

**COMPARATIVE EVALUATION OF THE EFFECT
OF BONE MARROW DERIVED STEM CELLS,
SE2 AND DHANWANTARAM KASHAYA IN
DIABETIC RATS**

*Thesis Submitted to
the University of Calicut in partial fulfilment of
the requirement for the award of the degree of*

Doctor of Philosophy in Biochemistry

Submitted by

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Under the Guidance of

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DECLARATION

I hereby declare that the work presented in the Thesis entitled **“COMPARATIVE EVALUATION OF THE EFFECT OF BONE MARROW DERIVED STEM CELLS, SE2 AND DHANWANTARAM KASHAYA IN DIABETIC RATS”** submitted to the University of Calicut, as partial fulfillment of the requirement for the award of the degree of Doctor of Philosophy in Biochemistry, is original and carried out by me under the supervision of Dr. Radhakrishna G Pillai, Assistant Professor Department of Life Sciences, University of Calicut. This has not been submitted earlier either in part or full for any degree or diploma of any university. .

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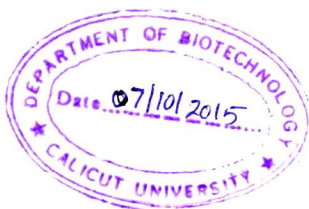
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Institutional Animal Ethics Committee (IAEC)

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Smitha Renganathan

PREFACE

Diabetes mellitus is defined as a group of metabolic diseases and associated with high morbidity due to a broad range of micro and macrovascular complications. This major public health problem was characterised by hyperglycaemia and affects all most all organs of the body. This chronic endocrine disorder is due to the defects in insulin secretion, insulin action, or both. There is no effective treatment and medicine for lifelong recovery from DM till now. Ayurveda, Indian traditional medicinal system offered several plants and methods for management of this lifestyle disease. Dhanwantaram Kashaya is a poly herbal formulation and versatile product of 40 herbal ingredients. It is mainly used for neuro regenerative disorders and also used as a growth stimulant in children. SE2 is another herbal product extracted from *Aphanizomenon flos aquae*, a unicellular prokaryotic microorganism included under the category of blue- green algae. The present study mainly focussed on the effect of Dhanwantaram Kashaya in diabetic rats with a comparative evaluation with the effect of Bone Marrow Derived Stem Cells and SE2 in similar situations.

The chapter 1 is a brief review of Diabetes Mellitus, Dhanwantaram Kashaya, stem cells and SE2. Materials and Methods employed in this study are well described in chapter 2. Chapter 3 to 10 describes the different investigations and the results along with detailed discussion of the results and each chapter concentrates on one aspect of the study. The Chapter 3 comprised of the observations on diabetic rats treated with DK with details on fasting blood sugar level,

HOMA IR index, oral glucose tolerance (OGT), serum insulin level, organ weight, body weight, haemoglobin levels and glycosylated haemoglobin levels, expression of insulin gene and histopathological changes in liver and pancreas. Chapter 4 comprised of preliminary analysis of phytochemicals and the *in vitro* free radical scavenging activity of Dhanwantaram Kashaya. Effect of Dhanwantaram on enzymatic and non-enzymatic antioxidants and serum total protein were included in chapter 5. Lipid profile (Total cholesterol, Free Fatty Acids, Phospholipids and Triglycerides) and lipid peroxidation (MDA, PPAR α and PPAR- δ) were included in chapter 6 and chapter 7 respectively. Chapter 8 highlights the effect of Dhanwantaram Kashaya on expression of various genes associated with diabetic complications (NF- κ B, TNF- α , Cox-2, Caspase 8 and Caspase 3). Chapter 9 comprised of effect of SE2 on diabetic rats. Chapter 10 highlights the effect of stem cells in diabetic rats. Summary and conclusion of the entire study is reported in chapter 11.

This thesis gives an overall idea on the effect of DK in diabetic condition. The results of this study reveal the advantage in using DK as an agent in the prevention or cure of diabetes over another plant derived formulation and a more complicated therapeutic process involving stem cell administration. I present this thesis with a dream to see DK in the near future as a less toxic and effective alternate therapeutic aid in curing or preventing diabetes.

