at 260 and 280 nm for determination of DNA concentration and purity. The purity of DNA has judged by OD ratio at 260:280 nm. The DNA having ratio between 1.8 to 2.0 was considered to be of good purity. The concentration of DNA was estimated using the formula:

$$\text{Conc. of DNA } (\text{mg/ml}) = OD_{260} \text{ x 50 x Dilution factor}$$

Qualities of DNA were again checked by agarose gel electrophoresis.

2.4.9 Composition of PCR Mix

a) 10x PCR buffer $MgCl_2$

b) 25 mM $MgCl_2$

c) 10 mM dNTP mix (2.5 mM each dNTP)

d) Taq Polymerase enzyme (1 U/µl)

e) Primers: The oligonucleotide primers used in this study were selected based on earlier studies. The primers were checked for their specificity in a BLAST search, available through the National Centre for Biotechnology Information Website (www.ncbi.nlm.nih.gov). The primer sequences were given in the Table 2:1(Yamada et al., 2004). PCR amplification profile was given in Table 2:2

f) 100 bp DNA ladder

Table 2. 1: *Azu* gene primer sequences used in this study

Gene	Sequence (5' - 3')	Amplicon size (bp)
Azu	F-5'GCCCAAGCTTACCTAGGAGGCTGCTCCATGCTA 3' R-5'TGAGCCCCTGTAGGCGCCCATGAAAAAGCCCGGC 3'	545

Table 2.2: PCR amplification profile

Initial Denaturation	**35 cycles**			**Final extension**
	Denaturation	**Annealing**	**Extension**	
94 °C for 5 min	94 °C for 30 s	60 °C for 45 s	72 °C for 3 min	72 °Cfor7 min

Amplification was carried out using different annealing temperatures (50 °C to 62 °C) and time (30 s to 60 s) using thermal cycler (Biorad T100^{TM}).

2.4.10 Agarose gel electrophoresis (Sambrook, 2001)

Reagents used for AGE

a) 10x Tris Borate EDTA (TBE) buffer (pH 8.0)

Tris Base	: 108 g
Boric acid	: 55 g
EDTA	: 9.3 g
Distilled water	: 1 liter

b) 2% agarose gel

2 g agarose in 100ml of 1x TBE buffer

c) Loading of Dye

30 % glycerol in distilled water.

0.25 % bromophenol blue.

0.25 % Xylene cyanol.

d) Ethidium bromide

10 mg of ethidium bromide in 10 ml of distilled water.

Procedure:

Agarose in 1xTBE buffer was melted to form agarose solution and while the solution was cooling, ethidium bromide was added such that the final concentrate would be 0.5 µg/ml of the solution. Agarose

gel of appropriate size was formed by pouring the solution into a gel casting tray and cooling it. Amplified products of both sets of reaction were mixed together and 30 µl of the amplified products were mixed with 6µl of loading dye and added to the wells. Amplicons were electrophoresed horizontally in 1xTBE buffer (prepared freshly from 10x TBE) at 100 volts for 90 min. The gel was then visualized under UV in a gel documentation system and DNA bands were analyzed visually by comparing with the DNA ladder standard which was run in the last well of the gel.

2.4.11 Protein Profile by SDS PAGE

SDS PAGE was performed using 15 % resolving gel containing 1.4 ml distilled water, 30 % Acryl amide, 1.5 M Tris (pH 8.8), 10 % SDS, 10 % APS and 2.0 µl TEMED was casted in the glass slab and kept it for 20 min for polymerization. After polymerization of the resolving gel, stacking gel (4 %) were loaded over the resolving gel which contains 0.68 ml distilled water, 30 % Acryl amide, 1M Tris (pH 6.8), 10 % APS, and 0.001 ml TEMED. After casting the gel, partially purified eight protein samples were loaded with standard protein molecular weight marker (SRL Biolit^{TM} Low Range 3-40 kDa). Glass slab gel was kept in the electrophoresis tank with tank buffer. This set up was connected with power pack initially in 80 V till the dye front crosses the stacking gel and then at 100 V. After that, the gel was removed and stained with 0.2 % Coomassie brilliant blue G-250 for overnight. Then the gel was destained with destaining solution

(45:45:10-methanol: water: acetic acid). The bands were observed using a transilluminator (Laemmli, 1970).

2.4.12 Molecular characterization and phylogenetic analysis

Genomic DNA was isolated using NucleoSpin® Tissue Kit (Macherey-Nagel) following manufacturer's instructions. The 16 S rRNA gene was amplified from genomic DNA by polymerase chain reaction with universal primers shown in Table 2.3 and PCR amplification profile was given in Table 2.4.

Table 2.3: Primer sequences used for 16S rRNA amplification

Gene	Sequence (5' - 3')	Amplicon size (bp)
16S-RS	F-5' CAGGCCTAACACATGCAAGTC 3' R-5' GGGCGGWGTGTACAAGGC 3'	1291

Table 2.4: PCR amplification 16S rRNA amplification profile

Initial Denaturation	35 cycles			Final extension
	Denaturation	Annealing	Extension	
94 °C for 5 min	94 °C for 30 s	60 °C for 40 s	72 °C for 60 s	72 °C for7 min

Amplified product was analyzed using agarose gel electrophoresis to confirm the targeted gene amplification (Mohamed, Fattah, & Mostafa, 2010).

The amplified product was excised from the gel and purified using ExoSAP-IT treatment. ExoSAP-IT (USB) consists of two

hydrolytic enzymes, Exonuclease I and Shrimp Alkaline Phosphatase (SAP), in a specially formulated buffer for the removal of unwanted primers and dNTPs from a PCR product mixture with no interference in downstream applications. Sequencing reaction was done in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems) using the Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) following manufactures protocol. The cleaned up air-dried product was sequenced in ABI 3730 DNA Analyzer (Applied Biosystems). The sequence quality was checked using Sequence Scanner Software V1 (Applied Biosystems). The 16s rRNA sequence was used to carry out BLAST with the nr database of NCBI Genbank. Based on the maximum identity score, best twenty sequences were selected and a phenogram was constructed with the selected strain. CLUSTAL W (Thompson, Higgins, & Gibson, 1994) was used for multiple sequence alignment and evolutionary history resolved by the neighbor-joining method (Saitou & Nei, 1987). The evolutionary distances and phylogenetic analysis were calculated using Kimura 2-parameter method (Kimura, 1980) and MEGA 5 (Tamura et al., 2011) respectively. Bootstrap resampling analysis for 1000 replicates was performed to evaluate the confidence of tree topologies (Felsenstein, 1985) and the tree generated was envisioned using FigTree v1.4.

2.4.13 PCR detection of azurin gene

The anticancer activity of this particular isolate may be due to the presence of azurin. This was confirmed by PCR using the azurin gene (*azu*) primers and comparison with standard strain. Standard strain *Pseudomonas aeruginosa* MTCC 2453 was obtained from the Microbial type culture collection center, Chandigarh, India (Sankar &

Mahitosh, 2011). Total genomic DNA was isolated according to modified Unal *et al.,* method. Azurin gene was amplified from the genome of selected bacterial strain and standard strain. The PCR was performed according to a previously described method.

2.4.14 Characterization of Partially purified azurin

2.4.14.1 FTIR

Infrared (IR) spectroscopy is one of the oldest and well established experimental techniques for the analysis of secondary structure of polypeptides and proteins (Elliott & Ambrose, 1950; Smith, 2011). Infrared spectroscopy experiments were performed using a Jasco 4000 spectrometer for *Pseudomonas aeruginosa* SSj and *Pseudomonas aeruginosa* MTCC 2453. Standard azurin analysed using PerkinElmer Spectrum Two FTIR spectrometer. The curves were deconvoluted and imported into software spectral manager and a Gaussian curve fitting was performed (Surewicz, Szabo, & Mantsch, 1987).

2.4.14.2 MALDI-TOF

MALDI-TOF is an effective method to analyze the molecular mass of proteins, peptides, sugars and large organic molecules (Fukuo *et al.,* 1998). The purified protein extract was analyzed using UltrafleXtreme MALDI–TOF from Bruker Daltonics at RGCB, Trivandrum, for molecular weight determination.

2.4.15 Hoechst staining

The breast cancer cells MCF-7 was grown on glass bottom plates for 24 h in DMEM medium containing 10 % Fetal Bovine

Serum. Then the cells were treated with azurin (1 µg/ml) for 24 h. The cells were stained with nuclear dye Hoechst at 1µg/ml concentration. The stained samples were observed under fluorescent microscope TiE (Nikon) using UV filter sets. The images were captured with Retig Exi camera using NIS element software (Nikon). The cells with intense, condensed nuclei were scored as apoptotic compared to control (Latt & Stetten, 1976; Latt, Stetten, Juergens, Willard, & Scher, 1975)

2.5 Results

2.5.1 Isolation and identification of *Pseudomonas aeruginosa* from various sources.

Several environmental samples were collected from the Malabar region of Kerala. Total of 43 isolates, in which 10 *Pseudomonas aerugi*nosa strains were identified by morphological and biochemical characteristics. Greenish mucoid colonies of *Pseudomonas* isolates were found on Kings B agar (Figure 2.1).

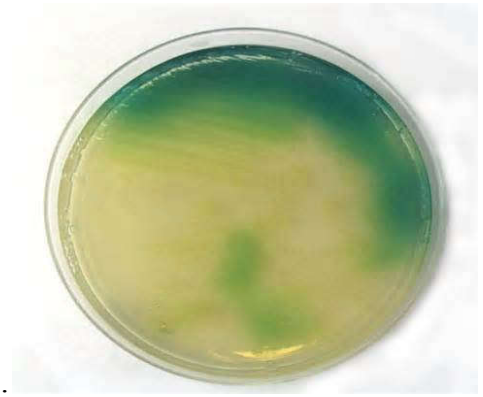


Figure 2.1: *Pseudomonas* isolates were found on Kings B agar.

All isolates *Pseudomonas* species were Gram-negative, motile rods producing oxidase and gelatinase enzymes and fermented most of the sugars used. It was urease negative.

2.5.2 Lowry assay for protein concentration

Pseudomonas isolates were assayed for determining protein concentration by Lowry's method (Table 2.5). In which isolate A showed the highest concentration of protein content than other strains.

Table 2.5: Protein concentration determined by Lowrys method.

No of samples	OD at 750nm	Concentration (g/ml)
1	1.377	0.0061
2	1.195	0.0048
3	1.164	0.0047
4	0.985	0.0038
5	1.126	0.0056
6	1.055	0.0038
7	0.904	0.0032
8	1.308	0.006
9	0.987	0.0038
10	1.022	0.004

2.5.3 Trypan blue exclusion assay (*In vitro* cytotoxicity assay)

Cytotoxicity activity of ten partially purified bacterial protein extracts were carried out against DLA cell line at different concentrations by trypan blue exclusion assay. Results of different concentrations of protein suspensions are tabulated in Table 2.6 and graphically represented in Figure 2.2. Out of ten protein suspensions

isolate A showed highest cytotoxicity effect on DLA cell lines. Isolates B and C have scarce activity when compared with other eight strains.

Table 2.6: Trypan blue exclusion assay of partially purified azurin.

	% of Cell death									
Con. of Extract	**A**	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**I**	**J**
200 µl	85	9	10	43	37	25	33	45	71	29
100 µl	60	4	6	20	18	12	16	22	35	14
50 µl	50	0	0	12	7	8	10	11	20	7
20 µl	40	0	0	7	4	2	5	5	10	2
10 µl	28	0	0	2	0	0	0	0	8	0

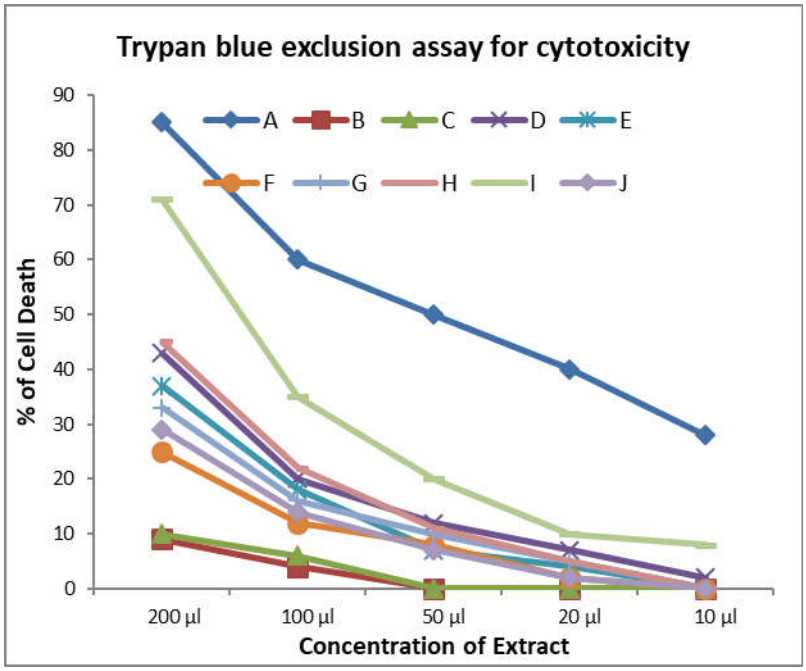


Figure 2.2: Trypan blue exclusion assay.

2.5.4 PCR detection of azurin gene

The whole genome was extracted from 10 *Pseudomonas aeruginosa* isolates according to modified Unal *et al.,* method**.** Total DNA obtained from all isolates analysed on 1 % agarose gel (Figure 2.3).

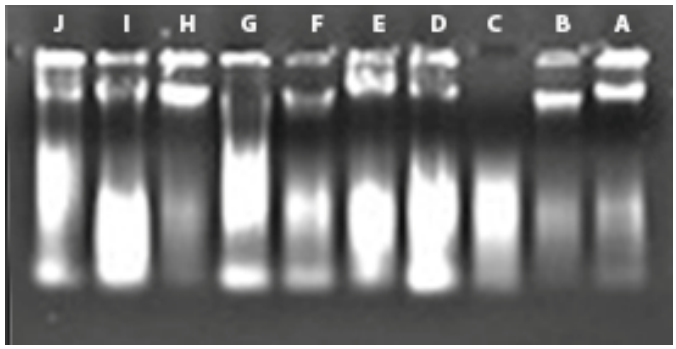


Figure 2.3: Total DNA extraction from *Pseudomonas* isolates loaded on 1 % agarose gel (run at 100 v for 90 min)

2.5.5 Results of PCR

PCR was used to amplify the azurin gene to detect azurin producing *Pseudomonas aeruginosa* samples. Alternative conditions for PCR amplification were employed using gradient PCR which includes various annealing temperatures and time. 60 °C at 45 s was found to be more specific for annealing. Standard amplification with the primer pair resulted in a 545 bp band as seen in ethidium bromide stained agarose gel electrophoresis (Figure 2.4). The azurin gene presence detected in 8 isolates out of 10 samples taken for this study.

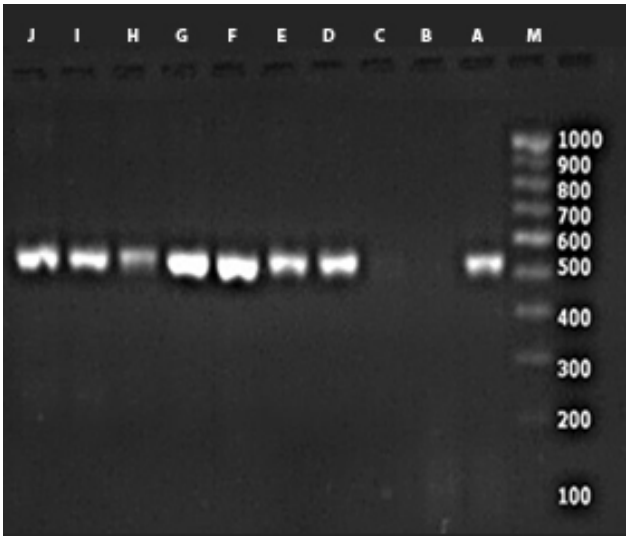


Figure 2.4:Agarose gel electrophoresis of PCR product (545 bp) loaded on 2 % agarose gel and run at 100 V for 120 min. (M=100 bp DNA ladder and A-J were 10 *Pseudomonas* isolates).

2.5.6 Protein extraction and SDS PAGE

Total partially purified protein extracted from 8 azurin producing isolates fractionated by SDS PAGE produced patterns with a wide range of discrete bands with molecular masses ranging from 6-100 KDa (Figure 2.5). All the lines appeared to be similar except in a few bands. A thick band approximately corresponding to 14 KDa Molecular weight was present in all eight azurin gene harboring isolates.

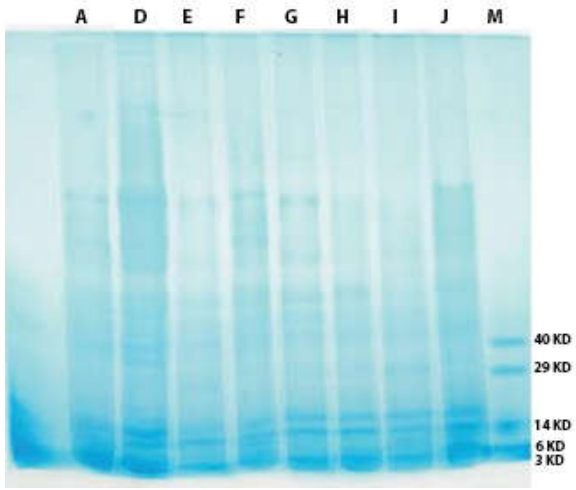


Figure 2.5: Protein profile of *Pseudomonas* isolates on SDS PAGE. Lane M contains Low Range molecular weight marker. Lane A-J were crude protein extracts from 8 *Pseudomonas* isolates.

2.5.7 Molecular characterization and phylogenetic analysis

The strain with the highest cytotoxic activity and protein content was chosen for further studies. The colonies of selected *Pseudomonas* strain on nutrient agar plates were irregular, flat, mucoid and opaque. Molecular and phylogenetic analysis were carried out to confirm the identity of *Pseudomonas aeruginosa* SSj. The 16S rRNA gene was amplified (Figure 2.6) and sequenced shown in. The consensus sequence of 1291 bp generated from forward and reverse sequence data was used for BLAST analysis and deposited in Genbank database with accession number KU821118. On 16S rRNA sequencing, the isolate was found to be 100 % similarity with *Pseudomonas aeruginosa* PAIRG. A phenogram reflecting the relationship among the strain and sequences of related strains obtained from the NCBI database are presented in Figure 2.7. The phylogenetic analysis showed close similarity with *Pseudomonas aeruginosa strain*

LOCK 0998 and *Pseudomonas aeruginosa strain PD29*. From results of the biochemical and molecular analysis, the isolate was affirmed to belong to *Pseudomonas aeruginosa*.

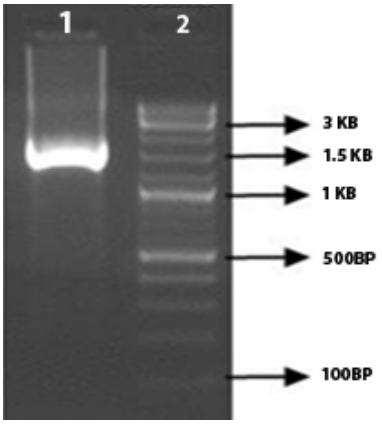


Figure 2.6: 16S rRNA gene sequence of *Pseudomonas aeruginosa* SSj: Lane1:16S r DNA amplicon, Lane2: DNA ladder

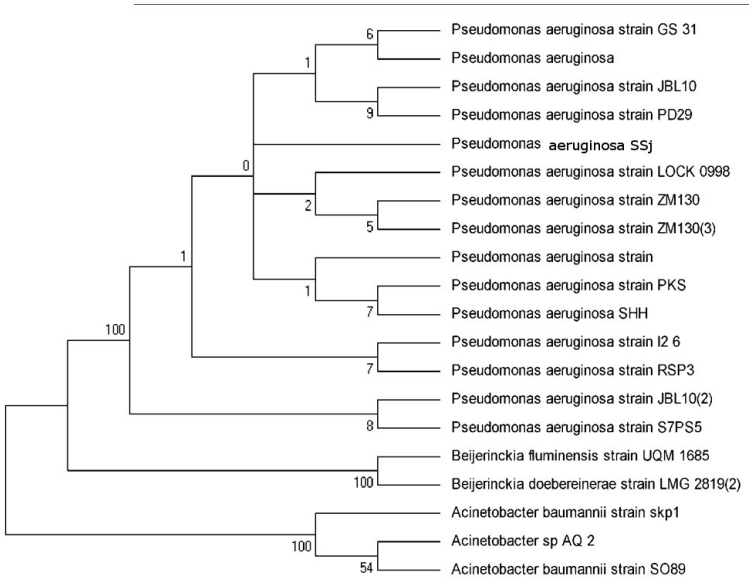


Figure 2.7: Phylogenetic tree based on 16S rRNA gene sequence, showing the relationship between the isolate SSj and closely related strains.

2.5.8 PCR detection of azurin gene

Standard amplification with azurin specific primer pair resulted in a 545 bp band as seen in Ethidium Bromide stained agarose gel electrophoresis. Both *Pseudomonas aeruginosa* SSj and *Pseudomonas aeruginosa* MTCC 2453 showed specific band concerning 545 bp (Figure 2.8). The presence of azurin gene was detected in selected isolate and *Pseudomonas aeruginosa* MTCC 2453 was taken as positive control for this study.

On azurin gene sequencing, sequence of the selected strain showed 100 % similarity with *Pseudomonas aeruginosa* NCGM 257. The sequence of azurin gene from *Pseudomonas aeruginosa* SSj also deposited on Genbank with accession number KU821119.

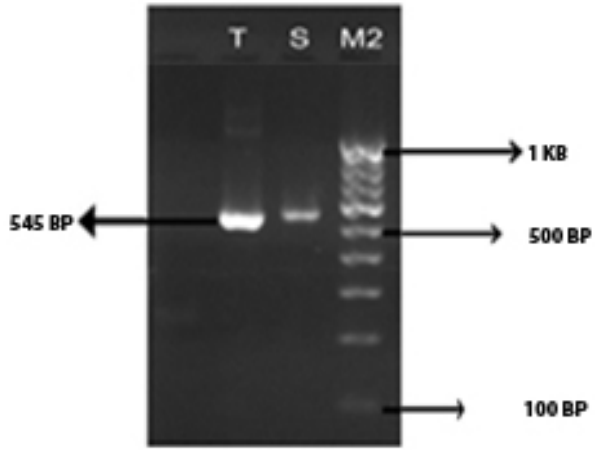


Figure 2.8: PCR amplification product run on agarose gel Lane T: *azu* gene amplicon from *Pseudomonas aeruginosa* SSj, Lane S: positive control, Lane M2: DNA ladder

2.5.9 FTIR

FTIR spectroscopy is one of the finest analysis method to provide the information about the secondary structure of proteins. The principle behind the FTIR spectroscopy is detecting the particular wavelengths of radiation in the infrared region of the spectrum are absorbed by the sample**.** The presence of the amide I band was indicated by the peak around 1650 cm^{-1} region. *Pseudomonas aeruginosa* SSj showed a peak around 1638 cm^{-1} region (Figure 2.9) and *Pseudomonas aeruginosa* 2453 showed a peak around 1637cm^{-1} region (Figure 2.10). Final confirmation was done in comparison with standard azurin which also showed a peak around 1641 cm^{-1} region (Figure 2. 11). All these peaks (Figure 2.12) signifies α-helix secondary structure of azurin. The significant differences in the value of peaks in the FTIR spectrum indicate differences in the amide I band and secondary structure of azurin. This may be the first study about the functional group characteristics of azurin using standard azurin.

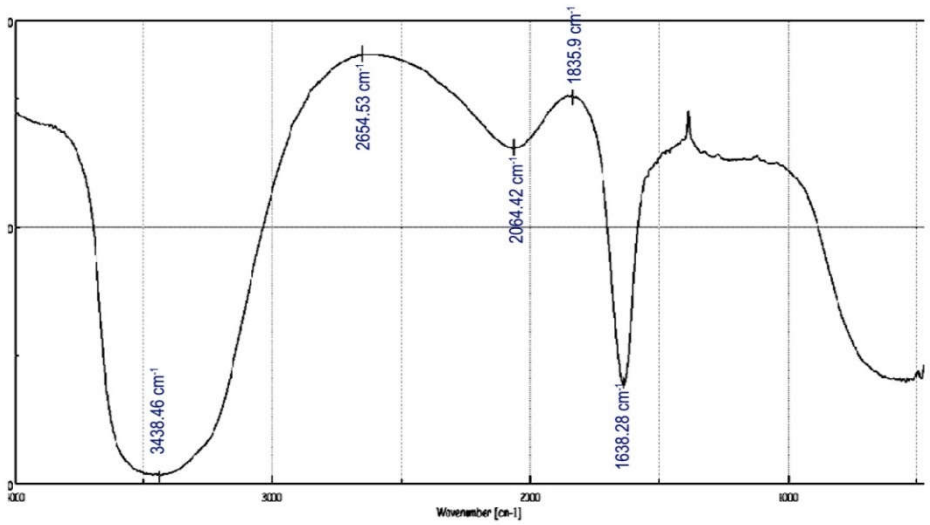


Figure 2.9: FTIR analysis of protein sample from *Pseudomonas aeruginosa* SSj showed a peak around 1638cm^{-1} region.

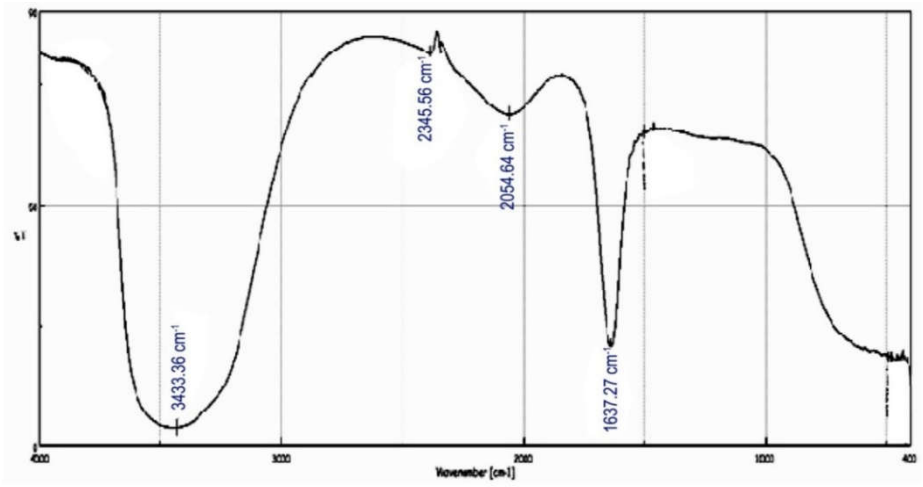


Figure 2.10: FTIR analysis of protein sample from *Pseudomonas aeruginosa* MTCC 2453 showed a peak around 1637cm^{-1}region .

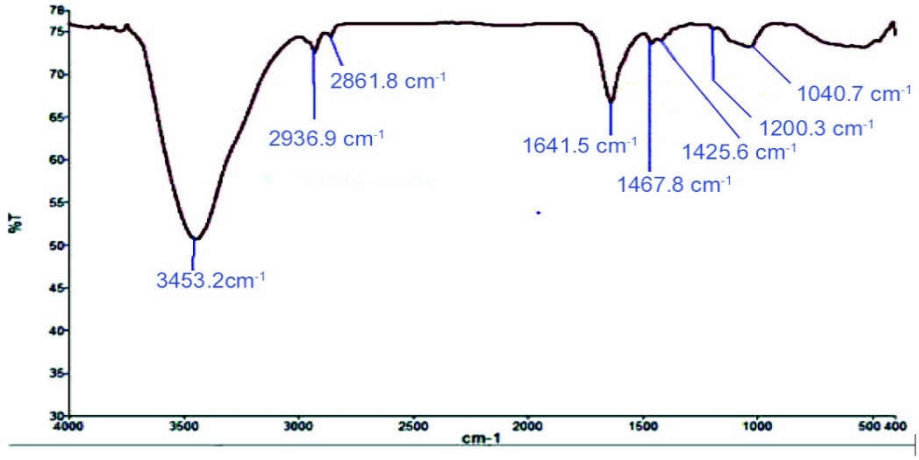


Figure 2.11:FTIR analysis of Standard azurin (Sigma Aldrich) showed a peak around 1641cm^{-1}region.

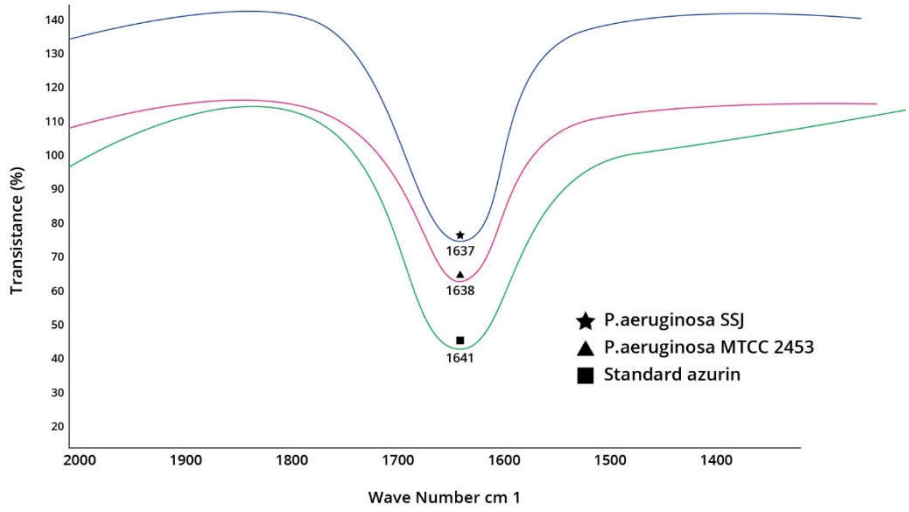


Figure 2:12: FTIR analysis results■ Standard azurin (1641 cm^{-1}), ★ protein sample from *Pseudomonas aeruginosa* SSj (1637 cm^{-1}) and ▲ protein sample from *Pseudomonas aeruginosa* MTCC 2453 (1638cm^{-1}).

2.5.10 MALDI -TOF

MALDI- TOF analysis of partially purified protein sample from *Pseudomonas aeruginosa* SSj, showed that it contains a mixture of proteins with a different range of molecular weight. The presense of a peak 14 KDa was confirmed in the spectrum, which denotes,the sample contains azurin with other unwanted proteins (Figure 2.13).

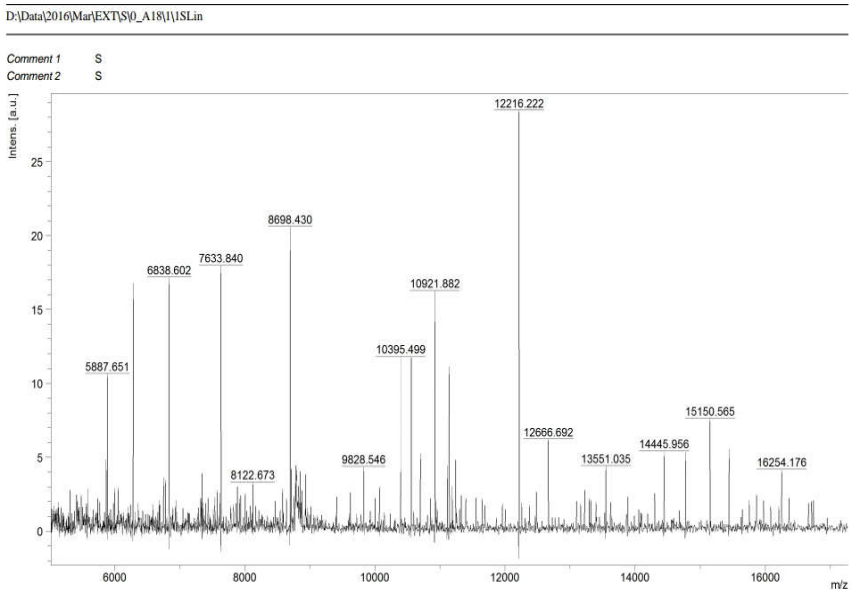
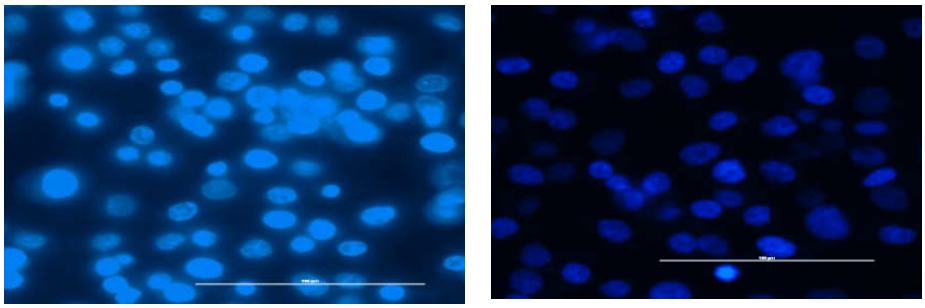


Figure 2.13:Partially purified azurin sample was analyzed on MALDI- TOF. The presense of a peak 14 KDa was confirmed in the spectrum.

2.5.11 Hoechst staining

Morphological characteristics of MCF breast cancer cells after partially purified azurin treatment was observed by Hoechst staining method (Figure 2.14). It was revealed that the treatment with azurin resulted in the condensation of the nucleus in cancer cell lines, and induced the nuclear condensation and cell fragmentation into apoptotic

bodies. Thus the mode of cell death triggered by microbial extract (azurin) might be the process of apoptosis, which is recognized as a novel strategy for the identification of anticancer drugs**.** The number of dead cells increased in azurin treated cancer cell line. Apoptosis inducing capability of azurin was more precisely studied in further works after purification.



Azurin (1 µg/ml) Control

Figure 2.14: Hoechst staining of MCF cell line with azurin.

2.6 Discussions

The effective discovery of new drugs with minimal side effect and high potentiality was directed through isolation of new bioactive compounds from microbes and testing its efficacy by both *in vitro* and *in vivo* studies. Azurin's cytotoxicity is reported only for specific cancer cells and not against healthy cells. It has fewer toxic side effects. The second most vital feature of azurin is that, tremendous number of therapeutic substance can link to azurin molecule, because of its ability to acts as a cargo protein (Mahfouz *et al.,* 2007).

The primary objective of this study was isolation, screening and identification of azurin gene harboring *Pseudomonas isolates* from

Malabar region. In the Indian scenario, research studies exploring the properties of native azurin producers were very rare in respect of its spectacular biomedical applications. Primarily, isolation of native *Pseudomonas* sp were done from environmental samples. Soil and water samples were collected aseptically from Malabar regions. More over 43 samples were analyzed and 10 *Pseudomonas* isolates were screened based on morphological and biochemical characteristics. Kings B agar is used for selecting *Pseudomonas* colonies. In another study by Ramachandran *et al.,* demonstrated the characteristics of azurin from four different MTCC strains of *Pseudomonas* (Sankar *et al.,* 2011). Osman *et al.,* (2013) reported the isolation of *Pseudomonas aeruginosa* from 95 clinical samples, azurin detection was done using PCR with specific *azu* gene primer (Osman *et al.,* 2013).

After protein extraction from these 10 isolates, partial purification with ammonium sulfate precipitation and dialysis was done and protein concentration was measured using Lowry's assay. Partially purified protein extract of these ten *Pseudomonas* samples were taken for anticancer studies. Anticancer activity is primarily measured by trypan blue exclusion assay using DLA (Daltons Lymphoma Ascites cells). Out of ten protein suspensions, isolate A showed highest cytotoxicity effect (85 %) on DLA cell lines. This particular isolate A was selected for further study because of increased cytotoxicity and protein concentration than other strain. Isolates B and C have limited activity when compared with other eight strains.

In this work screening of azurin containing *Pseudomonas sp* were done by molecular methods. Some researchers reported that different strains of *Pseudomonas aeruginosa* have the varying ability for azurin production due to their physiological variation (Sankar et al., 2011). In this study gradient, PCR was used which help to provide different temperatures and time combinations for annealing simultaneously. We tried 50 °C to 62 °C with different time (30 s to 60s) combinations and 60 °C for 45 s was found to be more specific for annealing, eight strains formed a single band particular to 545 bp on agarose gel electrophoresis. In both PCR and trypan blue exclusion assay samples, B and C are azurin negative strains. Partially purified protein samples were characterized by SDS page analysis. A thick band was presented according to 14 KDa among other several bands (Sankar *et al.,* 2012).

The bacterial isolate (A) showing highest anticancer activity, was characterized and identified as *Pseudomonas aeruginosa* SSj on the basis of 16S rRNA (accession number KU821118) and partial *azu* gene (accession number KU821119) sequence analysis. To confirm the gene identification results further, the *azu* gene sequence obtained was compared with sequences available in the gene bank. The primers already reported in the previous works were used for screening and sequencing of *azu* gene. On *azu* gene sequence analysis, the isolate showed 100 % similarity with *Pseudomonas aeruginosa* strain NCGM (accession number AP01465).

Azurin gene identification of *Pseudomonas aeruginosa* SSj was done with standard strain *Pseudomonas aeruginosa* MTCC 2453 by previously described methods. Both showed a conventional amplification with azurin specific primer and resulted in a 545 bp band was seen in agarose gel electrophoresis. The presence of azurin gene was detected in *Pseudomonas aeruginosa* SSj and *Pseudomonas aeruginosa* MTCC 2453 was taken as positive control for this study. Characterization study of partially purified azurin sample was done by FTIR and MALDI -TOF. Functional groups were studied by FTIR and standard azurin (Sigma Aldrich) was also used to compare with the sample. Sankar *et al.,* (2011) investigated the difference in the secondary structure of four different *Pseudomonas aeruginosa* strains by FTIR (Sankar & Mahitosh, 2011). Azurin showed a peak around 1650cm^{-1}, which determined the presence of amide 1 band. This peak also indicated the α-helix secondary structure of azurin. Protein sample from *Pseudomonas aeruginosa* SSj and *Pseudomonas aeruginosa* MTCC 2453 showed a peak around 1637 cm^{-1} and 1638cm^{-1} and standard azurin showed a peak around 1641 cm^{-1}. Current studies about azurin revealed that chemical modification of this protein helps to develop an oral formulation of azurin for treating and preventing the onset of cancer (A. M. Chakrabarty *et al.,* 2014). The FTIR analysis of azurin showed the presence of C=O (protein backbone) stretching, which indicates the presence of amide I band (Ali et al., 2017).

Partially purified azurin sample was profiled by MALDI -TOF. Ramachandran *et al.,* studied the purity profile of azurin on each stage of purification by MALDI TOF assays (Sankar *et al.,* 2012). In this study, azurin presence was confirmed with a mixture of proteins.

Because all of these factors further purification of azurin would be needed. Then MCF breast cancer cell lines were used to study the apoptosis death (Yang *et al.,* 2005). Hoechst staining method is used for describing nuclear condensation and cell fragmentations of cancer cell lines after azurin treatment (J. H. Cho et al., 2011).

Based on cytotoxicity analysis, PCR screening, partial gene sequencing and FTIR, it is evident that the *Pseudomonas aeruginosa* strains were harboring azurin gene. The efficacy of the isolate can be further improved by genetic modifications and make it a prospective candidate for medical applications.

2.7 Conclusions

The competent way of discovering new drugs was fed through isolation of new bioactive compounds from microbial sources and assayed its efficacy by both *in vitro* and *in vivo* studies. The studies on the detection of azurin producing strains from environmental sources are rare. This study provides evidence for the existence of azurin harboring *Pseudomonas* strains in Malabar region of Kerala. A novel strain of *Pseudomonas aeruginosa* (strain SSj), producing promising levels of active azurin was successfully isolated and characterized in this study.

Chapter 3

Cloning, expression, purification and characterization of azurin

3.1 Introduction

According to WHO, cancer is responsible for an estimated 9.6 million deaths and 18 million new cases in 2018. So the discovery of a drug with high potentiality and few side effects are very essential. Cloning and expression of azurin gene is essential for its characterization studies and use in cancer research. But the purification of this protein is difficult and require multiple steps which result in low yield. Besides, azurin is too expensive to obtain readily. So as to overcome these limitations, recombinant DNA technology was used for the commercial production of purified azurin at large scale. Many studies about cloning and expression of azurin gene in *E. coli* were done by previous workers like Karlsson and Canters (Canters, 1987; Karlsson, Pascher, Nordling, Arvidsson, & Lundberg, 1989).

Azurin act as an electron transfer shuttle in *Pseudomonas aeruginosa* and other bacteria. This novel protein has some different properties than other cuprous proteins such as high reduction potential, intense blue color, and a small parallel hyperfine coupling in the electron spin resonance spectrum (Solomon *et al.,* 1992). Microorganisms using azurin as an electron transport protein during electron transport cycle. It transports an electron between cyt c-551 and cyt oxidase in their respiration process (Kakutani *et al.,* 1981). The scaffold property is also due to its structural similarity with variable domains of immunoglobulins. So high-affinity interactions with various unrelated mammalian proteins relevant in cancers are possible (A. M. Chakrabarty *et al.,* 2014). For the specificity of azurin protein

in anticancer studies and spectroscopic studies cloning and expression of azurin gene is essential. Azurin can be hyper-expressed in *Escherichia coli*, which makes the process of production very economical (Bernardes, Chakrabarty, *et al*., 2013) and as a small protein it can be hypothesized that its expression may occur in different vectors, including some human cell types. Because of all these reasons, azurin can be used in cancer therapy. Selection of the recombinant host is an essential factor in the success of rDNA technology. *E.coli* is the most commonly used bacterial expression host as its genome is well characterized and easy to manipulate (J. J. Chang et al., 2012). In this study, cloning and expression of azurin gene and confirmation of purified azurin was done by different characterization technique.

3.2 Objectives of the study

- Cloning of *azu* gene in pET22b(+) vector and expression in *E.coli* BL-21 (DE3).
- Characterization of purified azurin using FTIR, MALDI-TOF and LC-MS/MS.
- Comparison studies of purified azurin with standard azurin (Sigma Aldrich).
- Docking studies of the azurin with some anticancer receptors using bioinformatics tools.

3.3 Review of Literature

Microbial therapy of cancer includes the use of bacterial products for cancer treatment. Azurin is a multi-targeted anticancer protein without any side effects. High expense and low level of production limits its use in the biomedical application. So cloning and expression of azurin gene in the suitable host is one of the best ways to produce high-level azurin protein. Bacteria are the most favored expression system for high-level production of enzymes because of their rapid growth on inexpensive substrates, well characterized genetics and availability of a large number of vectors and mutant strains (Terpe, 2006). *E.coli* continues to be the most attractive host due to its short generation time, quick and easy overexpression of the recombinant enzyme, low cost and easy handling compared to other hosts (Gopal & Kumar, 2013).

A suitable vector also contains the origin of replication, promoter, multiple cloning sites (MCS), terminators, selection markers, a fusion protein. Some of the *E. coli* promoters are *lac, trp, tac, trc, ara, cspA, lpp, phoA, recA, tetA,* T7, T7 *lac* operator, T3-*lac* operator, T5-*lac,* etc.(B. C. Joseph, Pichaimuthu, & Srimeenakshi, 2015). Though *E.coli'*s own promoters like *lac, trp, tac* have been used, T7 systems are the most influential promoters because they produce the desired protein to a level about 50 % of the total cell protein (Baneyx, 1999). Regularly short peptides (tags) or fusion proteins are attached to the N- or C- terminal of the heterologous protein. These affinity tags increase solubility, expression and make detection and purification of

recombinant protein simple (Nilsson, Stahl, Lundeberg, Uhlen, & Nygren, 1997). Some of the commonly used tags are poly-His, poly-Arg, FLAG, c-Myc, and Strep II- tags(Terpe, 2003). Commercial antibodies are available for detection of the tags by western blot technique. The 6xHis affinity tag is the most widely used tag as it is small, less immunogenic, uncharged at pH 8.

There is a variety of expression host available for *E. coli* depending on the purpose. The strain BL21 (DE3) and derivatives of K-12 lineage strains are widely used for first protein expression. BL21(DE3) strain was developed by Studier and Moffatt in 1986 (Studier & Moffatt, 1986) after several modifications to B line. BL21 (DE3) has a T7 system. Induction temperature, IPTG concentration and presence of inducers are some factors that influence the yield of recombinant protein. Prolonged induction at low temperature with decreased concentration of IPTG could increase the protein production (Steczko, Donoho, Dixon, Sugimoto, & Axelrod, 1991).

Karlsson *et al.,* (1989) reported the cloning of azurin gene on different expression plasmid vectors and highest expression obtained in plasmid pUC18 (Karlsson *et al.,* 1989). Another study on cloning was reported by Yamada *et al.,* (2002), in this work *Pseudomonas aeruginosa* strain PAO1 was used for DNA extraction, plasmid vector was pUC19 and expression host was *Escherichia coli* JM109 (Yamada, Goto, Punj, Zaborina, Kimbara, et al., 2002). In a previous study, three different azurin derived peptides: Azu 1-50, Azu 1-77 and Azu 80-128 were cloned into the pWH844 vector that encodes a polyhistidine tag

in the N-terminal of the target peptide, it helps for further purification. From the three constructions, Azu 1-50 and Azu 1-77 were successfully expressed in *E. coli* BL21 (DE3) (Ana Teresa Estevens, 2011).

Mohammed *et al.,* (2010) reported that azurin gene was cloned into pGEM-T easy vector system, then the (azurin/pGEM) construct was expressed in the Mach1™- T1® *E. coli* competent cells. Azurin gene was subcloned into the T7-inducible vector (pET-28a (+)) then azurin / pET-28a(+) construct was transformed into BL21 (DE3)-pLysS *E.coli* (Mohamed *et al.,* 2010).

Akhter *et al.,* (2016) reported the production of recombinant azurin through cloning rpTZ57R/T-azu vector into *E.coli* strain DH-5α and subcloning pET28-azu vector into *E.coli* BL21-Codon Plus (DE3). Optimization of azurin production was done by IPTG induction at different concentrations then standardized high expression level at 1mM concentration of IPTG for 5 h. Purification has been done by using Ni+2 affinity chromatography (Akhter, 2016). Thomas *et al.,* studied the expression of azurin gene, using pUC18 and *E.coli* (TG1) and mutagenesis also (T. K. Chang et al., 1991).

After cloning, expression and purification of azurin, characterization studies of this protein was done. According to Mandel, The functional groups of azurin were studied using FTIR spectrum (Sankar et al., 2011). Azurin showed a peak around 1650 cm^{-1}, which determined the presence of amide 1 band. This peak also indicates the α-helix secondary structure of azurin (Surewicz *et al.,* 1987). Azurin

synthesized from different bacterial strain showed a difference in secondary structure. After extraction and purification of azurin, its molecular weight determination is done by MALDI-TOF (Fukuo et al., 1998; Sankar et al., 2011). LC-MS/MS analysis of purified azurin was done to confirmed the successfulness of cloning. Bioinformatics studies about azurin docking with susceptible receptors were rare. Most of the bioinformatics work is based upon azurin interaction with p53(Apiyo & Wittung-Stafshede, 2005; De Grandis, Bizzarri, & Cannistraro, 2007; Nguyen & Nguyen, 2016). Therapeutic efficacy of azurin can be increased by docking studies with specific anticancer receptors.

3.4 Materials and methods

Bacterial strains and plasmid

The *E.coli* strains used for cloning and expression were *E.coli* DH5α and *E.coli* BL21 (DE3) respectively. The plasmid pET 22b (+), used for cloning was obtained from Promega, USA.

3.4.1 DNA Isolation

Total DNA from *Pseudomonas aeruginosa* SSj was isolated by N- Cetyl- N, N,N-Trimethyl-ammonium bromide (CTAB) method (Winnepenninckx, Backeljau, & De Wachter, 1993). 1 ml bacterial culture was centrifuged 1000 rpm for 2 min at 4 ^{o}C. Then the pellet was washed with sterile distilled water. After that 675 µl of extraction buffer was added and incubated at 37 ^{o}C for 30 min. Then, 100 µl of SDS (20 %) was added and incubated at 65 ^{o}C for 2 h. After

incubation, this mixture was centrifuged at 10000 rpm for 10 min at 4 ^{o}C, and supernatant was collected in another microcentrifuge tube. Centrifugation step was repeated with equivalent amount of Chloroform: Isoamyl alcohol (24:1). Then supernatant was transferred into a sterile microcentrifuge tube and added 600 µl of isopropyl alcohol. After 1 h, this mixture was centrifuged at 10000 rpm for 10 min and the pellet was washed in 500 µl of 70 % ethanol. Pellet was dried and dissolved in sterile distilled water.

3.4.2 Primer design and PCR

Specific primers were designed using Primer 3 software and they were incorporated with *Hind*III and *Bam*HI restrictions sites at the 5' end of the forward and reverse primers (Table 3. 1). PCR amplification was done in 20 µl reaction volume containing 2 µl 10X reaction buffer with $MgCl_2$ (1.5 mM), 0.25 µl Taq DNA polymerase (5U), 2 µl each of forward and reverse primer, 1 µl of genomic DNA, 2 µl dNTP mix (2.5 mM) and final volume was made upto 20 µl with nuclease free water. The PCR was performed in Eppendorf thermal cycler as per protocol in Table 3.2

Table 3.1: Sequences of designed primers

Primer	**Sequence**	**Tm (^{o}C)**	**GC %**	**Annealing Temp**
Azu-f	AAGCTTGCCCAAGCTTACCTAG GAGGCTGCTCCATGCTA	74.7	65.0	50.0
Azu_r	GGATCCTGAGCCCCTGTAGGCG CCCATGAAAAAGCCCGGC	69.7	54.0	

Table 3.2: PCR amplification profile

Initial Denaturation	35 cycles			Final extension
	Denaturation	Annealing	Extension	
94 °C for 3 min	94 °C for 60 s	50 °C for 45 s	72 °C for 60 s	72 °C for10 min

3.4.3 Purification of PCR product

The obtained PCR product was purified by PCR purification kit (Promega-wizard SV Gel & Gel PCR cleaning up system). To 300 µl of the PCR product, an equal volume of Membrane Binding Solution was added. An SV mini column was inserted into the collection tube, the prepared PCR product was transferred into it and incubated at room temperature for 1 min. This was centrifuged at 16,000 rpm for 1 min and supernatant was discarded. To the column 700 µl membrane wash solution was added and centrifuged at 16,000 rpm for 1 min. Centrifugation was repeated with 500 µl membrane wash solution. The minicolumn was transferred to a clean 1.5 ml microcentrifuge tube and 50 µl of nuclease-free water was added into the minicolumn. It was then centrifuged at 16,000 rpm for 1 min. The mini column was discarded and DNA stored at –20 ^{o}C . The purity of PCR product before and after PCR purification was checked using NanoDrop.

3.4.4 Restriction Digestion

Restriction digestion of pET22b (+) and the PCR product was done for ligation. The restriction was carried out separately in the 10µl volume containing 5µl vector/PCR product, 10 X buffer 1µl, BSA 0.1

µl, 2 U of each restriction enzyme (EcoR1/HindIII) 1µl. All the reaction mixtures were incubated at 37 ^{o}C in a water bath for 3 h and inactivated by incubating at 65 ^{o}C for 10 min.

3.4.5 Alkaline phosphatase Treatment

This is to prevent the re-ligation of the digested vector and the reaction was carried out in a total volume of 20 ul containing 10 ul of the digested vector, 2 ul of 10x Buffer and 1ul of Alkaline phosphatase. The mixture was incubated at 37 ^{o}C for 30 min. The enzymes were inactivated at 65 ^{o}C for 10 min.

The restricted products were run on 1% agarose gel, the region of interest was sliced with a clean scalpel and purified using a QIAquick Gel Extraction Kit (Qiagen). The gel slice was weighed in a clean tube and 3 volumes of Buffer QG was added to 1 volume of gel. The tube was incubated at 50 ^{o}C for 10 min and vortexed every 2-3 min to help dissolve gel. It was incubated at 50 ^{o}C for an additional 5 min. To this 1 volume of isopropanol was added and mixed well. A spin column was placed in a collection tube; the sample was applied to the column and centrifuged at 13,000 rpm for 1 min. 750 µl Buffer PE was added to the column and centrifuged at 13,000 rpm for 1 min. The column was placed in a clean 1.5 microcentrifuge tube and eluted with Buffer EB. 30 µl of EB was added to the center of the column, incubated for 4 min and centrifuged at 13,000 rpm for 1 min. The concentration and purity of eluted DNA was checked using NanoDrop.