CONTENTS

List of Tables

List of Figures

Abbreviations

	<i>Page No.</i>
Chapter – 1 Introduction and Review of Literature	i-42
I: Introduction	i
1.1: Review of Literature	1
1.2: Dhanwantaram Kashaya	7
1.3:Ingredients of Dhanwantaram Kashaya (Ashtanga Hrudayam)	8
1. Balamula	10
2. Yava	10
3. Kola	11
4. Kulattha	11
5. Kasmari	12
6. Vilwa	12
7. Patala	12
8. Shyonaka	13
9. Agnimantha	13
10. Shalaparni	14
11. Prinshnaparni	14
12. Brihati	14
13. Nidigdhika	15
14. Gokshura	15
15. Dwimeda	16
16. Daru	16
17. Manjishta	16
18. Dwikakoli	17
19. Chandana	17
20. Sariba	18
21. Kushtha	19

22. Tagara	19
23. Diwjivaka	19
24. Kalanusari	20
25. Shaileya	20
26. Vacha	21
27. Punarnava	21
28. Kshirasukla	22
29. Yashti	22
30. Haritaki	23
31. Amalaki	23
32. Vibhitaki	24
33. Rasa	25
34. Satahwa	25
35. Ela	26
36. Twak	26
37. Patra	26
1.4: Genes studied	30
1. Beta – actin	30
2. Insulin	31
3. Caspase 3	31
4. Caspase 8	32
5. iNOS	33
6. GPx1	34
7. GRd	35
8. TNF-alpha	35
9. NF-kB	36
10.PPAR- α	37
11.PPAR- δ	38
12.COX- 2	38
13.L-gulonolactone- oxidase	39
14. β 2- macroglobulin	
15.Y-Chromosome protein	
16.X-Chromosome protein	
1.5: Stem cells in diabetes	39
1.6: Objectives of the study	41

Chapter – 2 Materials and Methods	43-97
2.1: Experimental Animals	43
2.2: Induction of Diabetes in rats	43
2.3: Animals Groups & Experimental Design	44
2.3.1: Set I	44
2.3.2: Set II	45
2.3.3: Set III	46
2.4: Dhanwantaram Kashaya (DK)	46
2.5: Chemicals	46
2.6: Determination of Fasting Blood Sugar Level	47
2.7: Determination of Serum Insulin Level	47
2.8: Homeostasis model assessment (HOMA)	47
2.9: Oral glucose tolerance tests (OGTT)	48
2.10: Determination of haemoglobin and glycosylated haemoglobin	48
2.11: Histopathology of Liver and Pancreas	48
2.12: Estimation of total tannins	49
2.13: Total flavonoid content	50
2.14: Total Phenolic Content	51
2.15: Ferric reducing antioxidant power (FRAP) assay	52
2.16: 2'2-Diphenyl-1-Picryl Hydrazine (DPPH) Scavenging activity	53
2.17: ABTS radical scavenging assay	55
2.18: Phosphomolybdenum assay	56
2.19: Hydroxyl radical scavenging activity	57
2.20: Hydrogen peroxide-scavenging activity	59
2.21: Nitric oxide scavenging activity	60
2.22: Assay of Catalase (EC 1.11.1.6)	61
2.23: Assay of Superoxide Dismutase (SOD, EC 1.15.1.1)	62
2.24: Assay of Glutathione Peroxidase (EC 1.11.1.9)	63
2.25: Assay of Glutathione Reductase (EC 1.8.1.7)	64
2.26: Estimation of Glutathione Content	65
2.27: Estimation of vitamin C in Tissues	66
2.28: Estimation of vitamin E	67