3.4.6 Ligation

10 µl ligation reaction was setup in 3:1 ratio of eluted insert and eluted vector DNA as follows in Table 3.3. The insert and vector mixture was incubated for 1 h at room. After ligation, it kept on ice for further analysis.

Table 3.3: Ligation mixture

Component	Volume (µl)
Insert (50ng/µl)	1
5X ligation buffer	4
pET22b (+) -vector (150ng/µl)	1
T4 DNA ligase	1
Nuclease-free water	13

3.4.7 Competent cell preparation (BL 21 Strain)

The competent cells were prepared by the inoculation of a single colony of *E. coli* DH5α in to 5ml of Luria Bertani (LB) broth. It incubated overnight at 37 ^{o}C in an orbital shaker with an agitation of 200 rpm. From the overnight culture (12-16 h), 500 µl were added to 50 ml of LB broth and incubated at 37 ^{o}C with moderate agitation (200 rpm). OD was checked at A600 during different intervals until the O. D. reached about 0.375. Then the cultures were transferred to pre-chilled centrifuged tubes and incubated for 10 min on ice. The tubes were centrifuged at 3000 g for 10 min at 4 ^{o}C. The pellet was taken and resuspended in 10 ml of (0.1M) $MgCl_2$ solution without vortexing and the cells were incubated for 1 h on ice. After incubation the

centrifugation step was again repeated with similar conditions, pellet resuspended in 1ml of (0.1M) $CaCl_2$ solution and kept on ice for 1h. The competent cells were stored at -80 °C by mixing an equal volume of 40 % glycerol.

3.4.8 Transformation

5 µl of the ligated product was mixed with 200 µl of prepared competent cells and placed on ice for 30 min. Heat shock was given to this mixture at 42 ^{o}C for 2 min and suddenly transferred to ice for 2 min. Then 1000 µl, LB broth was added and the tubes were placed in an orbital shaker at 37 ^{o}C for 1 h. During the incubation period, 50 ml of LB agar with Ampicillin (50 µg/ml) was poured on to the sterile Petri plates. After that, the tubes (ligation and competent cell mixture) were centrifuged at 1000 rpm for 12 min and supernatant was removed. Then fresh LB broth was added to the pellet and 0.1 ml was spread on Petriplate using a L shaped glass rod and incubated at 37 ^{o}C overnight.

3.4.9 Colony PCR

After incubation few colonies were observed on the plate and the selected colonies were subjected to colony PCR for the confirmation on the presence of insert. The colonies were picked and suspended in 10 µl of sterile water. From this 2µl was used as a template for the PCR with the specific primers and the PCR was performed as mentioned above. The respective suspension for the

colony which showed the presence of bands was streaked onto the master plate for the further downstream process.

3.4.10 Plasmid isolation

Reagents for Plasmid isolation

1. Alkaline Lysis Solution I: 50 mM glucose, 25 mM Tris-Cl and 10 mM EDTA. The pH was adjusted to 8.0 and stored at 4 ^{o}C.

2. Alkaline Lysis Solution II: 0.2 N NaOH (freshly diluted from a 5N stock) and 1 % w/v SDS.

3. Alkaline Lysis Solution III: 60 ml of 5 M potassium acetate, 12.5 ml of glacial acetic acid are mixed and the volume is made up to 100 ml. The resulting solution is 3M with respect to potassium and 5M concerning acetate. The solution was stored at 4 ^{o}C .

Plasmid isolation by alkaline lysis method

The plasmid isolation protocol included harvesting and lysis of the cells by alkali. Each of the selected clones were inoculated separately into 2 ml of LB medium containing Ampicillin (50 μg/ml) in a 15 ml sterile tube. The cultures were incubated overnight at 37 ^{o}C with vigorous shaking. To a new sterile microcentrifuge tube, 1.5 ml of culture was poured and centrifuged at 12000 rpm for 30 s at 4 ^{o}C in a microcentrifuge. The medium was removed and the bacterial pellet was dried. The bacterial pellet was resuspended in 100 μl of cold solution I by vigorous vortexing. To the tubes, 200 μl of freshly

prepared solution II was added and were closed tightly. 150 µl of cold solution III was added to this mixture, and mixed well. Then the tubes were incubated on ice for 3-5 min and centrifuged at 12000 rpm for 5 min at 4 ^{o}C . Supernatant was mixed with equal volume of chloroform: isoamyl alcohol (24:1) and centrifuged at 12000 rpm for 2 min at 4 ^{o}C in a microcentrifuge. Plasmid DNA was precipitated with 0.6 volume of ice cold isopropanol and centrifuged at 12000 rpm for 10 min at 4 ^{o}C in a microcentrifuge. The supernatant was discarded. The pellet was washed using 500 µl of 70 % ethanol and centrifuged at 12000 rpm for 10 min. The pellet was re-suspended in 50 µl of deionized sterile distilled water.

The isolated plasmid was used for confirmation of insert by restriction as mentioned above.

3.4.11 Protein expression

The colony was inoculated in fresh LB media containing Ampicillin (50 µg/ml) and incubated overnight for starter culture. After incubation 200 µl of an overnight culture was added to a 10 ml LB media containing Ampicillin and incubated till the OD reaches 0.4 - 0.8 @ 600 nm. To this 7µl of IPTG (100 mg/1.5 L) was added and incubated for 3 h. After that, the culture was centrifuged at 5000 rpm for 12 min. To this 750 µl of lysis buffer (25 mM Tris HCl, 2mM EDTA) of pH 7.6 was added and vortexed followed by centrifuging at 10000 rpm for 10 min. The temperature was maintained at 4 ^{o}C throughout the procedure.

3.4.12 Purification of the recombinant protein by Ni-NTA column (Mohamed *et al.*, 2010)

For purification of protein, elution buffer (50 mM Tris PH 8,300 mM NaCl, 5 % Glycerol, 400 mM Imidazole) and wash buffer (50 mM Tris PH 8,300 mM NaCl,5 % Glycerol and 20 mM Imidazole*) were prepared. The fresh beads were washed three times with wash buffer at 2000 rpm. The beads were mixed with 10 ml protein sample and kept for overnight binding in a shaker incubator with low rpm. The beads were washed eight times with wash buffer and eluted overnight using elution buffer. The temperature was maintained at 4 ^{o}C throughout the procedure. The pellet was collected for further analysis (Waugh, 2005) and presence of azurin protein of 14 KDa size in there suspended pellet was confirmed by SDS- PAGE (Laemmli, 1970). Purified protein sample was assayed for determining protein concentration by Lowry's method. Purified protein was dissolved in PBS and concentration of protein was 1mg/ml.

*Imidazole should be prepared in fresh for elution buffer.

3.4.13 Characterization of azurin protein

Purified azurin is used for protein characterization studies and confirms the presence of protein.

3.4.13.1 FTIR

Infrared (IR) spectroscopy is one of the oldest and well established experimental techniques for the analysis of secondary

structure of polypeptides and proteins (Elliott & Ambrose, 1950; Smith, 2011). Infrared spectroscopy experiments were performed using a PerkinElmer Spectrum Two FT-IR spectrometer. FTIR spectrum of purified azurin was compared with spectrum of standard azurin (Sigma Aldrich). The curves were deconvoluted and imported into spectramanager software, and a Gaussian curve fitting was performed (Surewicz et al., 1987).

3.4.13.2 MALDI-TOF

MALDI-TOF is an effective method to analyze the molecular mass of proteins (Fukuo et al., 1998). The purified protein extract was analyzed using UltrafleXtreme MALDI –TOF from Bruker Daltonics at RGCB, Trivandrum, for molecular weight determination.

3.4.13.3 LC-MS/MS

LC-MS/MS is a method to directly identify proteins in mixtures. The protein mixture to be identified was digested by proteolytic enzymes to produce its short peptides. These protein peptides were analyzed using AB SCIX 3200 QTRAPLC-MS/MS system and mass spectrum of each peptides was obtained (McCormack et al., 1997).

3.4.14 Interaction studies between azurin (PDBID: 1VLX) and selected receptors by molecular docking (rigid body docking approach)

Molecular interaction between azurin and certain selected receptors such as p53 (1HS5), Apaf 1 (1CY5), Cyt C (1CGN), Bax (1F16), Caspase 7 (1K86) and Caspase 9 (2AR9) have been

demonstrated. These receptors were selected on the basis of their role in breast cancer. When MCF-7 cells were treated with azurin, it primarily travels to the nucleus and increases intracellular level of p53 and Bax. Then mitochondrial Cyt C is released into the cytosol. Then it activates caspase cascade and it leads to apoptosis (Punj *et al.,* 2004). Tools for molecular docking were used PatchDOCK & FireDock: Molecular docking algorithm based on shape complementarity principles (Mashiach et al., 2010; Schneidman-Duhovny, Inbar, Nussinov, & Wolfson, 2005; Shatsky, Dror, Schneidman-Duhovny, Nussinov, & Wolfson, 2004). Tools used for visualisation of docked structures by PyMOl 2.1 (Warren L. DeLano, 2009).

3.5 Results

3.5.1 Genomic DNA extraction

The genomic DNA was extracted and quantitatively and qualitatively analyzed. The full amount of DNA obtained in this study refers to the successful method of DNA extracted from *Pseudomonas* isolate which depend on using the protocol of the CTAB method (Doyle & Doyle, 1987). On running agarose gel, a single high molecular weight DNA was visible without smear formation (Figure 3.1).

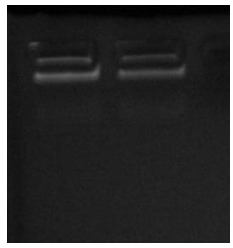


Figure 3.1 DNA isolated from *Pseudomonas aeruginosa* SSj.

3.5.2 PCR amplification of azurin gene

Azurin gene from *Pseudomonas aeruginosa* SSj was amplified using specific primers (Primer 3). After amplification the products were analyzed using agarose gel electrophoresis (Figure3.2).

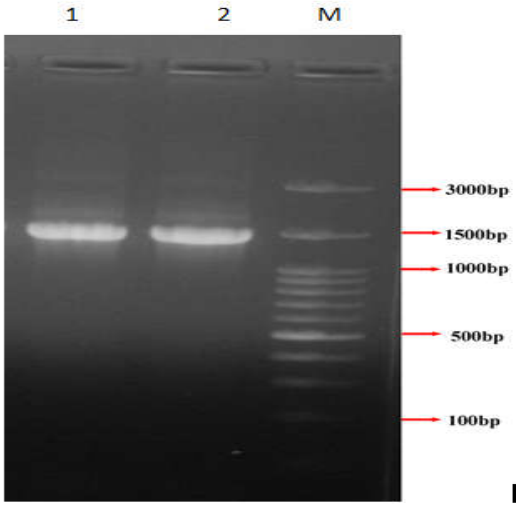


Figure 3.2: PCR product for azurin (M: Marker; 1, 2: DNA samples isolated from *Pseudomonas aeruginosa*)

3.5.3 Ligation and Transformation

In this study, pET22b (+) was used to express the azurin gene. A large volume of double digest of the vector (pET22b (+)) and the insert (purified PCR product) was prepared for ligation. Both pET 22 b(+) vector and purified PCR product were digested using the restriction enzymes EcoRI/HindIII. PCR product was digested by the restriction enzymes, and the isolated azurin gene was inserted into pET22b (+). Then, it was transformed into *E.coli* DH5α strain (Figure 3.3).

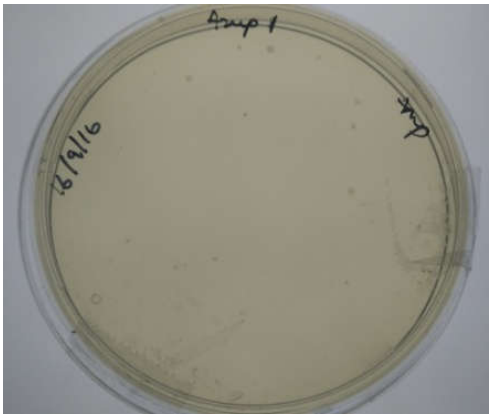


Figure 3.3:Transformed colonies of *E. coli* DH5α containing the azurin gene.

3.5.4 Colony PCR

Colony PCR of few randomly selected colonies were performed as mentioned earlier to confirm transformation. The positive colony which showed the presence of bands (Figure 3.4) were streaked on plates and also stored as the glycerol stock.

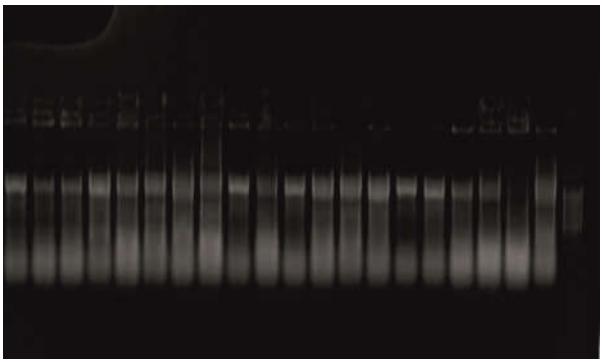
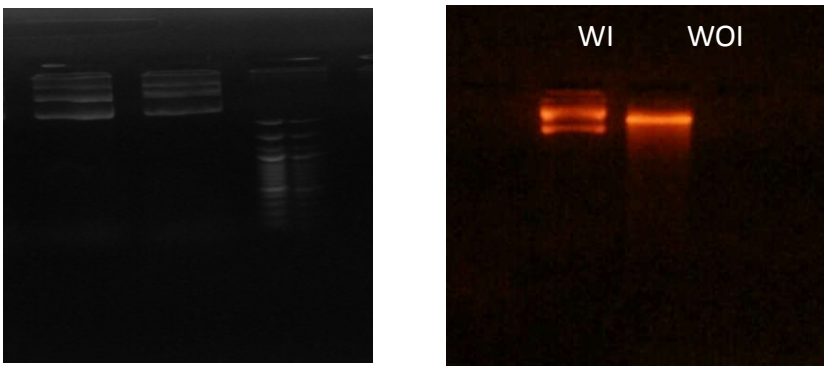


Figure 3.4: Colony PCR of Transformed colonies

3.5.5 Plasmid isolation and clone confirmation

Plasmid DNA from *E.coli* DH5α strain was isolated and the presence of the azurin gene was confirmed by restriction digestion of isolated plasmid. The positive colony which showed the presence of bands (Figure 3.4) were selected as successful transformants and stored as lyophilized cultures. The plasmids from the transformed colonies were isolated (Figure 3.5.A) and digested with *ECOR1*/*HindIII* for confirmation of insert. On double digestion of the recombinant plasmid, two bands corresponding to the vector and insert were observed (Figure 3.5.B).



(A) **(B)**

Figure 3.5 (A) Plasmid isolation from transformed colonies; (B) Restriction digestion of the plasmid (WI: with insert; WOI: without insert)

3.5.6 Gene expression and purification

For the expression of azurin gene from *E.coli* BL21 (DE3), the recombinant colony was grown in LB broth and induced with IPTG. LB broth and induced with IPTG. Purification of soluble protein was done by affinity chromatography on Ni-NTA agarose beads.

The SDS PAGE analysis on purified samples showed that a band with the apparent molecular weight of about 14 KDa was detected (Figure 3.6) after staining with Coomassie brilliant blue. Standard azurin was taken as positive control.

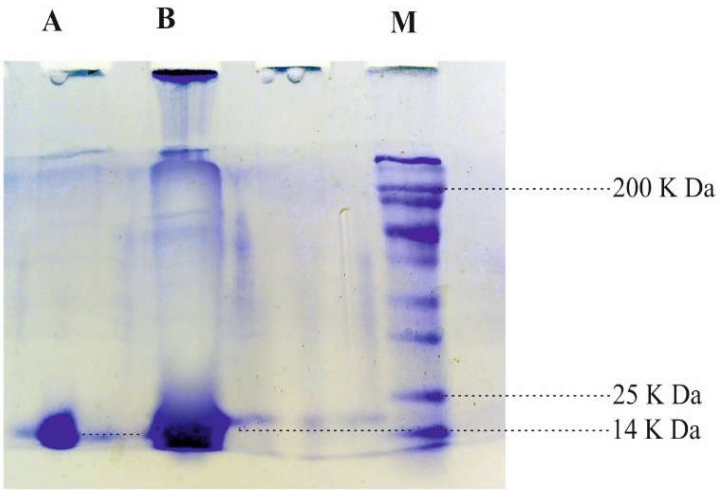


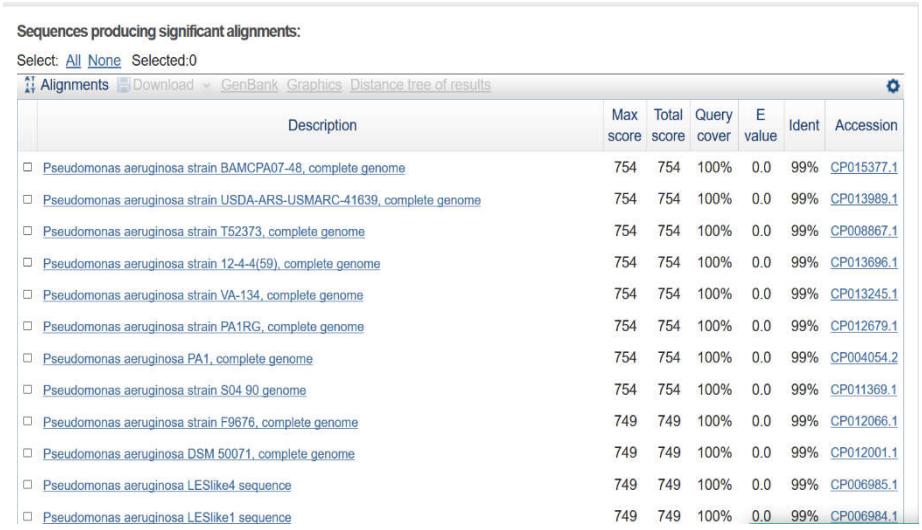
Figure 3.6: SDS-PAGE gel electrophoresis showing purification of Azurin: **A**-standard azurin**, B**-purified azurin ,**M** –Protein marker,

3.5.7 Gene sequencing of azurin gene

The azurin gene from recombinant plasmid was sequenced, the vector contamination removed and BLAST analysis was carried out. The amplicon of 1224 bp was sequenced and flanking regions were removed to get the *azu* sequence of 418bp. BLASTn analysis the *azu* gene sequence showed 99 % similarity to the many *Pseudomonas aeruginosa* strains (Figure 3.7). The Genscan analysis of this sequence showed a coding region for 126aa peptide. The sequence was deposited in Genbank with accession number MK120121.

>Azu (418bp)

CTGTGCCTGCTCAGTGCGCCGACTGCTGGCTGCCGAGTGCTCGGTGGACA
TCCAGGGTAACGACCAGATGCAGTTCAACACCAATGCCATCACCGTCGAC
AAGAGCTGCAAGCAGTTCACCGTCAACCTGTCCCACCCCGGCAACCTGCC
GAAGAACGTCATGGGCCACAACTGGGTACTGAGCACCGCCGCCGACATGC
AGGGCGTGGTCACCGACGGCATGGCTTCCGGCCTGGACAAGGATTACCTG
AAACCCGACGACAGCCGTGTCATCGCCCACACCAAGCTGATCGGCTCGGG
CGAGAAGGACTCGGTGACCTTCGACGTCTCCAAGCTGAAGGAAGGCGAG
CAGTACATGTTCTTCTGCACCTTCCCGGGCCACTCCGCGCTGATGAAGGGC
ACCCTGACCCTGAAGTGA



Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Pseudomonas aeruginosa strain BAMCPA07-48, complete genome	754	754	100%	0.0	99%	CP015377.1
Pseudomonas aeruginosa strain USDA-ARS-USMARC-41639, complete genome	754	754	100%	0.0	99%	CP013989.1
Pseudomonas aeruginosa strain T52373, complete genome	754	754	100%	0.0	99%	CP008867.1
Pseudomonas aeruginosa strain 12-4-4(59), complete genome	754	754	100%	0.0	99%	CP013696.1
Pseudomonas aeruginosa strain VA-134, complete genome	754	754	100%	0.0	99%	CP013245.1
Pseudomonas aeruginosa strain PA1RG, complete genome	754	754	100%	0.0	99%	CP012679.1
Pseudomonas aeruginosa PA1, complete genome	754	754	100%	0.0	99%	CP004054.2
Pseudomonas aeruginosa strain S04 90 genome	754	754	100%	0.0	99%	CP011369.1
Pseudomonas aeruginosa strain F9676, complete genome	749	749	100%	0.0	99%	CP012066.1
Pseudomonas aeruginosa DSM 50071, complete genome	749	749	100%	0.0	99%	CP012001.1
Pseudomonas aeruginosa LESlike4 sequence	749	749	100%	0.0	99%	CP006985.1
Pseudomonas aeruginosa LESlike1 sequence	749	749	100%	0.0	99%	CP006984.1

Figure 3.7: BLASTn analysis of azurin gene

The probable protein sequence of azurin gene given below (translated sequence in functional form).

>Azurin(126aa)

CSVDIQGNDQMQFNTNAITVDKSCKQFTVNLSHPGNLPKNVM
GHNWVLSTAADMQGVVTDGMASGLDKDYLKPDDSRVIAHTK
LIGSGEKDSVTFDVSKLKEGEQYMFFCTFPGHSALMKGTLTLK

Similarity search was done by BLASTp. Protein sequence shows 100 % similarity with PDB ID: 1VLX, an already existing structure was used for docking studies and other analysis.

3.5.8 Characterization of azurin protein

Purified azurin is used for protein characterization studies and confirms the presence of protein.

3.5.8.1FTIR

The functional groups of azurin were analyzed by FTIR spectrum. Purified azurin and standard azurin showed a peak around 1637 cm^{-1} and 1641 cm^{-1}region (Figure 3.8).

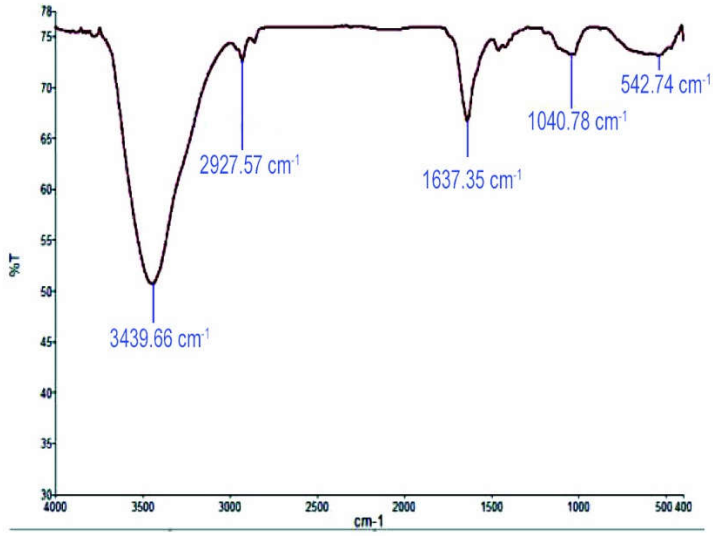


Figure 3.8**: A.** FTIR spectra of purified azurin showed a peak around 1637.35 cm^{-1}

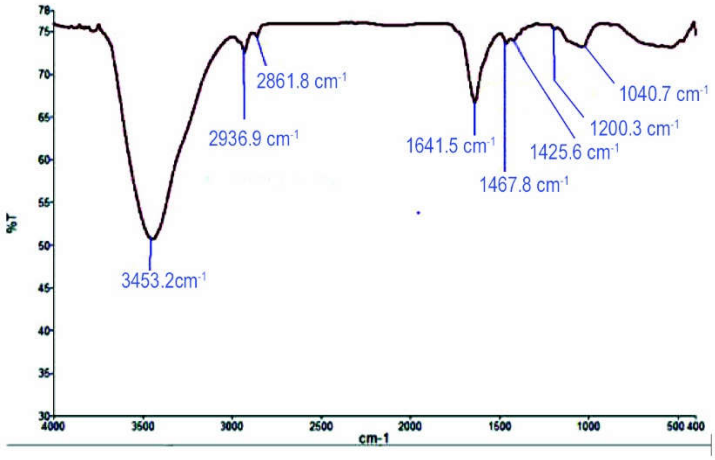


Figure 3.8**:B.** FTIR spectra of standard azurin showed a peak around 1641.5 cm^{-1}

3.5.8.2 MALDI-TOF

The purified protein was analyzed by MALDI-ToF (Figure 3.9) to confirm the presence of azurin. A peak was observed around 14 KDa molecular weight which confirmed the presence of azurin.

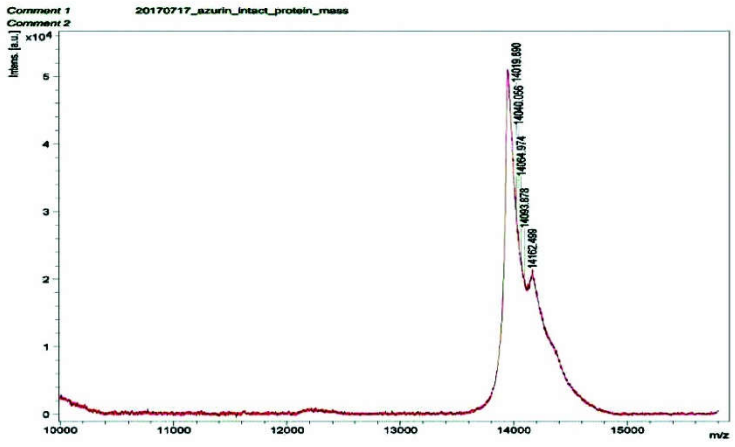


Figure 3.9:Purified azurin was analyzed in MALDI-ToF

3.5.8.3 LC-MS/MS

Purified azurin sample was analyzed using LC-MS/MS analysis (Figure 3.10) and confirmed that the sample contains azurin. MS spectrum analysis of azurin (Figure3.11-3.14) showed four short peptide fragments of azurin (Table 3.4). The protein sequence of these short peptides were compared with azurin sequence obtained in this study (MK120121) (Figure 3.15).

Table 3.4: MS/MS spectrum of selected ions azurin protein

Seq No.		Sequence	Mass
1	Figure 3.11	HPGNLPK	997.48
2	Figure 3.12	LIGSGEK	703.4
3	Figure 3.13	LIGSGEKDSVTFDVSK	1681.868
4	Figure 3.14	LKEGEQYMFFCTFPGHSALMK	2521.1685

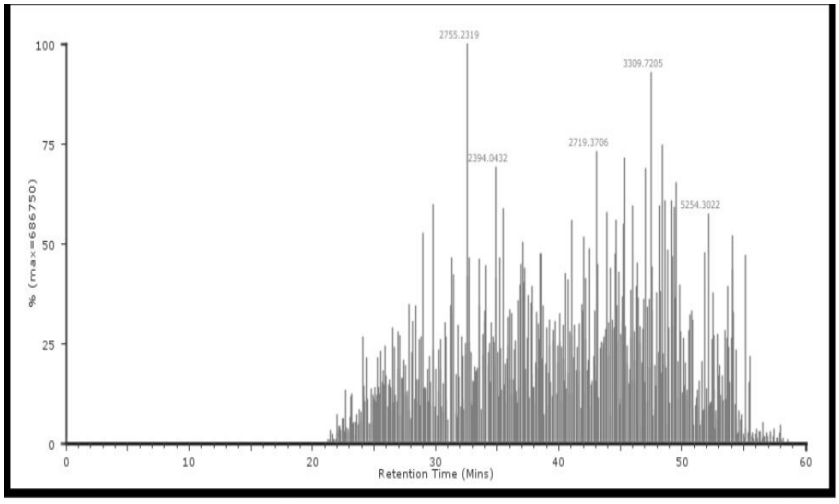


Figure 3.10:LC-MS analysis of purified azurin. (x-axis is retention time and Y axis mass)

The four files are MS/MS (fragmentation) spectrum of selected ions (peptide) of azurin protein, used for sequencing and identifying the azurin protein.

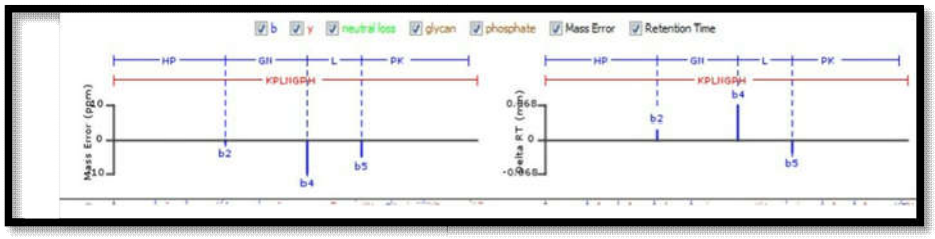


Figure 3.11: Fragment 1: HPGNLPK (mass 997.48)

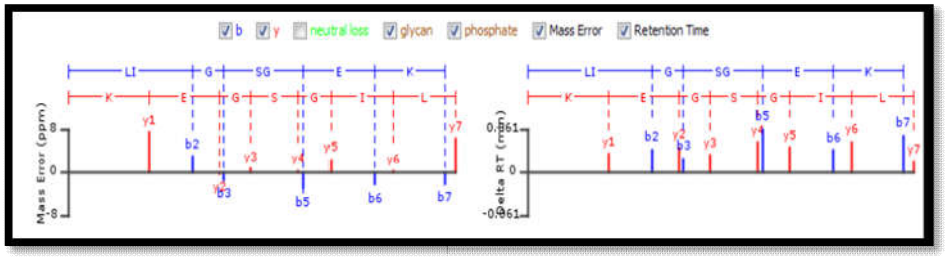


Figure 3.12: Fragment 2: LIGSGEK (mass 703.4)

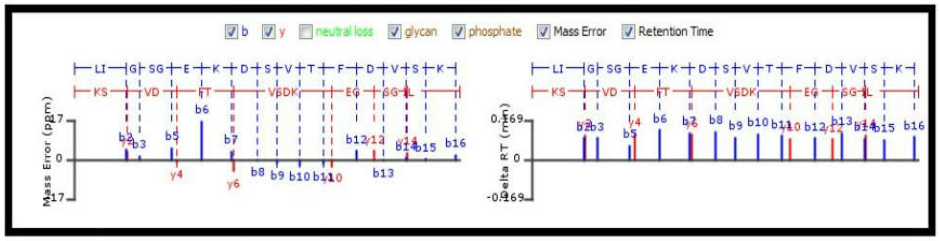


Figure 3.13: Fragment 3: LIGSGEKDSVTFDVSK (mass 1681.868)

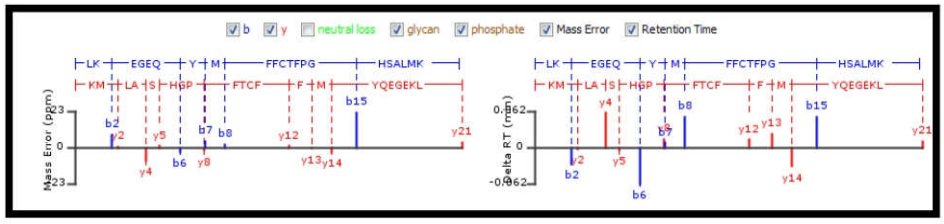


Figure 3.14: Fragment 4: LKEGEQYMFFCTFPGHSALMK (mass 2521.1685)

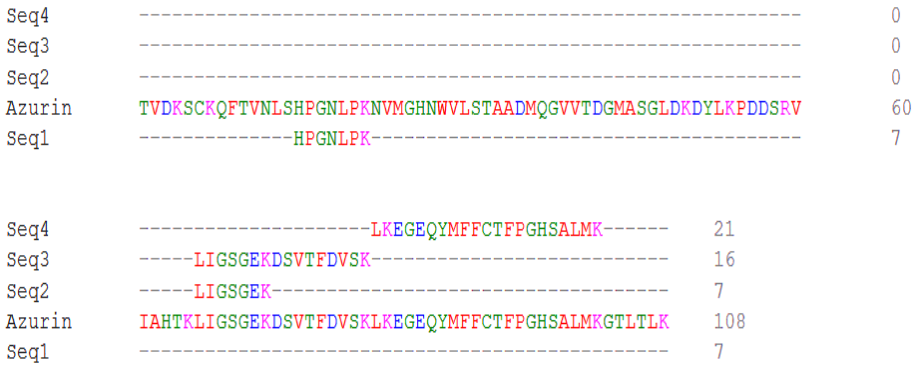


Figure 3.15: LC-MS/MS fragments aligned with azurin reference sequence (MK120121)

3.9 Interaction studies between Azurin (PDBID: 1VLX) and selected receptors by molecular docking results (rigid body docking approach)

Docking studies of azurin were done by rigid body docking approach. Azurin and certain receptors such as p53 (1HS5), Apaf 1 (1CY5), Cyt C (1CGN), Bax (1F16), Caspase 7 (1K86) and Caspase 9 (2AR9), in breast cancer pathway, were done (Table 3.5 & Figure 3.16). Bioinformatics approach for Docking studies of azurin and cancer cell receptor in a breast cancer pathway was found to be the first attempt.

Table 3.5: Docking results-Interaction between Azurin (PDBID: 1VLX) and selected receptors

Ligand	Receptor (PDB ID)	Docking score (Geometric shape complementarity score)	ACE (Atomic contact energy)	Area (Approximate interface area of the complex)	Cluster RMS
Azurin (1VLX)	1HS5(p53)	13646	467.73	1940.80	4.0
	1CY5 (Apaf1)	11152	462.11	1663.70	4.0
	1CGN (Cyt C)	14714	248.80	1974.50	4.0
	1F16 (Bax)	15264	426.93	2427.00	4.0
	1K86 (Caspase 7)	15984	469.80	2227.00	4.0
	2AR9 (Caspase 9)	17944	436.66	2883.20	4.0

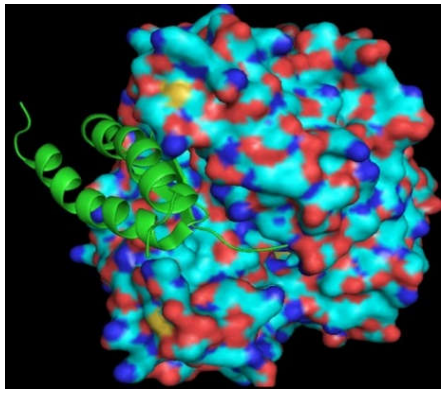


Figure 3.16a: 1HS5(p53) and 1VLX

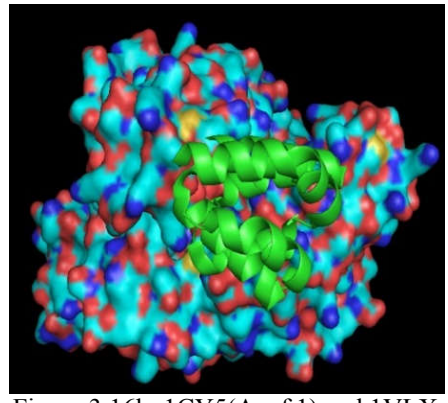


Figure 3.16b: 1CY5(Apaf 1) and 1VLX

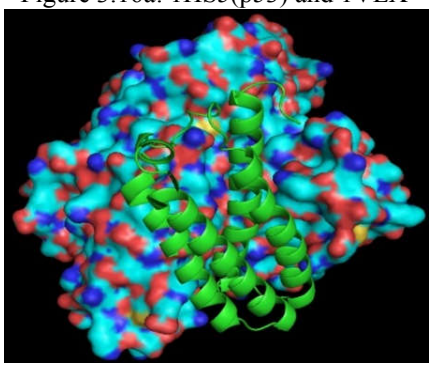


Figure 3.16c: 1CGN(Cyt C) and 1VLX

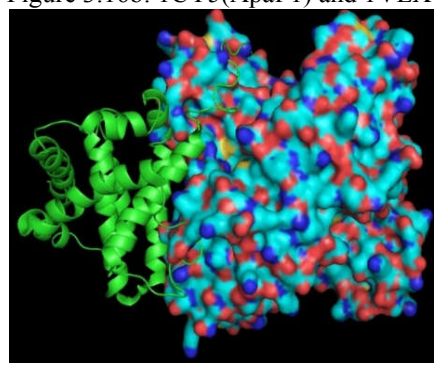


Figure 3.16d:1F16(Bax) and 1VLX

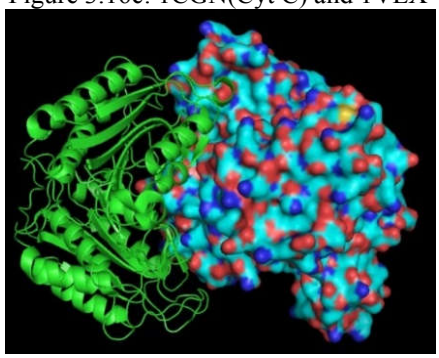


Figure 3.16e: 1K86(Caspase 7) and 1VLX

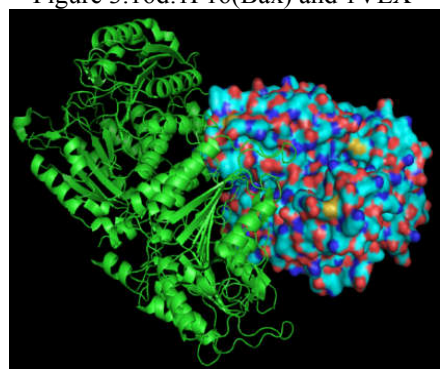


Figure 3.16f: 2AR9(Caspase 9) and 1VLX

Figure 3.16. Interaction between receptors (ribbon display) and azurin (1VLX): Visualization of Docked complex by PyMOL.

3.6 Discussion

The strain *Pseudomonas aeruginosa* SSj was recognized as an active azurin producer from the findings of chapter 2, but the yield and purity of azurin produced was low. However, for biomedical applications it is important to enhance the production. Increased production can be obtained by over-expression of azurin in heterologous hosts. *E. coli* is the most widely used host for recombinant protein production. Azurin is too expensive to obtain readily. In addition to all this, azurin can be hyper-expressed in *Escherichia coli*, which makes the process of production very economical (Bernardes, Chakrabarty, *et al.,* 2013). Azurin has a great potential to be used in the molecular electronics and biomedical research. We have cloned the protein and purified it in a single step and got a better yield of the protein.

In this work we were successful in cloning the *azu* gene from strain SSj in pET22b (+) vector and expressed it in *E.coli* BL-21 (DE3). The amplicon of 1224 bp was sequenced and flanking regions were removed to get the *azu* sequence of 418bp (MK120121). BLASTn analysis of the *azu* gene sequence showed 99 % similarity to the *Pseudomonas aeruginosa* strain SP4528. The Genscan analysis of this sequence showed a coding region for 126 amino acid peptide. Several researchers reported about 128 amino acid containing azurin from different species of *Pseudomonas* (Ambler & Brown, 1967; Barber, Trimboli, & McIntire, 1993; Fuentes, Oyola, Fernandez, & Quinones, 2004). The recombinant azurin was purified using Ni-NTA agarose affinity chromatography. In a similar study, Mohammed *et al.,* (2010) purified recombinant azurin using Ni+2 affinity

chromatography. Several reports explain the recombinant protein purified by column chromatographic methods (Yamada, Goto, Punj, Zaborina, Chen, *et al.,* 2002). SDS-PAGE analysis was carried out to demonstrate the over-expression and to determine the molecular weight of the protein (Figure 3.6). The successful over-expression of azurin protein was confirmed by the presence of a thick band corresponding to 14kDa.

Functional groups of azurin was studied using FTIR and compared with standard azurin. Purified azurin and standard azurin showed a peak around 1637.35 cm^{-1} and 1641.5 cm^{-1}region respectively. In the FTIR spectrum, the presence of a peak around 1650 cm-1 region indicated the amide I band, and also signifies α-helix secondary structure of azurin, which ascends because of the stretching and vibration of the main chain of carbonyl groups. It is coupled with the in-plane N-H bending and C-N stretching modes (Surewicz et al., 1987). Ali *et al.,* (2017) also studied about the functional group of azurin by FTIR (Ali *et al.,* 2017). Azurin synthesized from different bacterial strains showed difference in secondary structure (Sankar *et al.,* 2011). Molecular weight determination was done by MALDI-TOF which confirmed the presence of 14 KDa azurin protein. In another characterization study of azurin, by Dinarieva *et al.,* (2012) reported the 13.920 Da molecular mass of azurin by MALDI -TOF (Dinarieva et al., 2012). The confirmation studies of azurin by MALDI-TOF analysis was previously done by Sankar *et al.,* in 2012 (Sankar Ramachandran *et al.,* 2012). Fukou *et al.,* (1998) also reported the

MALDI -TOF analysis of azurin for molecular mass analysis (Fukuo *et al.,* 1998).

The works which include both about cloning and characterization of purified protein are rare. Finally, the presence of azurin was corroborated by LC-MS/MS analysis. Short peptide sequences analyzed from MS spectra were compared with azurin protein sequence (MK120121) (Figure 3.15). Gorman *et al.,* (2010) reported a method to analyse the presence of p28 (a short peptide derived from azurin) in mice serum using LC-MS/MS (Gorman *et al.,* 2010). After the computational translation of nucleotide sequences it shows 100 % similarity with already existed structure 1VLX. Hence there is no need for structure modeling and this existed structure was taken for docking studies. We selected six receptors of breast cancer path way. Molecular interaction between azurin and certain selected cancer related receptors such as p53 (1HS5), Apaf 1 (1CY5), Cyt C (1CGN), Bax (1F16), Caspase 7 (1K86) and Caspase 9 (2AR9) have been demonstrated. Azurin performs in a proper way and all six receptors gave good docking score and atomic contact energy. Hence azurin or derived proteins or peptides could be scaffolds against these proteins in breast cancer cells models, being an exciting new therapeutic tool. Presently azurin is available commercially with very high price which counteracts its direct application in research and development. This study provides an economical source for the production of purified azurin that can be used in cancer research.

3.7 Conclusions

In this chapter, we report the successful cloning and expression of azurin gene from *Pseudomonas aeruginosa* SSj in *E. coli*. The recombinant azurin ensures a high degree of purity and yield. Characterization of the azurin was done by FTIR, MALDI-TOF and LC-MS/MS. Docking studies of azurin were also done with selected breast cancer cell receptors by bioinformatics tools. The recombinant purified azurin could be used for anticancer studies in further works.

Chapter 4

Study of growth suppression by azurin on various cancer cell lines.

4.1 Introduction

Cancer is a critical condition, in which almost every organs or tissues of our body may be affected with malignant growths (G.A., P., & S., 2007). It is the second major cause of mortality and morbidity worldwide after cardiac diseases (Ovesna & Horvathova-Kozics, 2005). Live or attenuated pathogenic bacteria or their products were used in the treatment of cancer (A. M. Chakrabarty, 2003; da Rocha, Lopes, & Schwartsmann, 2001). In certain conditions the use of live bacteria may cause some undesired infections. So, the discovery of novel approaches are necessary for anticancer therapies include the use of purified products of microbial origin (A. M. Fialho *et al.,* 2007; Schulz, 2005). Researchers have been looking to engineer microorganism to produce medicinally important compounds and improve their activity through protein engineering, so that cancer treatments may become more efficient and cost effective (Punj et al., 2003). Here, we concentrate on recombinant azurin, a novel anticancer protein mainly produced from *Pseudomonas aeruginosa* SSj expressed on pET 22b (+).

Azurin's cytotoxicity is only for specific cancer cells and does not affect healthy cells and it has fewer toxic side effects. The second most vital feature of azurin is the tremendous number of therapeutic substance can link to azurin molecule, so it also acts as cargo protein (Mahfouz *et al.,* 2007). Azurin is reported to have antiviral and antiparasitic activity along with anticancer activity (Chaudhari et al., 2006; Valli S & T, 2018). Azurin is a multi-targeted anticancer agent

that acts through three pathways; first, induction of apoptosis through intracellular stabilization of P53 protein, (azurin-P53 complex formation stabilizing it and enhancing its intracellular level, which leads to apoptosis via caspase-mediated mitochondrial cytochrome c release pathways). Second, inhibition of cell cycle progression through intracellular binding to Eph receptor tyrosine kinase and finally, prevention of angiogenesis through inhibition of VEGFA (Vascular endothelium growth factor A (Apiyo & Wittung-Stafshede, 2005; Chaudhari *et al.,* 2007; Miyashita & Reed, 1995; Pasquale, 2010). In this study, the anticancer activity of recombinant azurin and standard azurin were analyzed on the different cancer cell lines and normal cell line. *In vivo* studies of azurin was also done. Apoptotic effect of azurin was confirmed using Hoechst staining and FRET based assay on SW480cell line. The hemolytic activity of purified azurin was also studied.

4.2 Objectives of the study

- Detection of the cytotoxicity of recombinant azurin in comparison with standard azurin using MTT assay on different cell lines such as human breast cancer cell line (MCF7), human colon carcinoma cell line (HCT15) and African green monkey kidney normal cells (VERO).
- Determination of apoptotic efficacy of azurin using Hoechst staining and FRET based assay.
- *In vivo* antitumor assay of azurin was done by ascites antitumor modeling.

- Evaluation of hemolytic potential of purified azurin against human blood erythrocytes.

4.3 Review of Literature

The anticancer activity of azurin was primarily detected by Zaborina *et al.,* They observed that an extracellular pathogen such as *Pseudomonas aeruginosa* elaborated a water-soluble, copper containing redox protein azurin that had cytotoxic activity against a macrophage cell line J774. Cytotoxicity was due to the presence of a unknown cytotoxic factor, participated in the conversion of procaspase-3 to active caspase-3 in an ATP-independent manner (Zaborina et al., 2000). There is a sensing mechanism in *Pseudomonas aeruginosa* so when exposed to a cancer cell, *Pseudomonas aeruginosa* cells release azurin to the outside medium (Mahfouz *et al.,* 2007).

4.3.1 Mechanisms behind cytotoxicity of azurin

Azurin is a multi-targeted anticancer agent that acts through three pathways. These pathways are independent and together lead to the inhibition of cancer growth:

1) Inhibition of cell cycle progression through extracellular binding to Eph receptor tyrosine kinases,

2) Induction of apoptosis through intracellular stabilization of p53 protein.

3) Prevention of angiogenesis through inhibition of VEGFA.

4.3.1.1, Inhibition of Cell Cycle progression:

Ephrins are proteins, included in the subfamily of receptor protein tyrosine kinases (RTKs) and act as lignds of Eph receptors. They are generating signals at sites of cell-cell contact, controlling cell morphology, adhesion, migration and invasion (Kullander & Klein, 2002). Ephrins are divided into two types: A and B. Type A are cell membrane linked and type B contains intracellular and transmembrane domain (Eph Nomenclature, 1997). These proteins, over expressed in many types of cancers including melanoma, breast, prostate, pancreatic, gastric, esophageal, colon and hematopoietic tumors (Easty, Herlyn, & Bennett, 1995; Kiyokawa *et al.,* 1994; Wicks, Wilkinson, Salvaris, & Boyd, 1992) and promote cancer growth and angiogenesis (Kuijper, Turner, & Adams, 2007). C-terminal domain of azurin (Azu 96-113) that exhibits structural similarity to ephrinB2 at the G-H loop region, and it involved in the EphrinB2-Fc receptor binding (Chaudhari *et al.,* 2007). The peptide (Azu 96-113) interfere in EphrinB2 signaling pathway by competing with EphrinB2 on binding to the tyrosine kinase. For the initiation of cell signaling, EphrinB2, binds its cognate receptor tyrosine kinase EphrinB2. This interference contributes to interfering in the upstream cell signaling and initiation of cancer cell death and tumor regression. Chaudhari *et al.,* (2007) reported the structural similarity of azurin with the extracellular domains of some ephrins (Chaudhari *et al.,* 2007; Pasquale, 2010).

4.3.1.2, Induction of apoptosis:

Azurin is highly cytotoxic to cancer cells having p53 but is less cytotoxic toward p53-negative or nonfunctional p53 cell lines. P53 is a 393 residue protein, contains four functional domains: an N-terminal domain (NTD), a sequence-specific DNA-binding domain (DBD), a tetramerization domain (TD) and a C-terminal regulatory domain (CTD) (Y. Cho, Gorina, Jeffrey, & Pavletich, 1994; C. Klein et al., 2001). P53 also known as guardian of the genome and provide an attractive target for anticancer drugs they can stabilize it, and enhance its tumor-suppressor function in cancer cells (Bossi & Sacchi, 2007; C. Klein & Vassilev, 2004). Azurin, is the only bacterial protein can enter cancer cells and complex with p53 and promoting cell death. It binds to p53 with nanomolar affinity in a four-to-one stoichiometry (pH 7.5, 25 ^{o}C) (Apiyo & Wittung-Stafshede, 2005).

Azurin enters into breast cancer cell line MCF-7 cells, firstly reaches the nucleus, and form a complex with p53 and stabilizing it. The p53 level is increased and leads to consequent down-regulation of bcl2 and increase of bax genes. P53 is a transcriptional regulator of Bax (proapoptotic) and Bcl2 (antiapoptotic) proteins (Punj et al., 2004). The down-regulation of Bcl2 levels and up-regulation of Bax levels triggered Bax translocation to mitochondria and release of cytochrome c into the cytosol (Yamada, Goto, Punj, Zaborina, Chen, *et al.,* 2002). Cytochrome c functions as a cofactor with Apaf-1 to promote the cleavage of pro-caspase-9 (Skulachev, 1998). Pro caspases

activates the caspase-9 and caspase-7, and initiating the apoptotic process (Punj et al., 2004).

4.3.1.3. Inhibition of Angiogenesis:

New blood vessel formation (angiogenesis) is essential to tumor growth, invasion, and metastatic dissemination. Angiogenesis is regulated by vascular endothelial growth factor (VEGF), fibroblast growth factor (Lieu, Heymach, Overman, Tran, & Kopetz, 2011) and platelet-derived growth factor (Hellberg, Ostman, & Heldin, 2010). Vascular endothelial growth factor (VEGF) signaling pathway plays vital roles in regulating tumor angiogenesis by the presence of a receptor VEGFA-2. It also activates extracellular matrix proteins that take part in angiogenesis. Azurin's preferential entry into cancer cells is mediated by p28 (amino acids 50-77), a peptide fragment from azurin. P28 plays a significant role in antiangiogenesis. Because p28 interferring with many receptors especially VEGFA-2, responsible for cell attachment and movement. Hence p28 decrease cell motility and increase cell rigidity, therefore inhibits angiogenesis (R. R. Mehta *et al.,* 2011).

4.3.2 Azurin as a scaffold protein

Scaffold proteins are regulators of many key signaling pathways. Scaffold proteins act in mainly four ways: binding signaling components, localizing signaling components to specific areas of the cell, regulating signal transduction and insulating correct signaling proteins from competing proteins (Shaw & Filbert, 2009). Azurin

shows structural similarity with certain proteins, so it displays high-affinity interactions with several proteins such as p53, EphB2, ICAM-3, DC-SIGN, gp120, MSP-1 and SAG1 and form complexes (Arsénio M Fialho & Chakrabarty, 2010).

Azurin's interaction with EphB2 and with DC-SIGN is due to the presence of a peptide of the 88-113 region, which is responsible for the complex formation with EphB2 (Arsenio M Fialho, Das Gupta, & Chakrabarty, 2008). Azurin interact with p53 through the hydrophobic region (aminoacids Met-44 and Met-64) (Yamada, Goto, Punj, Zaborina, Chen, *et al.,* 2002). Azurin also showed structural similarity with the variable domains of immunoglobulins (Fab fragment). Anti-viral effect of azurin is due to the structural similarity of azurin with the amino-terminal domain of CD4 and the extracellular domain of two host proteins involved in HIV-1 binding and entry into host cells. Anti-parasitic effect is due to an structural similarity with MSP1-19 of the parasite *Plasmodium falciparum* and the surface antigen SAG1 of the parasite *Toxoplasma gondii* (Chaudhari et al., 2006; Chaudhari et al., 2007).