2.29: Estimation of Nitric oxide levels in liver tissue	68
2.30: Estimation of Protein	69
2.31: Lipid Profile in Tissue and Serum	70
2.31.1: Extraction of Tissues for Lipid Analysis	70
2.31.2: Extraction of serum for lipid estimation	70
2.31.3: Estimation of cholesterol	71
2.31.4: Estimation of Free fatty acids	72
2.31.5: Estimation of Phospholipids	74
2.31.6: Estimation of Triglycerides	75
2.32: Estimation of Malondialdehyde	77
2.33: RNA isolation	78
2.33.1: Materials required	78
2.33.2: Phase separation	79
2.33.3: RNA precipitation	80
2.33.4: RNA wash	80
2.33.5: RNA re-suspension	81
2.33.6: Preparation of DEPC Water	81
2.33.7: Determining yield and purity of RNA	81
2.33.8: cDNA synthesis	82
2.33.9: Gene Expression Studies	83
2.33.10: Primers selected for the genes are following.	83
2.34: Real Time PCR Analysis	84
2.34.1: Standardization of internal control	86
2.34.2: PCR protocol	86
2.34.3: Primer designing and synthesis	87
2.35: Isolation and Culture of Rat Bone Marrow Derived Mesenchymal Stem Cells (BMDSCs)	88
2.36: Cell Culture	89
2.37: Checking viability of cells and counting	91
2.38: MTT assay	91
2.39: Effect of administration of bone marrow cells to diabetic rats	94
2.40: Blood Cell Analysis.	94
2.41: Determining the presence of BMSCs in the pancreas	94

2.42: Electrophoresis of PCR product	95
2.42.1: Reagents and preparation	95
2.42.2: DNA loading buffer (6x)	96
2.43: Statistical analysis	97
Chapter – 3 Effect of Dhanwantaram Kashaya on Glucose Metabolism	99-124
3.1: Introduction	99
3.1.1: Effect of insulin on protein synthesis	99
3.2: Results	102
3.2.1: Fasting blood sugar level and Effect of DK on HOMA –IR index	103
3.2.2: Effect of DK on oral glucose tolerance (OGT)	105
3.2.3: Serum insulin level and Organ weight	107
3.2.4: Body weight and Haemoglobin levels	109
3.2.5: Glycosylated haemoglobin levels	112
3.2.6: Expression of insulin gene	113
3.2.7: Histopathology of liver and pancreas	114
3.3: Discussion	118
3.4: Conclusion	124
Chapter – 4 Phytochemical composition and <i>in vitro</i> antioxidant power of Dhanwantaram Kashaya	125-
4.1: Introduction	125
4.1.1: Molecular structure of tannins.	127
4.1.2: Molecular structure of the flavone backbone (2-phenyl-1,4-benzopyrone)	128
4.1.3: Structure of polyphenols.	129
4.2: Results	130
4.2.1: Phytochemicals	130
4.2.2 Total Tannins	130
4.2.3: Total Flavonoids	131
4.2.4: Total Phenolic Content	131
4.2.5: Antioxidant Power Assays	132
4.2.5.1: Ferric Reducing Antioxidant Power (FRAP)	133
4.2.5.2: 2’2-Diphenyl-1-Picryl Hydrazine	134

(DPPH) scavenging activity	
4.2.5.3: ABTS radical scavenging assay	135
4.2.5.4: Phosphomolybdenum assay	136
4.2.5.5: Hydroxyl radical scavenging activity	136
4.2.5.6: Hydrogen peroxide-scavenging activity	137
4.2.5.7: Nitric oxide scavenging activity	138
4.3: Discussion	139
4.4: Conclusion	148
Chapter – 5 Antioxidants and Serum total protein	149-179
5.1: Introduction	149
5.2: Results	154
5.2.1: Enzymatic Antioxidants	154
5.2.1.1: Catalase	154
5.2.1.2: Super Oxide Dismutase (SOD)	156
5.2.1.3: Glutathione Peroxidase (GPx)	158
5.2.1.4: Glutathione Reductase (GRd)	162
5.2.2: Non - Enzymatic Antioxidants	165
5.2.2.1: Reduced Glutathione (GSH)	165
5.2.2.2: Vitamin C	166
5.2.2.3: Vitamin C synthesis at mRNA level	168
5.2.2.4: Vitamin E	169
5.2.3: Nitric oxide levels in liver tissue of normal and experimental rats	171
5.2.4: Serum Total Protein	174
5.3: Discussion	175
5.4: Conclusion	179
Chapter – 6 Effect of Dhanwantaram Kashaya on Lipid profiles	181
6.1: Introduction	181
6.2: Results	185
6.2.1: Total Cholesterol	186
6.2.2: Free Fatty Acids	187
6.2.3: Phospholipids	188