4.3.3 Azurin derived peptides

The major peptide fragments involved in the entry of azurin into cancer cells are p28 and p18. Amino acids 50 to 77 of azurin, p28 which contains a putative protein transduction domain (PTD) responsible for the endocytotic and nonendocytotic- penetration of azurin into cancer cells, stabilizing p53 and raising its intracellular level. Protein transduction domains are two types they are cationic and

amphipathic α-helix. Cationic peptides enter normal and malignant cells by interacting with negatively charged surface glycoproteins (R. W. King, Jackson, & Kirschner, 1994). Amphipathic peptides disrupt cell membranes and alter mitochondrial permeability or attach to specific receptors to produce a cytotoxic effect (Leuschner & Hansel, 2004). P28 is a unique agent possessing both antiangiogenic and cytostatic properties. The first-human, phase I clinical trial of p28 (NSC745104), was reported in 2013. Different factors such as safety, tolerability, pharmacokinetics and preliminary activity of p28 in patients with $p53^{+}$ metastatic solid tumors were investigated. A total of 15 patients were administered p28. Nobody shows toxicity symptoms. Hence p28 can be used as a good therapeutic drug (Warso *et al.,* 2013). *In vivo* studies of p28 on male mice show good result without any toxicity symptoms (Jia *et al.,* 2011).

P28 can enter cancer cells at 37 ^{o}C easily, but hydrophilic (67-77) and hydrophobic (50-66) domains fails to enter cells either at 37 ^{o}C or at 4 ^{o}C. P28 can transport cargo protein by an energy-dependent process via an endocytic mediated pathway to cells (Yamada et al., 2005). Yamada *et al.,* (2005) studied the mode of action of p28 in p53-positive breast cancer cells. Confocal microscopy showed that p28 preferentially enter tumourigenic cells (adenocarcinoma MCF-7, ductal T47D and carcinoma ZR75-1) comparing to non-tumourigenic cells (epithelia MCF-10A cells). *In vivo* analysis with p28 on MCF-7 tumor xenograft in athymic mice with intraperitoneal injection, showed a significant decrease in tumor growth without reducing the body weight and changing mice behavior. P28 can promote uptake of heterologous

proteins such as green fluorescent protein and glutathione S-transferase into cultured cells (B. N. Taylor *et al.,* 2009; Yamada *et al.,* 2005).

P18 causes the cell death of most K562 cells by depolarizing plasma membrane potential and enhancing membrane permeability, other than activating the classical apoptosis pathway. These studies revealed that disrupting plasma membrane is an alternative method to kill cancer cells (Tang *et al.,* 2010). Azurin p18 and p28 have high affinity towards cancer cells compared to other anticancer cationic peptides (Kirkham & Parton, 2005).

4.3.4 Azurin action on different cell lines

4.3.4.1, Breast cancer cell lines

Azurin is able to form a complex with p53 in certain breast cancer cell lines. It is effective to p53 –positive breast cancer cell lines (MCF-7), and less cytotoxic toward p53-negative breast cancer cell line (MDA-MB- 157) or cell lines with non-functional p53 (MDD2 and MDA-MB-231). Punj *et al.,* (2004) reported the *in vivo* experiments of azurin on athymic mice induced with MCF-7 breast cancer cells. The treated animals did not show any toxicity symptoms and also showed significant decrease in the size of tumor (Punj et al., 2004).

In a previous study by Bernards *et al.,* (2013), reported the action of azurin on three different breast cancer cell lines, MCF-7/AZ. Mock, MCF-7/AZ.Pcad and SUM149 (Bernardes, Ribeiro, *et al.,* 2013). Yamada *et al.,* (2009) also reported the action of p28 on

different human breast cancer cell lines MCF-7, ZR-75-1, and T47D through a caveolin-mediated pathway (Yamada *et al.,* 2009). Marcelli *et al.,* (1998) reported that treatment of MCF-7 cells with azurin causes Bax mediated apoptosis than caspase-3 action (Marcelli et al., 1998). Drugs involved in breast cancer such as etoposide, cisplatin, doxorubicin were found sensitive to apoptosis when cells were reconstituted with caspase-3 (Mooney, Al-Sakkaf, Brown, & Dobson, 2002). Azurin can also induces an alternate apoptosis pathway independent of caspase-3 in combination with other chemotherapeutic agents (Punj *et al.,* 2004).

4.3.4.2 Oral cancer

Studies about azurin's action on oral cancer cell lines are few. Oral cancer is the most common type among Indian men (Rajeev, Choudhary, Panda, & Gandhi, 2012). When azurin is used as single or along with anticancer drugs such as 5-fluorouracil or etoposide, have a strong synergistic anticancer effect on oral cancer cells. Cho *et al.,* (2011) reported the action of azurin on YD-9 (p53positive) human oral squamous carcinoma cells showed decreased cell viability (J. H. Cho et al., 2011).

4.3.4.3 Prostate cancer

Prostate cancer begins when cells in the prostate gland start to grow uncontrollably. As we discussed before, azurin has structural similarity to a ligand known as ephrinB2, which binds its cognate receptor tyrosine kinase EphB2 to initiate cell signaling. When azurin

binds to the EphB2-Fc receptor with high affinity, inhibits signal pathway and leads to cell death.

Chaudhari *et al.,* (2007) reported the action of azurin on a prostate cancer cell line DU145, lacking functional EphB2, So azurin or its GST-fusion derivatives (p27) had a little cytotoxic effect (Chaudhari *et al.,* 2007). EphB2 that is hyper-produced at the surface of many cancer cells such as breast, prostate, lung, etc (A. M. Chakrabarty et al., 2014). Higher levels of cyclin B1 is present in prostate cancer cells may be a good analytical marker for chemotherapy. The increased expression of cyclin B1 leads to increased sensitivity to apoptosis because of the low level of Bcl-2 and high level of p53 (Gomez, de Las Pozas, Reiner, Burnstein, & Perez-Stable, 2007). Clinical trials with p28 in prostate cancer patients shows that p28 could consider as a good therapeutic drug for prostate cancer (A. M. Chakrabarty *et al.,* 2014).

4.3.4.4 Colon cancer

Colorectal cancer begins as a growth called a polyp on the inner lining of the colon or rectum. Certain polyps converted into cancers, but all polyps do not become cancers. Experimental studies of azurin action on colon cancer cell lines were scares. Action of azurin derived hexapeptide on colon cancer (HCT116) cell lines showed apoptosis, determined by fluorescence microscopy and MTT assays (Nishita & Manonmani, 2015). Another study on same colon cancer cell lines was done by Mohammed *et al.,* (2010), they used sulfur rhodamine B staining and cytotoxicity was measured. The study showed that the

proliferation of a colon carcinoma cell line was strongly inhibited with a doze dependent manner (Mohamed *et al.,* 2010).

The first-in-human, phase I clinical trial of p28 (NSC745104), was done in 4 colon cancer patients and act as a good anticancer drug in patients with $p53^{+}$ metastatic solid tumors (Warso et al., 2013). Ali *et al.,* (2017) also reported the antitumor activity of azurin on colon cancer with the replacement of metals in azurin structure (Ali *et al.,* 2017).

4.3.4.5 Leukemia

Azurin's activity studies against liquid cancers such as leukemia are less. Azurin enters into leukemia cell lines K562(chronic myelogenous leukemia) and HL60 (acute myelogenous leukemia) cells and showed a good cytotoxic effect on both K562 and HL60 cells, azurin causes cell cycle arrest in G2/M phase in K562 cell line(A. M. Chakrabarty et al., 2014; Kwan et al., 2009).

4.3.4.6 Ovarian cancer

Azurin showed significant cytotoxicity towards adeno carcinoma cell line SKOV3 (Naffouje, Yamada, & Gupta, 2017) and less cytotoxic to normal ovarian cell line HOSE6-3 cells (Kundu *et al.,* 2009). Azurin and p28 possess, both cancer therapeutic activity and cancer preventive activity.

4.3.4.7 Bone cancer

Osteosarcoma is a type of malignant bone cancer. It is most prevalent in adolescents and younger ones. Azurin action on two

different osteosarcoma cell lines such as U2OS and MG 63 were studied in a doze dependent manner. U2OS containing P53 and MG63 is P53 negative cell line (Yang *et al.,* 2005). One more studies also showed that azurin could induce apoptosis of human osteosarcoma U2OS cells with down-regulation of Bcl-2, up-regulation of Bax, and activation of Caspase-3 (Z. M. Ye *et al.,* 2005). On the contrary when U2OS cells treated with P53 si RNA, causes a lower apoptosis rate, lower caspase-3 activity, and up-regulation of Bcl-2, down-regulation of bax (Yan *et al.,* 2011).

Azurin showed considerable potential in the treatment of osteosarcoma. Azurin concentration directly influence the rate of apoptosis, but it is too expensive to obtain sufficiently large amounts of active, purified azurin protein. Hence, a plasmid containing azurin cDNA construct was made to transfect U2OS cells (Yang et al., 2005). U2OS cells showed a higher rate of apoptosis than they are treated directly with azurin protein. The induction of apoptosis by pcDNA3.1(+)/azurin is due to the down-regulation of Bcl-2 and the up-regulation of Bax, hence confirmed the increased expression of p53 (Z. Ye, Peng, Fang, Feng, & Yang, 2007).

4.3.4.8 Melanoma

Melanoma is usually cancer of the skin. So it is easier to detect in its early stages. Yamada *et al.,* (2002) described the action of azurin on melanoma cancer cell on both *in vitro* and *in vivo*. They firstly reported that azurin forms a complex with p53 and helps in cancer regression. They reported the action of azurin on UISO-Mel-2 and

UISO-Mel-6 cell lines. Azurin forms a complex with p53 and cause a increased level of synthesis of Bax and other proapoptotic proteins. *In vivo*, experiments were done on athymic mice, were treated with azurin for 22 days, tumor growth inhibition was observed with compared to untreated group. UISO-Mel-6 cells are p53 negative type, so there is a loss of cytotoxicity of azurin toward these cells (Yamada, Goto, Punj, Zaborina, Chen, *et al.,* 2002).

4.3.4.9 Brain tumor

Brain tumors are highly invasive and painful to treat. In clinical trials, p28 a small peptide from azurin showed significant efficacy in pediatric patients with brain tumors. Azurin does not break blood-brain barrier, but p28 can penetrate into glioblastoma, because it does not possess the inhibitory potential of full-length azurin (N. Mehta *et al.,* 2017).

4.3.4.10 Liver cell line

Mohammed *et al.,* (2010) studied the activity of azurin on the Liver cell line (HEPG2) and result showed fluctuating activity, but it shows its cytotoxicity efficacy (Mohamed *et al.,* 2010).

4.3.5 Current status of azurin anticancer research

CDG Therapeutics inc, an American biotechnology company, is developing a drug consist of synthetic p28 peptide for the treatment of p53 –positive cancer. Several patents have been issued on this matter and at the date p28 ended Phase I clinical trials (CDG,2011). Fialho *et al.,* (2008) reported that azurin is a protein with multiple domains and

working differently from the other dry compounds. It will also suppress tumor, researchers named the protein as 'Drug of the Future'(Arsenio M Fialho et al., 2008). In Mexico, plant biotechnologist Miguel Gomez was able to express azurin in tomatoes. The plant extract was found to have the anti-cancer activity and now they are trying to find out whether the plant extract can kill tumor cells in mice.

Roh *et al.,* (2014) studied azurin's ability to incorporate into plant leaves. In this study tobacco plants are transformed with the azurin gene harboring chloroplast expression vector and also examined the expression level of azurin on leaves. Younger leaves showed a high expression level of 5.7 % of total soluble protein in contrast to a low expression level of 0.72 % in fully mature leaves (Roh, Choi, Kwak, Seo, & Lee, 2014).

The Indo American scientist AM Chakrabarty proposed a new idea of delivery of anticancer drug to patients via genetically modified fruits and vegetables open a new way in cancer drug research. He made collaboration with scientists in New Delhi, Japan, Mexico and Portugal, and examined the prospects of genetically engineering plants so that they can manufacture the bacterial protein as part of their metabolism (A M Chakrabarty, September 29, 2015).

4.4 Materials and methods

Anticancer activity of the purified protein from the recombinant strain developed was studied by *in vitro* and *in vivo* methods.

4.4.1.Trypan blue exclusion assay (*In vitro* cytotoxicity assay)

In vitro cytotoxicity of the purified recombinant azurin and standard azurin were studied using trypan blue exclusion assay on Dalton's lymphoma ascites cells (Elia *et al.,* 1993; Sereena .M.C, 2018). The tumor cells were extracted from the peritoneal cavity of tumor-bearing mice and washed repeatedly with PBS. Viable cells (10^6 cells/100 µl) were added to tubes containing different concentrations (200 µg/ml,100 µg/ml,50 µg/ml,20 µg/ml and 10 µg/ml) of purified and standard azurin protein in PBS. After incubation for 3 h at 37 ^oC, 100 µl of trypan blue (1%) was mixed to this cell suspension and loaded on a hemocytometer. Then separately counted the number of dead cells (blue colour) and live cells (Elia *et al.,* 1993).

$$Cytotoxicity\,(\%) = \frac{No.of\ dead\ cells}{No.of\ living\ cells + No.of\ dead\ cells} x100$$

4.4.2 MTT assay

The human colorectal cancer cells (HCT-15), human breast cancer cells (MCF-7) and African green monkey kidney normal cells (VERO) were obtained from National Centre for Cell Science, Pune, India. The cells were cultured in a T25 flask (Nunc, Thermoscientific), in DMEM media (Gibco) supplemented with 10 % FBS at 37 ^oC in a humidified incubator with 5 % CO_2 concentration. Upon reaching 60-70 % confluence, the cells were washed with PBS and trypsinized. After detachment of cells, the trypsin was neutralized with 1ml of DMEM media and gently mixed with pipetting. The single cells thus

obtained were counted in a hemocytometer and adjusted to a final concentration of 1 lakh cells/ ml by diluting with complete DMEM medium. Further, the cells were plated in a 48 well plate by pipetting 0.5 ml/ well (50,000 cells) to each well and incubated at standard conditions for 24 h. The media was replaced with fresh media after 24 h and drugs (purified azurin and standard azurin) at different concentrations (0-50 µg/ml) were added. The cells were further incubated for 48 h in a CO_2 incubator. After incubation, the media was replaced with DMEM containing MTT (5mg/ ml) and incubated for another 4 h. In the end, the formazan crystals formed were dissolved in dimethyl sulfoxide and the absorbance was measured at 570 nm in a UV/ Vis spectrophotometer (Mosmann, 1983).

The percentage of cytotoxicity was calculated using the equation;

$$\%\ Cytotoxicity = \frac{\text{Absorbance of Control-Absorbance of Test}}{\text{Absorbance of Control}} x\,100$$

4.4.3 *In vivo* ascites antitumor model

This study was conducted at Amala Cancer Research Institute Kerala, India. Swiss albino mice of 70-day age and weight of about 20±2 g were used as experimental animals for this study. The mice were divided into three groups (six mice per group) and maintained in polyacrylic cages at a temperature of 25±2 °C, dark/light cycle, with feed and water (ad-libitum) and suitable humidity. The study was approved by the Institutional Animal Ethical Committee: (ACRC/IAEC/2017[5]) about good laboratory practice on animal

experimentation. DLA cells maintained *in vivo* in Swiss albino mice by intraperitoneal transplantation of 10^6 cells/mouse were used for the induction of lymphoma. The DLA cells extracted from the peritoneal cavity of the mice were washed with saline and given intraperitoneally to the experimental animals to develop ascitis tumor (Jose *et al.,* 2016). Purified azurin and cyclophosphamide were administrated intraperitoneally for ten consecutive days. Group A served as a control group; Group B mice induced with cyclophosphamide (2.5 mg/kg); Group C mice induced with azurin (1.25 mg/kg). The death pattern of the animals were noted and calculated the percentage of increase in lifespan (ILS) (S. Ramachandran & Mandal, 2011).

Calculation

$$\% \, ILS = \frac{(T - C)}{C} x \, 100$$

T : Mean survival of treated group.

C: Mean survival of control group.

4.4.4, Apoptosis study

4.4.4.1 Cell lines

Human colorectal cancer cell line – SW480 was procured from NCI, USA. The cells were maintained in RPMI1640/DMEM containing 10 % FBS and incubated at 37 ^oC at 5 % CO_2 in a humidified CO_2 incubator. The medium was replaced every four days.

Revival protocol:

Around 5ml of RPMI1640/DMEM containing 20 % serum was taken in sterile falcon tubes and each tube was labeled properly. The cells which are to be revived are taken out from -80 ºC or liquid nitrogen and kept on ice. The cells were thawed at 37 ºC and then they were transferred into the respective labeled tubes containing the medium. The culture tubes were then centrifuged at 1000 rpm for 5 min. Then the pellet was mixed with 1ml of 20 % culture media in each tube. The contents of the tube were then transferred onto sterile culture flasks which were labeled accordingly. Next, an appropriate amount of 20 % RPMI1640/DMEM was added into each of the flasks. Finally, the flasks were maintained in a humidified CO_2 incubator, after observing under a microscope.

4.4.4.2, Hoechst 33342 Staining method

Human colorectal cancer cell line (SW480) procured from NCI, USA and maintained as per standard protocol was used for cell cycle analysis by Hoechst staining method. The cells were maintained in RPMI1640/DMEM containing 10 % FBS and incubated at 37 ^{o}C at 5 % CO_2 in a humidified CO_2 incubator. The medium was replaced every 4 days. Colon carcinoma cells SW480 was treated with different concentration of azurin (0.05 µg/ml, 1.0 µg/ml and 5.0µg/ml) for 24 h. The cells were trypsinised and centrifuged at 1000 rpm for 5 min at 4 ^{o}C. The pellet was taken and re suspend in 10 % DMEM containing 10 µg/ml Hoechst 33342. Incubated the cells in the dark for 30 min in a CO_2 incubator at 37 ^{o}C. After centrifugation with same condition, the

pellet was resuspended in 500 µl of 10 % DMEM and filtered through a 40 µm cell strainer. After that, the cells were analysed using a flow cytometer equipped with a DAPI filter set. From the scatter plot, only the healthy cells (SSC-A Vs. FSC-A) after elimination of doublets (SSC-W Vs. SSC-A and FSC-W Vs. FSC-A) were used for analysis (Engin Ulukaya, 2011).

4.4.4.3, Fret-Based Caspase Sensor Probe assay

A fret-based technique is a quantitative real-time approach to imaging caspase activation to detect apoptosis (Lekshmi et al., 2017). The SW480 cells stably transfected with the ECFP-DEVD-EYFP expression vector (SCAT3 NLS) using Lipofectamine LTX plus reagent was used in this study. These cells express ECFP-DEVD-VENUS fusion protein. Caspases are cysteine proteases that play a major role in apoptosis; upon activation, caspases cleave multiple proteins leading to apoptosis. The fluorescent protein ECFP acts the FRET donor and mVenus acts as the acceptor linked by the tetrapeptide sequence DEVD. Effector caspases such as caspase 3, upon activation, recognize the tetrapeptide DEVD and cleaved it; resulting in the loss of FRET in caspase activated cells.

The cells were transferred on to 96 well plates, after attaining 50 % - 60 % confluence, cells were treated with three different concentrations of the purified azurin (0.05 µg/ml, 1.00 µg/ml and 2.0 µg/ml) and incubated overnight. After 24 h the cells were imaged using BD Pathway TM 435 Bioimager (BD Biosciences) using AttoVisionTM software (version 1.6/435; BD Biosciences). For

imaging of ECFP-DEVD-EYFP the pathway bioimager was configured with Ex: 438 ± 12 nm band-pass filter for ECFP, 458 LP dichroic and dual emission at 483 ± 15 and 542 ± 27 nm in ratio mode. The higher ratio of cells indicate the apoptotic cells and the cells with lower ratio are live cells. The percentage of apoptotic cells was calculated.

$$\% \text{ of apoptotic cells} = \frac{\text{Dead cells}}{\text{Total no of cells}} x\,100$$

4.4.5 Hemolytic assay

Many anticancer proteins might have a hemolytic effect on human erythrocytes. Hence, they may cause serious adverse effects like hemolytic anemia after their application. So, the drugs need to be evaluated for their potential hemolytic activity. 200 µl of blood was mixed with various concentrations (0-50 µg/ml) of purified azurin sample. It was incubated at 37 ^{o}C for 30 min and the absorbance of the mixture was measured at 450 nm. AAPH (2,2'-azobis (2-amidinopropane) hydrochloride) was used as a positive control, which is known to induce hemolysis.

4.5 Results

4.5.1, Trypan blue exclusion assay

Cytotoxicity test of standard azurin and purified azurin were carried out against DLA cell line at different concentrations using trypan blue exclusion assay. Results of different concentrations of

protein suspensions (200 µg, 100 µg, 50 µg, 20 µg and 10 µg per ml) are graphically represented in Figure: 4.1 and tabulated on Table 4.1.

Table 4.1:Cytotoxicity of standard and purified azurin on trypan blue exclusion assay.

Conc of protein (µg/ml)	% of cell death	
	Standard azurin	Purified azurin
200	100 %	100 %
100	100 %	98 %
50	100 %	80 %
20	65 %	60 %
10	32 %	30 %

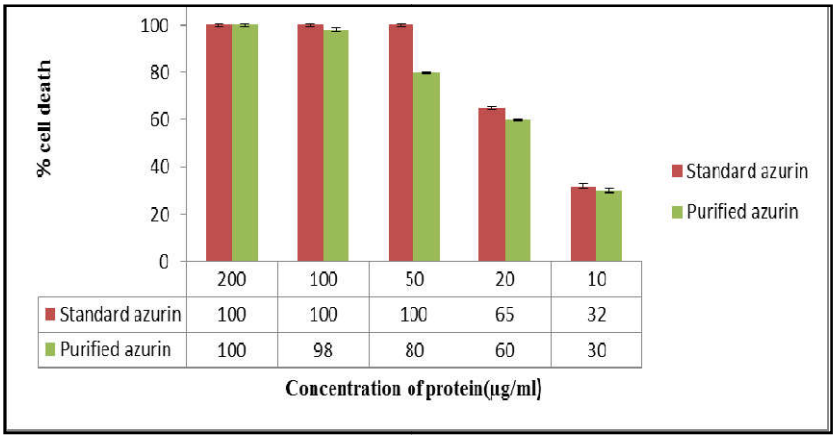


Figure 4.1: Trypan blue exclusion assay of purified and standard azurin.

4.5.2, MTT assay

The cytotoxicity of the purified azurin and standard azurin were measured by MTT cell viability assays, that were performed in two tumor cell lines MCF-7 and HCT15 in comparison with VERO cells. The cytotoxicity of purified azurin and standard azurin on various cell

lines with various concentrations were shown in (Figure 4.2). Graphical representation of the cytotoxicity of purified azurin and standard azurin on HCT15, MCF-7 and VERO cell lines were s shown in Figure 4.3 ,4.4 and 4.5 respectively and IC50 values tabulated on Table 4.2.

Table 4.2: Inhibitory concentration of purified and standard azurin on different cell lines.

Compound	**IC 50(µg/ml)**		
	HCT 15	MCF 7	VERO
Purified azurin	110.42	102.22	227.58
Standard azurin	97.07	70.93	120.69

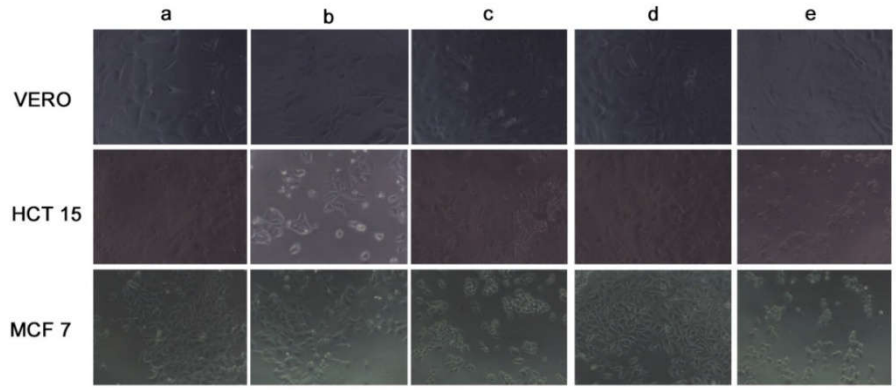
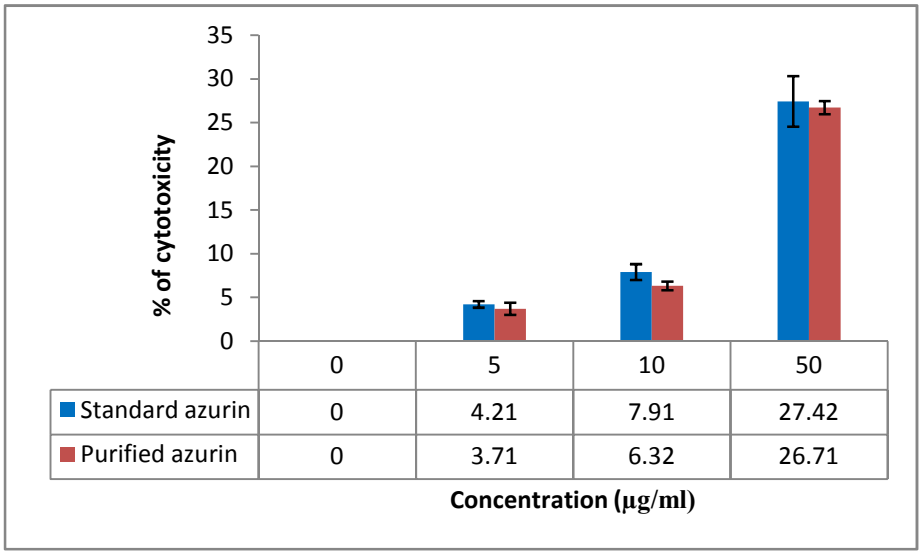
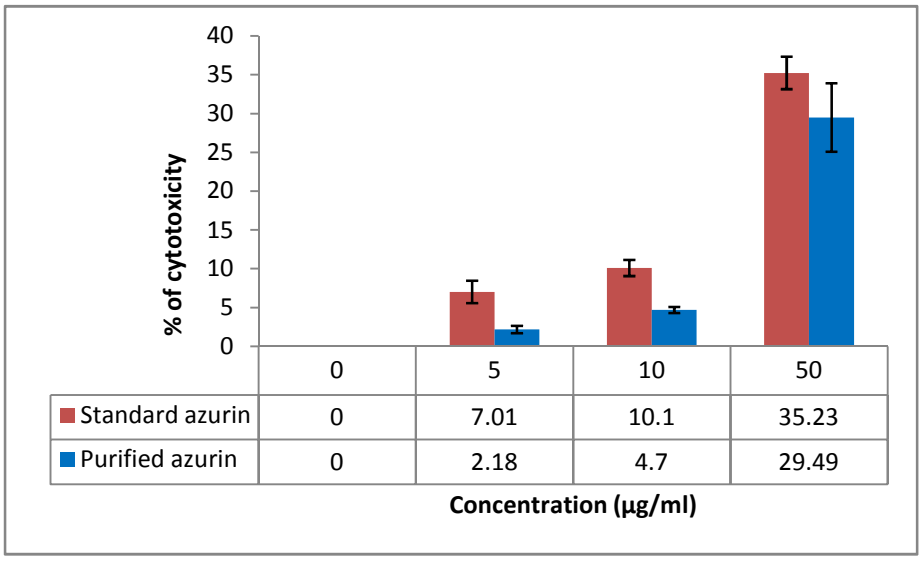


Figure 4. 2**:** MTT assay of purified azurin and standard azurin was done on various cell lines.**a,** control**; b,** standard azurin **(5** µg/ml); **c**, standard azurin (50 µg/ml); **d**, purified azurin (5 µg/ml); **e**, purified azurin (100 µg/ml).



	0	5	10	50
Standard azurin	0	4.21	7.91	27.42
Purified azurin	0	3.71	6.32	26.71

Figure 4.3: MTT assay of azurin on HCT 15 cell lines.



	0	5	10	50
Standard azurin	0	7.01	10.1	35.23
Purified azurin	0	2.18	4.7	29.49

Figure 4.4: MTT assay of azurin on MCF 7 cell lines.

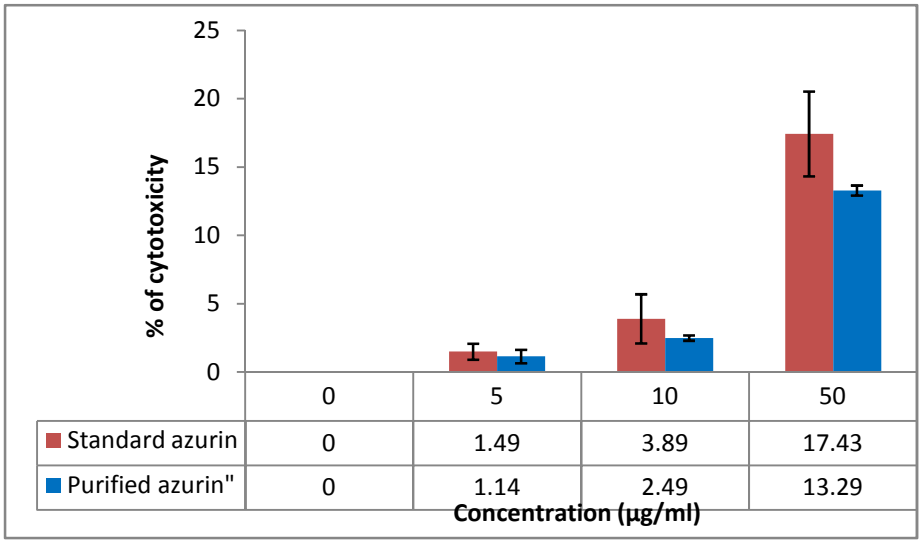


Figure 4.5: MTT assay of azurin on VERO cell lines.

4.5.3, *In vivo* ascites model

The ascites model of tumor study employed for studying the increase in life span of tumor-bearing animals for 50 days revealed that, the azurin treated animals showed an increase in lifespan when compared to control group (Table 4.3) and graphically represented in Figure 4. 6. Azurin treated mice shows 140.96 % increase in lifespan when compared with control.

Table 4.3: Azurin treated mice shows increased percentage of lifespan on *in vivo* ascites model

Group	Lifespan (days)	% Increase in lifespan
Control	18.5	
Cyclophosphamide (Standard)	28.6	54.59
Azurin (Treated)	44.5	140.54

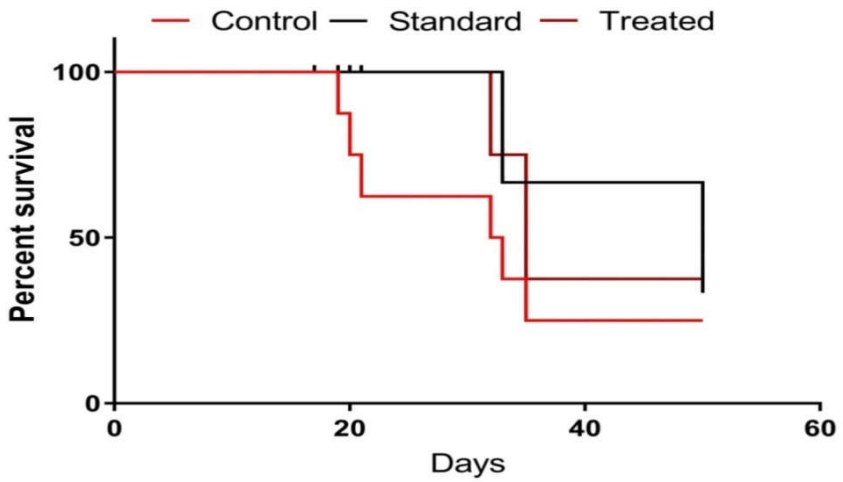


Figure 4. 6: Treated group shows Increase in lifespan on *in vivo* ascites model

4.5.4, Apoptosis assay

4.5.4.1 Hoechst staining method

Apoptosis analysis of azurin treated cells shows various changes in the cell cycle phases with compared to control. Cancer cell lines treated with 0.05 µg/ml, 1.0 µg/ml and 5.0 µg/ml of azurin showed a typical DNA pattern that represented sub-G0, G1, S, and M phases of the cell cycle as shown in Figure 4.7 and graphically represented on Figure 4.8. The relative percentage of apoptotic cells were shown in Table 4.4 and graphically represented in Figure 4.9.

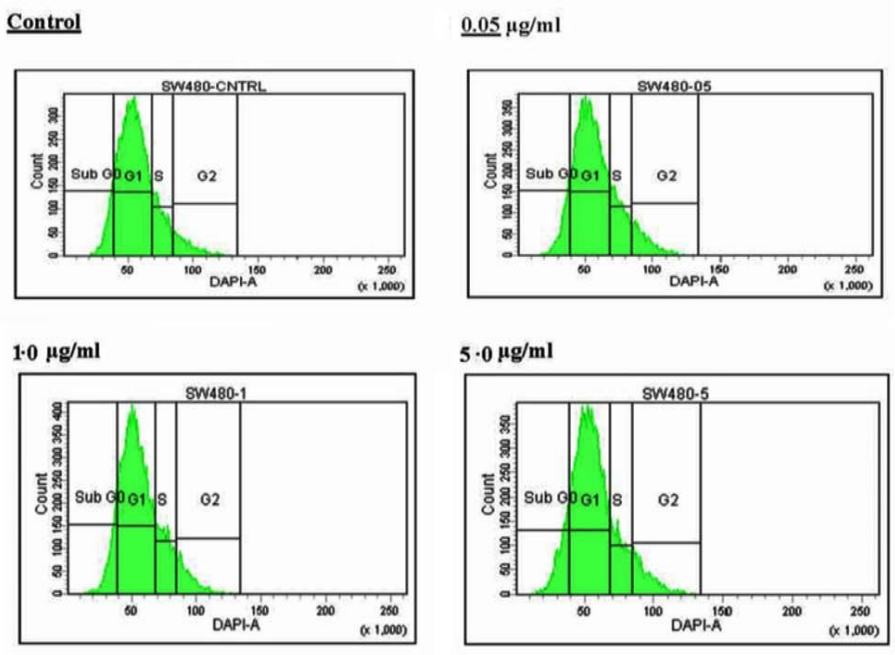
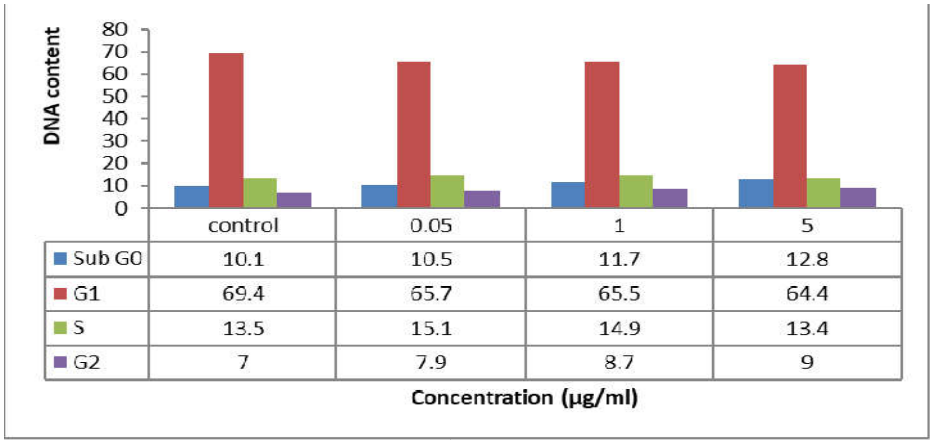


Figure 4.7 **:** Apoptosis assay of azurin against SW480 cancer cells. Colon carcinoma cells SW480 was treated with different concentration of azurin(0,0.05 µg/ml,1.0 µg/ml and 5.0 µg/ml) for 24 h and cell cycle analysis was performed using Hoechst staining method.



	control	0.05	1	5
Sub G0	10.1	10.5	11.7	12.8
G1	69.4	65.7	65.5	64.4
S	13.5	15.1	14.9	13.4
G2	7	7.9	8.7	9

Figure 4.8: Graphical representation of cancer cell lines treated with 0.05 µg/ml, 1.0 µg/ml and 5.0 µg/ml of azurin showed a typical DNA pattern that represented sub-G0, G1, S, and M phases of the cell cycle.

Table 4.4: Percentage of cells exhibiting apoptosis in SubG0 phase of cell cycle

Concentration (µg/ml)	% of cells exhibiting apoptosis(SubG0)
0	10.1
0.5	10.5
1.0	11.7
5.0	12.8

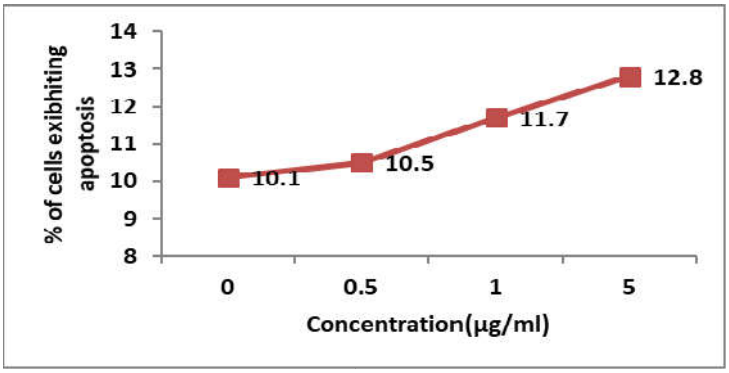


Figure 4.9: Graphical representation of apoptosis induced by azurin on SW480 cells by Hoechst staining method.

4.5.4.2, FRET based assay

In this study we used, a FRET probe (ECFP-DEVD-EYFP) contains a ECFP (donor) and EYFP (acceptor) which are linked together by activated caspase-3 and caspase-7 cleavable amino acid sequence "DEVD". When caspases are activated, the linker DEVD sequence gets cleaved and ECFP-EYFP bond cleaved. Separation of both fluorophores leads to the FRET loss between the probes. Loss of FRET during caspase activation will be reflected as a change in ratio of ECFP/EYFP in ratio imaging platform (Sobhan et al., 2013)(Figure 4.10 B). When azurin concentration increases from 0.05 µg/ml to 1.0

μg/ml and 2.0 μg/ml the number of apoptotic cells were also increased. There is a FRET loss occurred due to caspase activation and it reflected as increasing ratio of ECFP/EYFP with increasing concentration of azurin (Figure 4.10 A). The staining pattern indicated the cause of cell death in colon cancer cell lines was apoptosis than necrosis. The percentage of apoptotic cells also calculated and graphically represented (Figure 4.10 C).

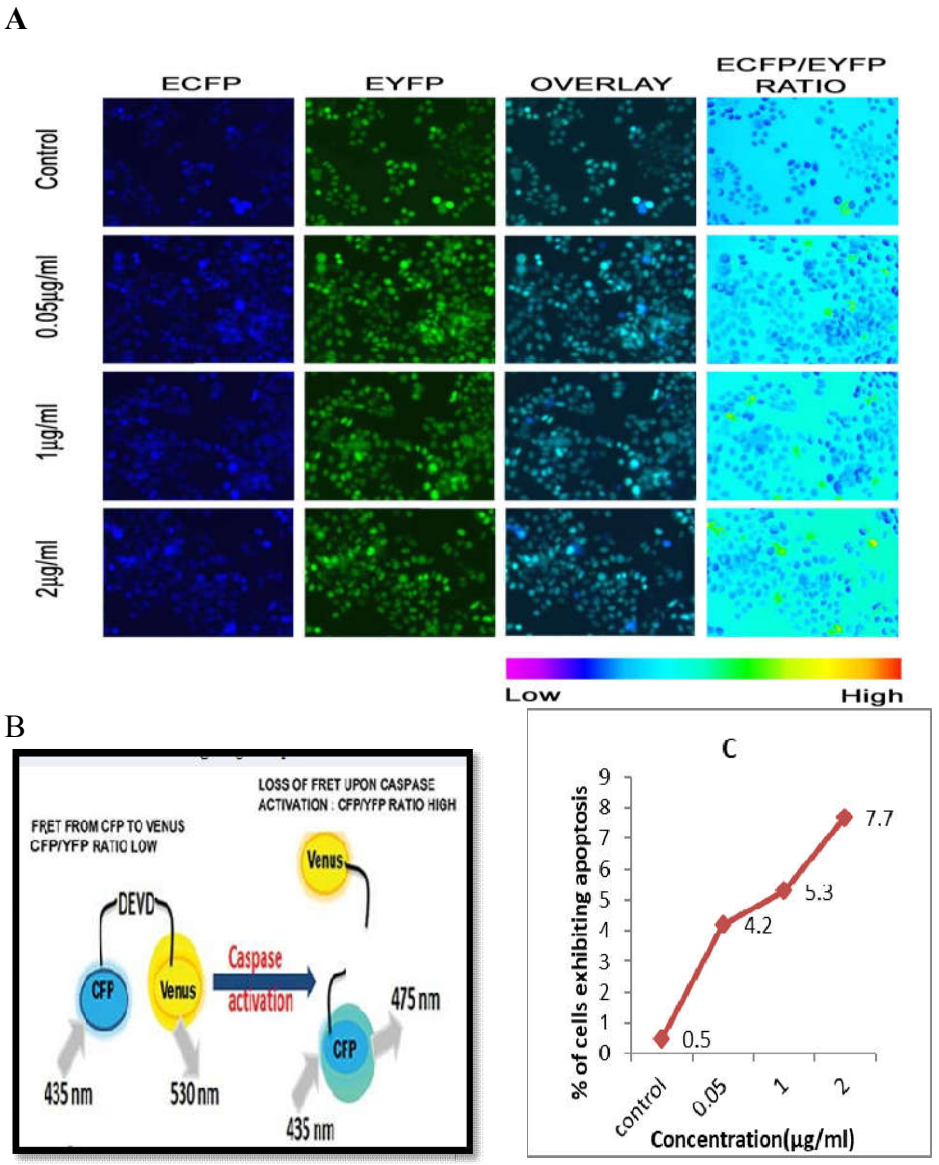


Figure 4.10:**A,** Colon cancer cells SW420 cells expressing ECFP-DEVD-EYFP probe ,was treated with azurin and image acquisition was done using BD pathway bioimager ; 4.10:**B,** Schematic representation of caspase sensor FRET probe (SCAT3-NLS) to detect caspase mediated apoptosis;4.10:**C,** SW480 cells expressing FRET probe were treated with azurin and ECFP/EYFP were calculated. The cells with FRET loss were calculated and represented as graph.

4.5.4, Hemolytic assay

Hemolytic activity of purified azurin protein was screened against normal human erythrocyte (Table 4.5) and it showed negligible hemolysis compared to control AAPH (B. N. Taylor *et al.,* 2009).

Table 4.5: Hemolytic assay of azurin on human erythrocyte.

	Absorbance		
Concentration (µg/ml)	I	II	Mean
0	0.089	0.097	0.093
5	0.081	0.077	0.079
10	0.088	0.056	0.072
20	0.105	0.094	0.0995
30	0.11	0.088	0.099
50	0.079	0.101	0.09
AAPH	0.824	0.851	0.8375

4.6 Discussion

Azurin is a novel molecule for cancer treatment and researchers termed it as a drug for the future (Yamada, Goto, Punj, Zaborina, Kimbara, *et al.,* 2002). The anticancer activity of azurin is primarily detected by Zaborina *et al.,* They observed that *Pseudomonas aeruginosa* produced a cuprous protein in the medium, that had cytotoxic activity against a macrophage cell line J774 (Zaborina *et al.,* 2000). Purified azurin sample and standard azurin were used for anticancer studies in this work. The purified azurin in this study showed a similar activity as that of standard azurin when tested with trypan blue exclusion assay on DLA cell lines. Both samples showed

100% cell death proving the efficacy of expressed azurin. In MTT assay, azurin had a moderate toxicity to HCT 15 and significant toxicity shown against MCF 7 cell lines. There was a significant decline in cell proliferation with an increase in the concentration of azurin as shown in Figure 4.3 and 4.4. Mervat *et al.,* (2010) studied the cytotoxic effect of a recombinant azurin on different cancer cell lines, such as on breast cancer cell, colon cancer cell line, hepatocellular carcinoma cell line and normal melanocytes were also studied and results showed strong inhibitory action on cancer cell except hepatocellular carcinoma cell lines (Mohamed et al., 2010). Ali *et al.,* (2017) reported about the replacement of metals in azurin used for treatment of breast cancer cell lines and colon cancer cell lines, and Ni- Az form has the most extensive anti-antitumor effect on breast- and colon cancer cell line (Ali et al., 2017). In a previous study, crude and purified azurin extracted from several clinical isolates of *Pseudomonas aeruginosa* were used to detect cytotoxicity in MCF 7 breast cancer cell lines and compared with a standard strain (Osman *et al.,* 2013).

In vivo studies showed increased lifespan of azurin treated mice group. In this study 140 % increase in lifespan is observed in azurin treated group. Ranachandran *et al.,* (2011) reported 94.19 % increase in life span of azurin treated mice (S. Ramachandran & Mandal, 2011).Yamada *et al.,* (2002) also demonstrated the cytotoxicity of azurin using both *in vivo* and *in vitro* methods (Yamada, Goto, Punj, Zaborina, Chen, *et al.,* 2002). Jia *et al.,* (2011) reported about the *in vivo* studies of p28 on male mices, showed good result without any

toxicity symptoms (Jia et al., 2011). The hemolytic assay showed azurin could be safely used as an anticancer drug with low or nonhemolytic effect. The *in vitro* and *in vivo* studies of azurin strongly suggest its potential applications in the treatment of human cancer. The unique potentiality of azurin in the inhibition of cancer cell proliferation would make it a very attractive drug for cancer chemotherapy.

Apoptotic effect of azurin was studied by, Hoechst 33342 staining on SW480 cells. Each stage of cell cycles was analysed for the detection of the DNA content at various stages of cell cycle with different concentrations of azurin (0.05 µg/ml, 1.0 µg/ml and 5.0 µg/ml). DNA content of azurin treated colon cancer cell lines were examined using flow cytometry, the result revealed an elevated cell population in G0/G1 phase. Azurin treated cells showed decreased DNA content in G1 phase and increased in Sub G0 phase which indicated azurin induces apoptosis in these cells. Ramachandran *et al.,* (2011) previously reported the apoptotic effect of azurin using cell cycle analysis of breast cancer cells (Sankar *et al.,* 2011).

Apoptotic potential of azurin was confirmed in colon cancer cell line SW480 with FRET based assay. After 24 h of treatment, SW480 cells showed increase in ECFP/EYFP ratio indicating that the probe is cleaved by activated caspases. Several workers reported the apoptotic effect of azurin on breast cancer cell line (Punj *et al.,* 2004), human osteosarcoma cell lines (Yang *et al.,* 2005) and human melanoma cell line (Yamada, Goto, Punj, Zaborina, Chen, *et al.,* 2002).

All these studies confirmed the efficacy of azurin as a drug to induce apoptosis in cancer cell lines. Cell cycle control and apoptosis are considered to be two major regulatory mechanisms for cell growth. In all these aspects azurin can be used as an ideal drug for future in cancer treatment.

4.7 Conclusions

In this study, we reported the anticancer activity of azurin from *Pseudomonas aeruginosa* SSj expressed on pET22b(+) vector using both *in vivo* and *in vitro* assays. Cell cycle analysis, apoptosis and hemolytic assays were used to confirm azurin's potential to be used as a good therapeutic agent in cancer therapy.

Chapter 5

Summary and conclusions

Azurin is considered as "Drug for future" because of its multi-targeted action to cancer regression pathway. Azurin is too expensive to obtain readily. Azurin's cytotoxicity is only for specific cancer cells and does not affect healthy cells and it has fewer toxic side effects. A second most vital feature of azurin is the tremendous number of therapeutic substance can link to azurin molecule, so it also acts as cargo protein. Azurin has antimalarial and anti-HIV activity, so it acts as potential therapeutic agents in the treatment of malaria and HIV-1 infections. The main aim of the study was to isolate an effective azurin producing bacterium, improve its efficiency by cloning and expression of *azu* gene and find its applicability in cancer treatment.

The major finding of the study can be summarized as follows:

- Among the 43 environmental samples isolated from different soil and water samples, ten isolates were selected and identified as *Pseudomonas aeruginosa* using morphological and biochemical characters. All of these strains were obtained from Malabar region.
- Cytotoxicity study of isolated strains were done by Trypan blue exclusion assay and found that eight strains (A, D, E, F, G, H, I and J) out of ten showed cytotoxicity towards DLA cell lines. Isolates B and C does not show any cytotoxicity towards DLA cell lines.
- Total DNA was extracted from these isolated strains by Unal method.

- PCR was done with specific *azu* gene primers to detect the presence of anticancer protein azurin gene in isolated strains. Similarly, out of ten strains eight strains showed the presence of azurin gene.
- Azurin protein was extracted from azurin gene harboring ten strains and partially purified by 70 % ammonium sulfate precipitation, dialysis and DEAE cellulose treatment.
- Protein profile was done by 12 % SDS page and a band with corresponding to 14 KDa was obtained in azurin gene harboring eight strains.
- The isolate which showed the highest cytotoxicity and good yield was taken for further studies.
- The best bacterial isolate was selected based on anticancer activity, was characterized and identified as *Pseudomonas aeruginosa* SSj on the basis of 16S rRNA (accession number KU821118) and partial *azu* gene (accession number KU821119) sequence analysis.
- A phylogenetic tree was constructed based on the 16S rRNA gene sequence of *Pseudomonas aeruginosa* SSj.
- The presence of azurin gene was detected in selected isolate and *Pseudomonas aeruginosa* MTCC 2453, taken as positive control for this study. Both showed a standard amplification with azurin specific primer and resulted in a 545 bp band was seen in agarose gel electrophoresis.

- Characterization of partially purified azurin protein was done. FTIR analysis of partially purified azurin from *Pseudomonas aeruginosa* SSj, *Pseudomonas aeruginosa* MTCC 2453 and standard azurin showed a peak around 1638 cm^{-1}region, 1637 cm^{-1} region and 1641 cm^{-1} region respectively.

- The molecular weight of partially purified azurin was analyzed by MALDI-TOF.

- Partially purified azurin was used to detect the apoptosis in MCF breast cancer cell lines by Hoechst staining method. More intense color nuclei with chromatin condensation appeared in azurin treated cells.

- In this work, we were successful in cloning the azurin gene from strain SSj in pET22b (+) vector and expressing it in *E.coli* BL21 (DE3).

- The amplicon of 1224 bp was sequenced and flanking regions were removed to get the *azu* sequence of 418bp (MK120121) BLASTn analysis of the *azu* gene sequence showed 99 % similarity to several strains of *Pseudomonas aeruginosa*.

- Purification of recombinant protein was done by Ni NTA agarose affinity chromatography.

- Purified azurin and standard azurin showed a peak around 1637 cm^{-1} and 1641cm^{-1} on FTIR analysis.

- Purified azurin profiled by MALDI TOF analysis and a peak which corresponding to 14 KDa was obtained.

- Purified azurin sample was analyzed using LC-MS/MS analysis and confirmed that the sample contains azurin. MS spectrum analysis of azurin showed four short peptide fragments of azurin. The protein sequence of these short peptides were compared with azurin sequence obtained in this study (MK120121).
- The successful over expression and purification of azurin was confirmed by the presence of a thick band corresponding to 14 KDa was produced on SDS page.
- Docking studies of azurin with selected receptors such as p53 (1HS5), Apaf 1 (1CY5), Cyt C (1CGN), Bax (1F16), Caspase 7 (1K86) and Caspase 9 (2AR9) were done. Azurin performed in a good way and all six receptors gave good docking score and binding energy.
- Purified azurin sample and standard azurin were used for anticancer studies. Primarily the anticancer activity was determined by trypan blue exclusion assay and showed good result.
- The cytotoxicity was confirmed by MTT assay on two cancer cell lines such as Breast carcinoma cell line (MCF 7), Colon carcinoma cell line (HCT 15) and one normal African green monkey kidney cell line (VERO).
- Cytotoxicity evaluation of the purified azurin and standard azurin against the HCT-15 line were IC 50=110.424 μg/ml and IC 50=97.07 μg/ml respectively.

- Results of the cytotoxicity evaluation against MCF 7 line of the purified azurin and standard azurin were IC 50=102.2271µg/ml and IC 50=70.93 µg/ml.

- Results of the cytotoxicity evaluation against VERO cell line of the purified azurin and standard azurin were IC 50=227.58 µg/ml and IC 50=120.69 µg/ml.

- *In vivo* antitumor assay also done by ascites antitumor model, by induction of Dalton's lymphoma cells in Swiss albino mice and azurin treated mice shows 140 % increased lifespan than controlled group.

- Hemolytic activity of azurin was done against human normal erythrocyte and azurin showed non hemolytic activity to compared with positive control AAPH.

- Apoptosis assay of azurin was done on colon cancer cells SW480 using Hoechst staining with varying concentration of azurin (0.05 µg/ml, 1.0 µg/ml and 5.0 µg/ml). Azurin treated cells showed decreased DNA content in G1 phase which indicates azurin induces apoptosis in these cells.

- Apoptosis study of azurin was confirmed by Using Fret-Based Caspase Sensor Probe**.** When azurin concentration increases from 0.05 µg/ml, 1.0 µg/ml and 2.0 µg/ml the number of apoptotic cells were also increased. The staining pattern indicated the cause of cell death in colon cancer cell lines was apoptosis than necrosis.

Conclusions

The unique features of azurin in cancer regression has prompted the researchers to look for effective azurin producers from unexplored environments. In this study we were successful in isolating azurin producing strains from environmental sources of Malabar region, indicating that azurin gene harboring isolates were present in the natural environment of India. Although, partially purified azurin from strain SSj was characterized and compared with standard strain. For biomedical applications recombinant proteins are preferred, thus the azurin gene was cloned and over-expressed in *E.coli* BL21. The anticancer activity of purified azurin was done on different cancer cell lines by comparison with standard azurin using MTT assay. Apoptotic effect of recombinant azurin was done by Hoechst staining and confirmed by FRET based assay. All these studies confirm the efficacy of azurin as a drug to induce apoptosis in cancer cell lines. The hemolytic assay shows azurin could be safely used as an anticancer drug with low or nonhemolytic effect. Finally we concluded that azurin from *Pseudomonas aeruginosa* SSj expressed on pET22b(+) vector has anticancer potentialities and could be used as a ideal drug for cancer treatment.

Bibliography

Adman, E. T. (1991). Copper protein structures. *Adv Protein Chem, 42*, 145-197.

Adman, E. T., Canters, G. W., Hill, H. A., & Kitchen, N. A. (1982). The effect of pH and temperature on the structure of the active site of azurin from Pseudomonas aeruginosa. *FEBS Lett, 143*(2), 287-292.

Adman, E. T., Stenkamp, R. E., Sieker, L. C., & Jensen, L. H. (1978). A crystallographic model for azurin a 3 A resolution. *J Mol Biol, 123*(1), 35-47.

Akhter, M. (2016). *Cloning and Expression of Azurin: A Protein Having Antitumor and Cell Penetrating Ability*. Paper presented at the ICBSO 2016 : 18th International Conference on Biomedical Sciences and Oncology World Academy of Science, Engineering and Technology International Journal of Medical and Health Sciences London, United Kingdom

Ali, M. A., Mohamed, M. S., & Sedek, R. G. (2017). Can metal replacement increase the antitumor activity of azurin?(in vitro study). *Gen Med Open, 1*(2), 1-7. doi: 10.15761/GMO.1000111

Ambler, R. P., & Brown, L. H. (1967). The amino acid sequence of Pseudomonas fluorescens azurin. *Biochem J, 104*(3), 784-825.

Ana Teresa Estevens. (2011). *Expression, purification and characterization of azurin derived peptides as target sequences for P-cadherin overexpressing breast cancer cells* Retrieved from https://www.omicsonline.org/open-access/an-overview-of-the-parameters-for-recombinant-protein-expression-in-escherichia-coli-2157-7013-1000221.php?aid=61257

Apiyo, D., & Wittung-Stafshede, P. (2005). Unique complex between bacterial azurin and tumor-suppressor protein p53. *Biochem Biophys Res Commun, 332*(4), 965-968. doi: 10.1016/j.bbrc.2005.05.038

Baba, A. I. C., Cornel. (2007). Tumor Cell Morphology. In A. I. B. a. C. Câtoi (Ed.), Comparative Oncology. Bucharest: The Publishing House of the Romanian Academy.

Baneyx, F. (1999). Recombinant protein expression in Escherichia coli. *Curr Opin Biotechnol, 10*(5), 411-421.

Barber, M. J., Trimboli, A. J., & McIntire, W. S. (1993). The amino acid sequence of Pseudomonas putida azurin. *Arch Biochem Biophys, 303*(1), 22-26. doi: 10.1006/abbi.1993.1250

Bernardes, N., Chakrabarty, A. M., & Fialho, A. M. (2013). Engineering of bacterial strains and their products for cancer therapy. *Appl Microbiol Biotechnol, 97*(12), 5189-5199. doi: 10.1007/s00253-013-4926-6

Bernardes, N., Ribeiro, A. S., Abreu, S., Mota, B., Matos, R. G., Arraiano, C. M., . . . Fialho, A. M. (2013). The bacterial protein azurin impairs invasion and FAK/Src signaling in P-cadherin-overexpressing breast cancer cell models. *PLoS One, 8*(7), e69023. doi: 10.1371/journal.pone.0069023

Bernardes, N., Seruca, R., Chakrabarty, A. M., & Fialho, A. M. (2010). Microbial-based therapy of cancer: current progress and future prospects. *Bioeng Bugs, 1*(3), 178-190. doi: 10.4161/bbug.1.3.10903

Birbrair, A., Zhang, T., Wang, Z. M., Messi, M. L., Mintz, A., & Delbono, O. (2015). Pericytes at the intersection between tissue regeneration and pathology. *Clin Sci (Lond), 128*(2), 81-93. doi: 10.1042/CS20140278

Blagosklonny, M. V. (2005). Carcinogenesis, cancer therapy and chemoprevention. *Cell Death Differ, 12*(6), 592-602. doi: 10.1038/sj.cdd.4401610

Bonander, N., Leckner, J., Guo, H., Karlsson, B. G., & Sjolin, L. (2000). Crystal structure of the disulfide bond-deficient azurin mutant C3A/C26A: how important is the S-S bond for folding and stability? *Eur J Biochem, 267*(14), 4511-4519.

Bossi, G., & Sacchi, A. (2007). Restoration of wild-type p53 function in human cancer: relevance for tumor therapy. *Head Neck, 29*(3), 272-284. doi: 10.1002/hed.20529

Canters, G. W. (1987). The azurin gene from Pseudomonas aeruginosa codes for a pre-protein with a signal peptide. Cloning and sequencing of the azurin gene. *FEBS Lett, 212*(1), 168-172.

Chakrabarty, A. M. (2003). Microorganisms and cancer: quest for a therapy. *J Bacteriol, 185*(9), 2683-2686.

Chakrabarty, A. M. (2016). Bacterial azurin in potential cancer therapy. *Cell Cycle, 15*(13), 1665-1666. doi: 10.1080/15384101.2016.1179034

Chakrabarty, A. M. (September 29, 2015). GM plants could be next anti-cancer drugs: Indian-American scientist *India west Best Indian news paper:IANS*.

Chakrabarty, A. M., Bernardes, N., & Fialho, A. M. (2014). Bacterial proteins and peptides in cancer therapy: today and tomorrow. *Bioengineered, 5*(4), 234-242. doi: 10.4161/bioe.29266

Chang, J. J., Ho, C. Y., Ho, F. J., Tsai, T. Y., Ke, H. M., Wang, C. H., . . . Li, W. H. (2012). PGASO: A synthetic biology tool for engineering a cellulolytic yeast. *Biotechnol Biofuels, 5*(1), 53. doi: 10.1186/1754-6834-5-53

Chang, T. K., Iverson, S. A., Rodrigues, C. G., Kiser, C. N., Lew, A. Y., Germanas, J. P., & Richards, J. H. (1991). Gene synthesis, expression, and mutagenesis of the blue copper proteins azurin and plastocyanin. *Proc Natl Acad Sci U S A, 88*(4), 1325-1329.

Chaudhari, A., Fialho, A. M., Ratner, D., Gupta, P., Hong, C. S., Kahali, S., . . . Chakrabarty, A. M. (2006). Azurin, Plasmodium falciparum malaria and HIV/AIDS: inhibition of parasitic and viral growth by Azurin. *Cell Cycle, 5*(15), 1642-1648. doi: 10.4161/cc.5.15.2992

Chaudhari, A., Mahfouz, M., Fialho, A. M., Yamada, T., Granja, A. T., Zhu, Y., . . . Chakrabarty, A. M. (2007). Cupredoxin-cancer interrelationship: azurin binding with EphB2, interference in EphB2 tyrosine phosphorylation, and inhibition of cancer growth. *Biochemistry, 46*(7), 1799-1810. doi: 10.1021/bi061661x

Cheesbrough, M. (2005). *District Laboratory Practice in Tropical Countries. Cambridge* Cambridge University Press.

Cheyne, W. W. (1886). *Recent essays by various authors on bacteria in relation to disease* (Vol. 115): New Sydenham Society.

Cho, J. H., Lee, M. H., Cho, Y. J., Park, B. S., Kim, S., & Kim, G. C. (2011). The bacterial protein azurin enhances sensitivity of oral squamous carcinoma cells to anticancer drugs. *Yonsei Med J, 52*(5), 773-778. doi: 10.3349/ymj.2011.52.5.773

Cho, Y., Gorina, S., Jeffrey, P. D., & Pavletich, N. P. (1994). Crystal structure of a p53 tumor suppressor-DNA complex: understanding tumorigenic mutations. *Science, 265*(5170), 346-355.

Clarke, S. K. (1953). A simplified plate method for detecting gelatine-liquefying bacteria. *J Clin Pathol, 6*(3), 246-248.

Coley, W. B. (1898). The treatment of inoperable sarcoma with the 'mixed toxins of erysipelas and bacillus prodigiosus.: Immediate and final results in one hundred and forty cases. *Journal of the American Medical Association, XXXI*(9), 456-465. doi: 10.1001/jama.1898.92450090022001g

Coley, W. B. (1910). The Treatment of Inoperable Sarcoma by Bacterial Toxins (the Mixed Toxins of the Streptococcus erysipelas and the Bacillus prodigiosus). *PrOR Soc Med, 3*(Surg Sect), 1-48.

Collins, C. H., Lyne, P. M., & Grange, J. M. (1995). *Collins and Lyne's Microbiological Methods, 7Ed*: Taylor & Francis.

Cowan, S. T. (1953). Micromethod for the methyl red test. *J Gen Microbiol, 9*(1), 101-109. doi: 10.1099/00221287-9-1-101

da Rocha, A. B., Lopes, R. M., & Schwartsmann, G. (2001). Natural products in anticancer therapy. *Curr Opin Pharmacol, 1*(4), 364-369.

De Grandis, V., Bizzarri, A. R., & Cannistraro, S. (2007). Docking study and free energy simulation of the complex between p53 DNA-binding domain and azurin. *J Mol Recognit, 20*(4), 215-226. doi: 10.1002/jmr.840

De Rienzo, F., Gabdoulline, R. R., Menziani, M. C., & Wade, R. C. (2000). Blue copper proteins: a comparative analysis of their molecular interaction properties. *Protein Sci, 9*(8), 1439-1454. doi: 10.1110/ps.9.8.1439

Deeudom, M., Huston, W., & Moir, J. W. (2015). Lipid-modified azurin of Neisseria meningitidis is a copper protein localized on the outer membrane surface and not regulated by FNR. *Antonie Van Leeuwenhoek, 107*(4), 1107-1116. doi: 10.1007/s10482-015-0400-z

Dennison, C. (2008). The role of ligand-containing loops at copper sites in proteins. *Nat Prod Rep, 25*(1), 15-24. doi: 10.1039/b707987g

Dinarieva, T. Y., Trashin, S. A., Kahnt, J., Karyakin, A. A., & Netrusov, A. I. (2012). Purification and characterization of azurin from the methylamine-utilizing obligate methylotroph Methylobacillus flagellatus KT. *Can J Microbiol, 58*(4), 516-522. doi: 10.1139/w2012-020

Doyle, J. J., & Doyle, J. L. (1987). A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochemical Bulletin, 19*, 11-15. doi: citeulike-article-id:678648

Easty, D. J., Herlyn, M., & Bennett, D. C. (1995). Abnormal protein tyrosine kinase gene expression during melanoma progression and metastasis. *Int J Cancer, 60*(1), 129-136.

Elahian, F., Moghimi, B., Dinmohammadi, F., Ghamghami, M., Hamidi, M., & Mirzaei, S. A. (2013). The anticancer agent prodigiosin is not a multidrug resistance protein substrate. *DNA Cell Biol, 32*(3), 90-97. doi: 10.1089/dna.2012.1902

Elia, M. C., Storer, R. D., Harmon, L. S., Kraynak, A. R., McKelvey, T. W., Hertzog, P. R., . . . Nichols, W. W. (1993). Cytotoxicity as measured by trypan blue as a potentially confounding variable in the in vitro alkaline elution/rat hepatocyte assay. *Mutat Res, 291*(3), 193-205.

Elliott, A., & Ambrose, E. J. (1950). Structure of synthetic polypeptides. *Nature, 165*(4206), 921-922.

Engin Ulukaya, C. A., Ferda Ari, Elif İkitimur, Yusuf Yilmaz. (2011). A Glance at the methods for detection of apoptosis qualitatively and quantitatively. *Turkish Journal of Biochemistry–Turk J Biochem], 36* (3), 261–269. Eph Nomenclature, C. (1997). Unified Nomenclature for Eph Family Receptors and Their Ligands, the Ephrins. *Cell, 90*(3), 403-404. doi: https://doi.org/10.1016/S0092-8674(00)80500-0

Farmer, J. J., 3rd, Fanning, G. R., Huntley-Carter, G. P., Holmes, B., Hickman, F. W., Richard, C., & Brenner, D. J. (1981). Kluyvera, a new (redefined) genus in the family Enterobacteriaceae: identification of Kluyvera ascorbata sp. nov. and Kluyvera cryocrescens sp. nov. in clinical specimens. *J Clin Microbiol, 13*(5), 919-933.

Felgner, S., Kocijancic, D., Frahm, M., & Weiss, S. (2016). Bacteria in Cancer Therapy: Renaissance of an Old Concept. *Int J Microbiol, 2016*, 8451728. doi: 10.1155/2016/8451728

Felsenstein, J. (1985). Confidence Limits on Phylogenies: An Approach Using the Bootstrap. *Evolution, 39*(4), 783-791. doi: 10.1111/j.1558-5646.1985.tb00420.x

Fialho, A. M., & Chakrabarty, A. M. (2010). Promiscuous anticancer drugs from pathogenic bacteria: rational versus intelligent drug design. *Emerging Cancer Therapy: Microbial Approaches and Biotechnological Tools, 2*, 181.

Fialho, A. M., Das Gupta, T., & Chakrabarty, A. M. (2008). Promiscuous drugs from pathogenic bacteria in the post-antibiotics era. In S. Roy & H. Colin (Eds.), *Patho-Biotechnology. Landes Bioscience* (pp. 145-162). Austin, Texas, USA: Landes Bioscience.

Fialho, A. M., Stevens, F. J., Das Gupta, T. K., & Chakrabarty, A. M. (2007). Beyond host-pathogen interactions: microbial defense strategy in the host environment. *Curr Opin Biotechnol, 18*(3), 279-286. doi: 10.1016/j.copbio.2007.04.001

Forbes, B. A., Sahm, D. F., Weissfeld, A. S., & Baron, E. J. (1998). Bailey & Scott's diagnostic microbiology *Bailey & Scott's diagnostic microbiology* (10th ed., pp. 60-63). St. Louis: Mosby.

Fuentes, L., Oyola, J., Fernandez, M., & Quinones, E. (2004). Conformational changes in azurin from Pseudomona aeruginosa induced through chemical and physical protocols. *Biophys J, 87*(3), 1873-1880. doi: 10.1529/ biophysj.104.042580

Fukuo, T., Kubota, N., Kataoka, K., Nakai, M., Suzuki, S., & Arakawa, R. (1998). Matrix-assisted laser desorption/ionization and

electrospray ionization mass spectrometry analysis of blue copper proteins. Azurin and mavicyanin. *Rapid Commun Mass Spectrom, 12*(23), 1967-1971. doi: 10.1002/(SICI)1097-0231(19981215)12:23<1967::AID-RCM422>3.0.CO;2-1

G.A., M., P., M., & S., J. (2007). Nanotechnology in Cancer prevention defection and treatment bright future lies ached. *World review of science technology and sustainable Development, 4*, 226 – 257. doi: https://doi.org/10.1504/WRSTSD.2007.013584

Gallup, D. G., & Talledo, O. E. (1987). Benign and malignant tumors. *Clin Obstet Gynecol, 30*(3), 662-670.

Garrison-Morton. (1937). The Papyrus Ebers : the greatest Egyptian medical document. In B. Ebbell (Ed.). Copenhagen :: Levin & Munksgaard ;.

Gerhardt, P. (1981). *Manual of methods for general bacteriology: by Philipp Gerhardt, editor-in-chief ; R.G.E. Murray, editor, I. Morphology ... [et al.]*: American Society for Microbiology.

Gessard, C. (1984). Classics in infectious diseases. On the blue and green coloration that appears on bandages. *Rev Infect Dis, 6 Suppl 3*, S775-776.

Gilardi, G., Mei, G., Rosato, N., Canters, G. W., & Finazzi-Agro, A. (1994). Unique environment of Trp48 in Pseudomonas aeruginosa azurin as probed by site-directed mutagenesis and dynamic fluorescence spectroscopy. *Biochemistry, 33*(6), 1425-1432.

Gomez, L. A., de Las Pozas, A., Reiner, T., Burnstein, K., & Perez-Stable, C. (2007). Increased expression of cyclin B1 sensitizes

prostate cancer cells to apoptosis induced by chemotherapy. *Mol Cancer Ther, 6*(5), 1534-1543. doi: 10.1158/1535-7163.MCT-06-0727

Gopal, G. J., & Kumar, A. (2013). Strategies for the production of recombinant protein in Escherichia coli. *Protein J, 32*(6), 419-425. doi: 10.1007/s10930-013-9502-5

Gorman, G. S., Coward, L. U., Freeman, L., Noker, P. E., Beattie, C. W., & Jia, L. (2010). A novel and rapid LC/MS/MS assay for bioanalysis of Azurin p28 in serum and its pharmacokinetics in mice. *J Pharm Biomed Anal, 53*(4), 991-996. doi: 10.1016/j.jpba.2010.06.006

Hanahan, D., & Weinberg, R. A. (2000). The hallmarks of cancer. *Cell, 100*(1), 57-70.

Hashimoto, W., Ochiai, A., Hong, C. S., Murata, K., & Chakrabarty, A. M. (2015). Structural studies on Laz, a promiscuous anticancer Neisserial protein. *Bioengineered, 6*(3), 141-148. doi: 10.1080/21655979.2015.1022303

Hay, M., Richards, J. H., & Lu, Y. (1996). Construction and characterization of an azurin analog for the purple copper site in cytochrome c oxidase. *Proc Natl Acad Sci U S A, 93*(1), 461-464.

Haynes, W. C. (1951). Pseudomonas aeruginosa--its characterization and identification. *J Gen Microbiol, 5*(5 Suppl.), 939-950. doi: 10.1099/00221287-5-5-939

Hellberg, C., Ostman, A., & Heldin, C. H. (2010). PDGF and vessel maturation. s*Recent Results Cancer Res, 180*, 103-114. doi: 10.1007/978-3-540-78281-0_7

Hoption Cann, S. A., van Netten, J. P., & van Netten, C. (2003). Dr William Coley and tumour regression: a place in history or in the future. *Postgrad Med J, 79*(938), 672-680.

Horio, T. (1958). Terminal oxidation system in bacteriaii. some physical and physiological properties of purified cytochromes of pseudomonas aeruginosa*. *The Journal of Biochemistry, 45*(4), 267-279. doi: 10.1093/oxfordjournals.jbchem.a126865

Inoue, T., Nishio, N., Kanamoto, K., Suzuki, S., Yamaguchi, K., Kataoka, K., . . . Kai, Y. (1999). Crystallization and preliminary X-ray study of iso-2 azurin from the methylotrophic bacterium, Methylomonas J. *Acta Crystallogr D Biol Crystallogr, 55*(Pt 1), 307-309. doi: 10.1107/S0907444998010245

Institute, N. C. (2018, February 26, 2018). Common Cancer Types. Retrieved November 27, 2018, from https://www.cancer.gov/types/common-cancers

Isenberg, H. D., & Sundheim, L. H. (1958). Indole reactions in bacteria. *J Bacteriol, 75*(6), 682-690.

Jain, R. K., & Forbes, N. S. (2001). Can engineered bacteria help control cancer? *Proc Natl Acad Sci U S A, 98*(26), 14748-14750. doi: 10.1073/pnas.261606598

Jia, L., Gorman, G. S., Coward, L. U., Noker, P. E., McCormick, D., Horn, T. L., . . . Beattie, C. W. (2011). Preclinical pharmacokinetics, metabolism, and toxicity of azurin-p28

(NSC745104) a peptide inhibitor of p53 ubiquitination. *Cancer Chemother Pharmacol, 68*(2), 513-524. doi: 10.1007/s00280-010-1518-3

Jose, J., Dhanya, A. T., Haridas, K. R., Sumesh Kumar, T. M., Jayaraman, S., Variyar, E. J., & Sudhakaran, S. (2016). Structural characterization of a novel derivative of myricetin from Mimosa pudica as an anti-proliferative agent for the treatment of cancer. *Biomed Pharmacother, 84*, 1067-1077. doi: 10.1016/j.biopha.2016.10.020

Joseph, Anthony, & Bateson. (1994). *The Expression of the Gene for Azurin fromAlcaligenes Denitrificans in E. coli* (MSc), Massey University.

Joseph, B. C., Pichaimuthu, S., & Srimeenakshi, S. (2015). An Overview of the Parameters for Recombinant Protein Expression in Escherichia coli. *Journal of Cell Science & Therapy, 06*(5), 221. doi: 10.4172/2157-7013.1000221

Kakutani, T., Watanabe, H., Arima, K., & Beppu, T. (1981). A blue protein as an inactivating factor for nitrite reductase from Alcaligenes faecalis strain S-6. *J Biochem, 89*(2), 463-472.

Karlsson, B. G., Pascher, T., Nordling, M., Arvidsson, R. H., & Lundberg, L. G. (1989). Expression of the blue copper protein azurin from Pseudomonas aeruginosa in Escherichia coli. *FEBS Lett, 246*(1-2), 211-217.

Karpiński, T. M., & Szkaradkiewicz, A. K. (2013). Anticancer peptides from bacteria. *Bangladesh Journal of Pharmacology, 8*(3). doi: 10.3329/bjp.v8i3.15704

Kianoosh, K., G, A. M., & Mehdi, R. (2010). Prospects for Cancer Nanotechnology Treatment by Azurin. *Dynamic Biochemistry, Process Biotechnology and Molecular Biology, 4* ((1)), 48-66

Kim, S. J., Chowdhury, F. N., Stryjewski, W., Younathan, E. S., Russo, P. S., & Barkley, M. D. (1993). Time-resolved fluorescence of the single tryptophan of Bacillus stearothermophilus phosphofructokinase. *Biophys J, 65*(1), 215-226. doi: 10.1016/S0006-3495(93)81070-1

Kimura, M. (1980). A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J Mol Evol, 16*(2), 111-120.

King, E. O., Ward, M. K., & Raney, D. E. (1954). Two simple media for the demonstration of pyocyanin and fluorescin. *J Lab Clin Med, 44*(2), 301-307.

King, R. W., Jackson, P. K., & Kirschner, M. W. (1994). Mitosis in transition. *Cell, 79*(4), 563-571.

Kirkham, M., & Parton, R. G. (2005). Clathrin-independent endocytosis: new insights into caveolae and non-caveolar lipid raft carriers. *Biochim Biophys Acta, 1745*(3), 273-286. doi: 10.1016/j.bbamcr.2005.06.002

Kirn, D. H. (2000). Replication-selective microbiological agents: fighting cancer with targeted germ warfare. *J Clin Invest, 105*(7), 837-839. doi: 10.1172/JCI9761

Kiyokawa, E., Takai, S., Tanaka, M., Iwase, T., Suzuki, M., Xiang, Y. Y., . . . Kino, I. (1994). Overexpression of ERK, an EPH family

receptor protein tyrosine kinase, in various human tumors. *Cancer Res, 54*(14), 3645-3650.

Klein, C., Planker, E., Diercks, T., Kessler, H., Kunkele, K. P., Lang, K., . . . Schwaiger, M. (2001). NMR spectroscopy reveals the solution dimerization interface of p53 core domains bound to their consensus DNA. *J Biol Chem, 276*(52), 49020-49027. doi: 10.1074/jbc.M107516200

Klein, C., & Vassilev, L. T. (2004). Targeting the p53-MDM2 interaction to treat cancer. *Br J Cancer, 91*(8), 1415-1419. doi: 10.1038/sj.bjc.6602164

Klein, C. A. (2008). Cancer. The metastasis cascade. *Science, 321*(5897), 1785-1787. doi: 10.1126/science.1164853

Koser, S. A. (1923). Utilization of the Salts of Organic Acids by the Colon-Aerogenes Group. *J Bacteriol, 8*(5), 493-520.

Kuijper, S., Turner, C. J., & Adams, R. H. (2007). Regulation of angiogenesis by Eph-ephrin interactions. *Trends Cardiovasc Med, 17*(5), 145-151. doi: 10.1016/j.tcm.2007.03.003

Kullander, K., & Klein, R. (2002). Mechanisms and functions of Eph and ephrin signalling. *Nat Rev Mol Cell Biol, 3*(7), 475-486. doi: 10.1038/nrm856

Kundu, M., Thomas, J., Fialho, A. M., Kwan, J. M., Moreira, L. M., Mahfouz, M., . . . Chakrabarty, A. M. (2009). The anticancer activity of the N-terminal CARD-like domain of arginine deiminase (ADI) from Pseudomonas aeruginosa. *Letters in Drug Design & Discovery, 6*(6), 403-412. doi: https://doi.org/10.2174/157018009789057580

Kwan, J. M., Fialho, A. M., Kundu, M., Thomas, J., Hong, C. S., Das Gupta, T. K., & Chakrabarty, A. M. (2009). Bacterial proteins as potential drugs in the treatment of leukemia. *Leuk Res, 33*(10), 1392-1399. doi: 10.1016/j.leukres.2009.01.024

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature, 227*(5259), 680-685.

Latt, S. A., & Stetten, G. (1976). Spectral studies on 33258 Hoechst and related bisbenzimidazole dyes useful for fluorescent detection of deoxyribonucleic acid synthesis. *J Histochem Cytochem, 24*(1), 24-33. doi: 10.1177/24.1.943439

Latt, S. A., Stetten, G., Juergens, L. A., Willard, H. F., & Scher, C. D. (1975). Recent developments in the detection of deoxyribonucleic acid synthesis by 33258 Hoechst fluorescence. *J Histochem Cytochem, 23*(7), 493-505.

Leckner, J., Bonander, N., Wittung-Stafshede, P., Malmstrom, B. G., & Karlsson, B. G. (1997). The effect of the metal ion on the folding energetics of azurin: a comparison of the native, zinc and apoprotein. *Biochim Biophys Acta, 1342*(1), 19-27.

Lee, C. H., Wu, C. L., Chen, S. H., & Shiau, A. L. (2009). Humoral immune responses inhibit the antitumor activities mediated by Salmonella enterica serovar choleraesuis. *J Immunother, 32*(4), 376-388. doi: 10.1097/CJI.0b013e31819d4ebc

Lekshmi, A., Varadarajan, S. N., Lupitha, S. S., Indira, D., Mathew, K. A., Chandrasekharan Nair, A., . . . Santhoshkumar, T. R. (2017). A quantitative real-time approach for discriminating

apoptosis and necrosis. *Cell Death Discov, 3*, 16101. doi: 10.1038/cddiscovery.2016.101

Leuschner, C., & Hansel, W. (2004). Membrane disrupting lytic peptides for cancer treatments. *Curr Pharm Des, 10*(19), 2299-2310.

Lieu, C., Heymach, J., Overman, M., Tran, H., & Kopetz, S. (2011). Beyond VEGF: inhibition of the fibroblast growth factor pathway and antiangiogenesis. *Clin Cancer Res, 17*(19), 6130-6139. doi: 10.1158/1078-0432.CCR-11-0659

Liu, P. V. (1974). Extracellular toxins of Pseudomonas aeruginosa. *J Infect Dis, 130 Suppl*(0), S94-99.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. *J Biol Chem, 193*(1), 265-275.

Lukasiewicz, K., & Fol, M. (2018). Microorganisms in the Treatment of Cancer: Advantages and Limitations. *J Immunol Res, 2018*, 2397808. doi: 10.1155/2018/2397808

M. Fialho, A., Bernardes, N., & M Chakrabarty, A. (2016). Exploring the anticancer potential of the bacterial protein azurin. *AIMS Microbiology, 2*(3), 292-303. doi: 10.3934/microbiol. 2016.3.292

Mac Faddin, J. F. (1976). *Biochemical tests for identification of medical bacteria*: Williams & Wilkins Co.

Mahfouz, M., Hashimoto, W., Das Gupta, T. K., & Chakrabarty, A. M. (2007). Bacterial proteins and CpG-rich extrachromosomal

DNA in potential cancer therapy. *Plasmid, 57*(1), 4-17. doi: 10.1016/j.plasmid.2006.11.001

Mandal, R. S. a. M. (2010). *Screening of Pseudomonas aeruginosa strain for enhanced synthesis of azurin induced by copper sulphate and potassiun nitrate and its characterization,,*. Paper presented at the 2010 International Conference on Systems in Medicine and Biology, Kharagpur. http://ieeexplore.ieee.org/stamp/stamp.jsp?tp=&arnumber=5735389&isnumber=5735330

Marcelli, M., Cunningham, G. R., Haidacher, S. J., Padayatty, S. J., Sturgis, L., Kagan, C., & Denner, L. (1998). Caspase-7 is activated during lovastatin-induced apoptosis of the prostate cancer cell line LNCaP. *Cancer Res, 58*(1), 76-83.

Martin, A. C., Facchiano, A. M., Cuff, A. L., Hernandez-Boussard, T., Olivier, M., Hainaut, P., & Thornton, J. M. (2002). Integrating mutation data and structural analysis of the TP53 tumor-suppressor protein. *Hum Mutat, 19*(2), 149-164. doi: 10.1002/humu.10032

Mashiach, E., Schneidman-Duhovny, D., Peri, A., Shavit, Y., Nussinov, R., & Wolfson, H. J. (2010). An integrated suite of fast docking algorithms. *Proteins, 78*(15), 3197-3204. doi: 10.1002/prot.22790

McCarthy, E. F. (2006). The toxins of William B. Coley and the treatment of bone and soft-tissue sarcomas. *Iowa Orthop J, 26*, 154-158.

McCormack, A. L., Schieltz, D. M., Goode, B., Yang, S., Barnes, G., Drubin, D., & Yates, J. R., 3rd. (1997). Direct analysis and

identification of proteins in mixtures by LC/MS/MS and database searching at the low-femtomole level. *Anal Chem, 69*(4), 767-776.

Mehta, N., Lyon, J. G., Patil, K., Mokarram, N., Kim, C., & Bellamkonda, R. V. (2017). Bacterial Carriers for Glioblastoma Therapy. *Mol Ther Oncolytics, 4*, 1-17. doi: 10.1016/j.omto.2016.12.003

Mehta, R. R., Yamada, T., Taylor, B. N., Christov, K., King, M. L., Majumdar, D., . . . Das Gupta, T. K. (2011). A cell penetrating peptide derived from azurin inhibits angiogenesis and tumor growth by inhibiting phosphorylation of VEGFR-2, FAK and Akt. *Angiogenesis, 14*(3), 355-369. doi: 10.1007/s10456-011-9220-6

Miyashita, T., & Reed, J. C. (1995). Tumor suppressor p53 is a direct transcriptional activator of the human bax gene. *Cell, 80*(2), 293-299.

Mohamed, M. S., Fattah, S. A., & Mostafa, H. M. (2010). Azurin as antitumor protein and its effect on the cancer cell lines. *Current Research Journal of Biological Sciences, 2*(6), 396-401.

Mooney, L. M., Al-Sakkaf, K. A., Brown, B. L., & Dobson, P. R. (2002). Apoptotic mechanisms in T47D and MCF-7 human breast cancer cells. *Br J Cancer, 87*(8), 909-917. doi: 10.1038/sj.bjc.6600541

Mosmann, T. (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods, 65*(1-2), 55-63.

Naffouje, S. A., Yamada, T., & Gupta, T. K. D. (2017, 017 Apr 1-5). *A peptide derived from a photosynthetic cupredoxin protein, auracyanin, induces a p53-independent apoptosis in cancer cells*.

Nguyen, C., & Nguyen, V. D. (2016). Discovery of Azurin-Like Anticancer Bacteriocins from Human Gut Microbiome through Homology Modeling and Molecular Docking against the Tumor Suppressor p53. *Biomed Res Int, 2016*, 8490482. doi: 10.1155/2016/8490482

Nilsson, J., Stahl, S., Lundeberg, J., Uhlen, M., & Nygren, P. A. (1997). Affinity fusion strategies for detection, purification, and immobilization of recombinant proteins. *Protein Expr Purif, 11*(1), 1-16. doi: 10.1006/prep.1997.0767

Nishita, K., & Manonmani, H. (2015). Changes in cell membrane morphology of colon cancer cells (HCT116) after treatment with azurin derived hexapeptide 4 by AFM and STEM. *International Journal of Innovative Research in Science, Engineering and Technology, 4*(11), 11550-11557.

Osman, Y. A., Douaa, R., & Younis, S. A. (2013). Azurin: A powerful anticancer from a local Pseudomonas aeruginosa Isolate. *Journal of American Science, 9*(12), 115-126.

Ovesna, Z., & Horvathova-Kozics, K. (2005). Structure-activity relationship of trans-resveratrol and its analogues. *Neoplasma, 52*(6), 450-455.

Parr, S. R., Barber, D., & Greenwood, C. (1976). A purification procedure for the soluble cytochrome oxidase and some other

respiratory proteins from Pseudomonas aeruginosa. *Biochem J, 157*(2), 423-430.

Parr, S. R., Barber, D., Greenwood, C., & Brunori, M. (1977). The electron-transfer reaction between azurin and the cytochrome c oxidase from Pseudomonas aeruginosa. *Biochem J, 167*(2), 447-455.

Pasquale, E. B. (2010). Eph receptors and ephrins in cancer: bidirectional signalling and beyond. *Nat Rev Cancer, 10*(3), 165-180. doi: 10.1038/nrc2806

Patyar, S., Joshi, R., Byrav, D. S., Prakash, A., Medhi, B., & Das, B. K. (2010). Bacteria in cancer therapy: a novel experimental strategy. *J Biomed Sci, 17*(1), 21. doi: 10.1186/1423-0127-17-21

Phumudzo, T., Ronald, N., Khayalethu, N., & Fhatuwani, M. (2013). Bacterial species identification getting easier. *African Journal of Biotechnology, 12*(41), 5975-5982. doi: 10.5897/ajb2013.12057

Priest, F. G. (1977). Extracellular enzyme synthesis in the genus Bacillus. *Bacteriol Rev, 41*(3), 711-753.

Punj, V., Bhattacharyya, S., Saint-Dic, D., Vasu, C., Cunningham, E. A., Graves, J., . . . Das Gupta, T. K. (2004). Bacterial cupredoxin azurin as an inducer of apoptosis and regression in human breast cancer. *Oncogene, 23*(13), 2367-2378. doi: 10.1038/sj.onc.1207376

Punj, V., Das Gupta, T. K., & Chakrabarty, A. M. (2003). Bacterial cupredoxin azurin and its interactions with the tumor

suppressor protein p53. *Biochem Biophys Res Commun, 312*(1), 109-114.

Rajeev, R., Choudhary, K., Panda, S., & Gandhi, N. (2012). Role of bacteria in oral carcinogenesis. *South Asian J Cancer, 1*(2), 78-83. doi: 10.4103/2278-330X.103719

Ramachandran, S., & Mandal, M. (2011). Induction of apoptosis of azurin synthesized from P. aeruginosa MTCC 2453 against Dalton's lymphoma ascites model. *Biomed Pharmacother, 65*(7), 461-466. doi: 10.1016/j.biopha.2011.03.006

Ramachandran, S., Singh, M., & Mandal, M. (2012). Purification of Azurin from Pseudomonas Aeuroginosa. In L. d. A. Calderon (Ed.), *Chromatography*: IntechOpen.

Ramasamy, S., Nattarayan, V., Jayaraj, G. G., Arulanandh, M. D., & Jaiswal, A. (2012). Bacterial infection-mediated anticancer activity (BIMAc) – Revisiting the molecular mechanisms. *Journal of Medical Hypotheses and Ideas, 6*(1), 19-22. doi: https://doi.org/10.1016/j.jmhi.2012.03.009

Rhee, Y. K., Bae, E. A., Kim, S. Y., Han, M. J., Choi, E. C., & Kim, D. H. (2000). Antitumor activity of Bifidobacterium spp. isolated from a healthy Korean. *Arch Pharm Res, 23*(5), 482-487.

Roh, K. H., Choi, S. B., Kwak, B.-K., Seo, S.-C., & Lee, S.-B. (2014). A single cupredoxin azurin production in transplastomic tobacco. *Plant Biotechnology Reports, 8*(6), 421-429. doi: 10.1007/s11816-014-0333-4

Rossello-Mora, R., & Amann, R. (2001). The species concept for prokaryotes. *FEMS Microbiol Rev, 25*(1), 39-67.

Saitou, N., & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol, 4*(4), 406-425. doi: 10.1093/oxfordjournals.molbev.a040454

Sambrook, J. (2001). *Molecular cloning: a laboratory manual*

(Vol. 1). New York: Cold Spring Harbor Laboratory.

Sambrook, J., Fritsch, E. F., & Maniatis, T. (1989). *Molecular cloning: a laboratory manual*: Cold spring harbor laboratory press.

Sankar, R., & Mahitosh, M. (2011). Copper sulphate enhanced synthesis of azurin from Pseudomonas aeruginosa MTCC 2453. *Research & Reviews: A Journal of Microbiology & Virology, 1*(1).

Sankar, R., Moganavelli, S., & Mahitosh, M. (2012). Purification of Azurin from Pseudomonas Aeuroginosa *Chromatography-The Most Versatile Method of Chemical Analysis*: InTech.

Sankar, R., Siddik, S., Abhijit, M., & Mahitosh, M. (2011). Azurin Synthesis from Pseudomonas Aeruginosa MTCC 2453, Properties, Induction of Reactive Oxygen Species, and p53 Stimulated Apoptosis in Breast Carcinoma Cells. *Journal of Cancer Science & Therapy, 03*(05). doi: 10.4172/1948-5956.1000069

Sausville, E. A., & Longo, D. L. (2005). Principles of Cancer Treatment: Surgery, Chemotherapy, and Biologic Therapy. In D. L. Kasper & T. R. Harrison (Eds.), *Harrison's principles of internal medicine* (16th ed., pp. 464 – 482). New York: McGraw-Hill, Medical Pub. Division.

Schneidman-Duhovny, D., Inbar, Y., Nussinov, R., & Wolfson, H. J. (2005). PatchDock and SymmDock: servers for rigid and symmetric docking. *Nucleic Acids Res, 33*(Web Server issue), W363-367. doi: 10.1093/nar/gki481

Schulz, W. A. (2005). *Molecular biology of human cancers : an advanced student's textbook*. Dordrecht, The Netherlands ; Norwell, MA: Springer.

Sereena .M.C, D. S. (2018). Characterization of anticancer compound azurin from indigenous isolate Pseudomonas aeruginosa SSj *International Journal of Life Sciences Research, 6*(4), 174-179.

Sereena, M. C., & Sebastian, D. (2016). Molecular Detection of Azurin: A Powerful Anticancer Protein from Native Pseudomonas Isolates. *Journal of Advances in Medical and Pharmaceutical Sciences, 5*(1), 1-7. doi: 10.9734/jamps/2016/20557

Shahabi, V., Maciag, P. C., Rivera, S., & Wallecha, A. (2010). Live, attenuated strains of Listeria and Salmonella as vaccine vectors in cancer treatment. *Bioeng Bugs, 1*(4), 235-243. doi: 10.4161/bbug.1.4.11243

Shatsky, M., Dror, O., Schneidman-Duhovny, D., Nussinov, R., & Wolfson, H. J. (2004). BioInfo3D: a suite of tools for structural bioinformatics. *Nucleic Acids Res, 32*(Web Server issue), W503-507. doi: 10.1093/nar/gkh413

Shaw, A. S., & Filbert, E. L. (2009). Scaffold proteins and immune-cell signalling. *Nat Rev Immunol, 9*(1), 47-56. doi: 10.1038/nri2473

Shetty, G., Shao, S. H., & Weng, C. C. (2008). p53-dependent apoptosis in the inhibition of spermatogonial differentiation in juvenile spermatogonial depletion (Utp14bjsd) mice. *Endocrinology, 149*(6), 2773-2781. doi: 10.1210/en.2007-1338

Skulachev, V. P. (1998). Cytochrome c in the apoptotic and antioxidant cascades. *FEBS Lett, 423*(3), 275-280.

Smith, B. C. (2011). *Fundamentals of Fourier transform infrared spectroscopy*: CRC press.

Sobhan, P. K., Seervi, M., Deb, L., Varghese, S., Soman, A., Joseph, J., . . . R, S. K. (2013). Calpain and reactive oxygen species targets Bax for mitochondrial permeabilisation and caspase activation in zerumbone induced apoptosis. *PLoS One, 8*(4), e59350. doi: 10.1371/journal.pone.0059350

Sohier M. Syame, A. S. H., Maryam H.Mugri,Nagah A Rashad,Ehak A R, Eman,A.Khairy. (2018). "Characterization and Purification of Azurin; A Protein Secreted FromPseudomonas Aeruginos Isolates, Promising A Novel Competing of Squamous Cell Carcinoma ". *IOSR Journal of Pharmacy and Biological Sciences (IOSR-JPBS), 13*(5), 50-57.

Solomon, E. I., Baldwin, M. J., & Lowery, M. D. (1992). Electronic structures of active sites in copper proteins: contributions to reactivity. *Chemical Reviews, 92*(4), 521-542. doi: 10.1021/cr00012a003

Stackebrandt, E., & Goebel, B. M. (1994). Taxonomic Note: A Place for DNA-DNA Reassociation and 16S rRNA Sequence Analysis in the Present Species Definition in Bacteriology. *International Journal of Systematic and Evolutionary*

Microbiology, 44(4), 846-849. doi: doi:10.1099/00207713-44-4-846

Steczko, J., Donoho, G. A., Dixon, J. E., Sugimoto, T., & Axelrod, B. (1991). Effect of ethanol and low-temperature culture on expression of soybean lipoxygenase L-1 in Escherichia coli. *Protein Expr Purif, 2*(2-3), 221-227.

Studier, F. W., & Moffatt, B. A. (1986). Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. *J Mol Biol, 189*(1), 113-130.

Surewicz, W. K., Szabo, A. G., & Mantsch, H. H. (1987). Conformational properties of azurin in solution as determined from resolution-enhanced Fourier-transform infrared spectra. *Eur J Biochem, 167*(3), 519-523.

Sutherland, I. W. (1966). The production of azurin and similar proteins. *Archiv für Mikrobiologie, 54*(4), 350-357. doi: 10.1007/bf00406717

Sutherland, I. W., & Wilkinson, J. F. (1963). Azurin: a copper protein found in Bordetella. *J Gen Microbiol, 30*, 105-112. doi: 10.1099/00221287-30-1-105

Suzuki, Y., Muto, M., & Kobayashi, A. (1986). Antitumor effect of formalin-fixed Toxoplasma gondii organisms on EL4 lymphoma in Toxoplasma-infected mice. *J Biol Response Mod, 5*(4), 288-293.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., & Kumar, S. (2011). MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and

maximum parsimony methods. *Mol Biol Evol, 28*(10), 2731-2739. doi: 10.1093/molbev/msr121

Tang, C., Shao, X., Sun, B., Huang, W., Qiu, F., Chen, Y., . . . Zhao, X. (2010). Anticancer mechanism of peptide P18 in human leukemia K562 cells. *Org Biomol Chem, 8*(5), 984-987. doi: 10.1039/b920762g

Taylor, B. N., Mehta, R. R., Yamada, T., Lekmine, F., Christov, K., Chakrabarty, A. M., . . . Das Gupta, T. K. (2009). Noncationic peptides obtained from azurin preferentially enter cancer cells. *Cancer Res, 69*(2), 537-546. doi: 10.1158/0008-5472.CAN-08-2932

Taylor, W. I., & Achanzar, D. (1972). Catalase test as an aid to the identification of Enterobacteriaceae. *Appl Microbiol, 24*(1), 58-61.

Terpe, K. (2003). Overview of tag protein fusions: from molecular and biochemical fundamentals to commercial systems. *Appl Microbiol Biotechnol, 60*(5), 523-533. doi: 10.1007/s00253-002-1158-6

Terpe, K. (2006). Overview of bacterial expression systems for heterologous protein production: from molecular and biochemical fundamentals to commercial systems. *Appl Microbiol Biotechnol, 72*(2), 211-222. doi: 10.1007/s00253-006-0465-8

Theys, J., & Lambin, P. (2015). Clostridium to treat cancer: dream or reality? *Ann Transl Med, 3*(Suppl 1), S21. doi: 10.3978/j.issn.2305-5839.2015.03.39

Thompson, J. D., Higgins, D. G., & Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res, 22*(22), 4673-4680.

Unal, S., Hoskins, J., Flokowitsch, J. E., Wu, C. Y., Preston, D. A., & Skatrud, P. L. (1992). Detection of methicillin-resistant staphylococci by using the polymerase chain reaction. *J Clin Microbiol, 30*(7), 1685-1691.

Valli S, A., & T, M. (2018). Bioinformatic study of an antitumor protein, azurin. *Asian Journal of Pharmaceutical and Clinical Research,*, 8. doi: 10.22159/ajpcr.2018.v11i6.23339

van de Kamp, M., Silvestrini, M. C., Brunori, M., Van Beeumen, J., Hali, F. C., & Canters, G. W. (1990). Involvement of the hydrophobic patch of azurin in the electron-transfer reactions with cytochrome C551 and nitrite reductase. *Eur J Biochem, 194*(1), 109-118.

Verhoeven, W., Takeda, T. (1956). *W. D. McElroy and B. Glass, eds.*

Walser, T., Cui, X., Yanagawa, J., Lee, J. M., Heinrich, E., Lee, G., . . . Dubinett, S. M. (2008). Smoking and lung cancer: the role of inflammation. *Proc Am Thorac Soc, 5*(8), 811-815. doi: 10.1513/pats.200809-100TH

Walter, R. L., Ealick, S. E., Friedman, A. M., Blake, R. C., 2nd, Proctor, P., & Shoham, M. (1996). Multiple wavelength anomalous diffraction (MAD) crystal structure of rusticyanin: a highly oxidizing cupredoxin with extreme acid stability. *J Mol Biol, 263*(5), 730-751.

Warren L. DeLano. (2009). PyMOL: An Open-Source Molecular Graphics Tool. from warren@delanoscientific.com

Warso, M. A., Richards, J. M., Mehta, D., Christov, K., Schaeffer, C., Rae Bressler, L., . . . Das Gupta, T. K. (2013). A first-in-class, first-in-human, phase I trial of p28, a non-HDM2-mediated peptide inhibitor of p53 ubiquitination in patients with advanced solid tumours. *Br J Cancer, 108*(5), 1061-1070. doi: 10.1038/bjc.2013.74

Waugh, D. S. (2005). Making the most of affinity tags. *Trends Biotechnol, 23*(6), 316-320. doi: 10.1016/j.tibtech.2005.03.012

Wicks, I. P., Wilkinson, D., Salvaris, E., & Boyd, A. W. (1992). Molecular cloning of HEK, the gene encoding a receptor tyrosine kinase expressed by human lymphoid tumor cell lines. *Proc Natl Acad Sci U S A, 89*(5), 1611-1615.

Wiehlmann, L., Wagner, G., Cramer, N., Siebert, B., Gudowius, P., Morales, G., . . . Tummler, B. (2007). Population structure of Pseudomonas aeruginosa. *Proc Natl Acad Sci U S A, 104*(19), 8101-8106. doi: 10.1073/pnas.0609213104

Willcox, W. R., Lapage, S. P., & Holmes, B. (1980). A review of numerical methods in bacterial identification. *Antonie Van Leeuwenhoek, 46*(3), 233-299. doi: 10.1007/bf00453024

Wilson, K. H. (1995). Molecular biology as a tool for taxonomy. *Clin Infect Dis, 20 Suppl 2*, S117-121.

Winnepenninckx, B., Backeljau, T., & De Wachter, R. (1993). Extraction of high molecular weight DNA from molluscs. *Trends Genet, 9*(12), 407.

Wood, P. M. (1978). Periplasmic location of the terminal reductase in nitrite respiration. *FEBS Lett, 92*(2), 214-218.

Woynarowska, B. A., & Woynarowski, J. M. (2002). Preferential targeting of apoptosis in tumor versus normal cells. *Biochim Biophys Acta, 1587*(2-3), 309-317.

Wu, H. J., Seib, K. L., Edwards, J. L., Apicella, M. A., McEwan, A. G., & Jennings, M. P. (2005). Azurin of pathogenic Neisseria spp. is involved in defense against hydrogen peroxide and survival within cervical epithelial cells. *Infect Immun, 73*(12), 8444-8448. doi: 10.1128/IAI.73.12.8444-8448.2005

Yamada, T., Fialho, A. M., Punj, V., Bratescu, L., Gupta, T. K., & Chakrabarty, A. M. (2005). Internalization of bacterial redox protein azurin in mammalian cells: entry domain and specificity. *Cell Microbiol, 7*(10), 1418-1431. doi: 10.1111/j.1462-5822.2005.00567.x

Yamada, T., Goto, M., Punj, V., Zaborina, O., Chen, M. L., Kimbara, K., . . . Chakrabarty, A. M. (2002). Bacterial redox protein azurin, tumor suppressor protein p53, and regression of cancer. *Proc Natl Acad Sci U S A, 99*(22), 14098-14103. doi: 10.1073/pnas.222539699

Yamada, T., Goto, M., Punj, V., Zaborina, O., Kimbara, K., Das Gupta, T. K., & Chakrabarty, A. M. (2002). The bacterial redox protein azurin induces apoptosis in J774 macrophages through complex formation and stabilization of the tumor suppressor protein p53. *Infect Immun, 70*(12), 7054-7062.

Yamada, T., Hiraoka, Y., Ikehata, M., Kimbara, K., Avner, B. S., Das Gupta, T. K., & Chakrabarty, A. M. (2004). Apoptosis or

growth arrest: Modulation of tumor suppressor p53's specificity by bacterial redox protein azurin. *Proc Natl Acad Sci U S A, 101*(14), 4770-4775. doi: 10.1073/pnas.0400899101

Yamada, T., Mehta, R. R., Lekmine, F., Christov, K., King, M. L., Majumdar, D., . . . Das Gupta, T. K. (2009). A peptide fragment of azurin induces a p53-mediated cell cycle arrest in human breast cancer cells. *Mol Cancer Ther, 8*(10), 2947-2958. doi: 10.1158/1535-7163.MCT-09-0444

Yan, X., Feng, J., Ye, Z., Miao, X., Li, W., & Yang, D. (2011). p53 siRNA inhibits apoptosis of U2OS cells treated with azurin. *Mol Med Rep, 4*(6), 1089-1094. doi: 10.3892/mmr.2011.544

Yang, D. S., Miao, X. D., Ye, Z. M., Feng, J., Xu, R. Z., Huang, X., & Ge, F. F. (2005). Bacterial redox protein azurin induce apoptosis in human osteosarcoma U2OS cells. *Pharmacol Res, 52*(5), 413-421. doi: 10.1016/j.phrs.2005.06.002

Ye, Z., Peng, H., Fang, Y., Feng, J., & Yang, D. S. (2007). The construction of the eukaryotic expression plasmid pcDNA3.1/azurin and the increased apoptosis of U2OS cells transfected with it. *Cell Mol Biol Lett, 12*(3), 407-421. doi: 10.2478/s11658-007-0012-3

Ye, Z. M., Miao, X. D., Yang, D. S., Xu, R. Z., Huang, X., & Ge, F. F. (2005). [Selective inducement effect of bacterial redox protein azurin on apoptosis of human osteosarcoma cell line U2OS]. *Ai Zheng, 24*(3), 298-304.

Zaborina, O., Dhiman, N., Ling Chen, M., Kostal, J., Holder, I. A., & Chakrabarty, A. M. (2000). Secreted products of a nonmucoid Pseudomonas aeruginosa strain induce two modes of

macrophage killing: external-ATP-dependent, P2Z-receptor-mediated necrosis and ATP-independent, caspase-mediated apoptosis. *Microbiology, 146 (Pt 10)*, 2521-2530. doi: 10.1099/00221287-146-10-2521

Zur Hausen, H. (1991). Viruses in human cancers. *Science, 254*(5035), 1167-1173.

Addendum