6.2.4: Triglycerides	189
6.3: Discussion	190
6.4: Conclusion	195
Chapter – 7 Effect of Dhanwantaram Kashaya on Lipid peroxidation	197-209
7.1: Introduction	197
7.2: Results	202
7.2.1: MDA levels	202
7.2.2: Expression of PPAR- α at mRNA level	204
7.2.3: Expression of PPAR- δ at mRNA level	205
7.3: Discussion	206
7.4: Conclusion	209
Chapter – 8 Effect of Dhanwantaram Kashaya on expression of various genes associated with diabetic complications	211-238
8.1: Introduction	211
8.1.1: Nuclear factor- κ B (NF- κ B)	211
8.1.2: Tumour necrosis factor - α (TNF- α)	213
8.1.3: Cox-2	214
8.1.4: Caspase 8	215
8.1.5: Caspase 3	216
8.2: Results	217
8.2.1: RNA extraction	217
8.2.2: Real Time PCR Analysis of gene expression at mRNA level	219
8.2.2.1 Expression of β actin gene used as reference gene in this study	219
8.2.2.2: Effect of DK on the expression of NF- κ B	220
8.2.2.3: Expression of TNF- α gene in diabetic and DK treated rats	223
8.2.2.4: Gene expression of COX-2 on treatment with DK	224
8.2.2.5: Expression of Caspase 8 in diabetic and DK treated rats	226
8.2.2.6: Expression of Caspase- 3 gene in	228

	diabetic and DK treated rats	
8.3: Discussion		230
8.4: Conclusion		238
Chapter – 9 Effect of Stem Enhance 2 (SE2) on diabetic rats		239-257
9.1: Introduction		239
9.2: Results		243
9.2.1: Effect of SE2 on serum insulin levels		243
9.2.2: Body weight of different groups of rats during the experimental period		245
9.2.3: Fasting Blood Sugar Level		246
9.2.4: MDA level in the different tissues of the rats of different groups on 21 st day.		247
9.2.5: NO levels in the liver of rats of different groups		249
9.2.6: Mobilisation of bone marrow stem cells		249
9.3: Discussion		251
9.4: Conclusion		257
Chapter 10 Effect of Stem cells in diabetic rats		259-272
10.1: Introduction		259
10.2: Results		262
10.2.1: Viability of bone marrow cells		262
10.2.2: Determining the presence of bone marrow cells in pancreas by PCR		263
10.2.3: Blood cells		265
10.2.4: Change in body weight of rats treated with bone marrow stem cells		267
10.2.5: Blood glucose level and serum insulin level in bone marrow cells injected rats		268
10.3: Discussion		269
10.4: Conclusion		272
Chapter 11 Summary and Conclusion		273-278
References		279-356
Publications and Presentations		

LIST OF TABLES

<i>Table No.</i>	<i>Title</i>	<i>Page No.</i>
1.1	Dhanwantaram Kashaya Ingredients	8
2.1	Showing the list of the genes studied along with the sequence of primers and the annealing temperatures	83
3.2.1	Fasting blood sugar level	103
3.2.2	Effect of DK on serum insulin ELISA.	107
3.2.3	Organ weight	108
3.2.4	Body weight	109
3.2.5	Effect of DK on expression of insulin gene	113
5.2.1.1	Glutathione Peroxidase	158
5.2.1.2	Expression of GPx-1 at mRNA level in liver	161
5.2.1.3	Expression of GRd at mRNA level in liver	164
5.2.2.1	Reduced Glutathione (GSH)	165
5.2.2.2	Vitamin E	169
5.2.3.1	Expression of iNOS at mRNA level in liver.	173
5.2.4	Serum Total Protein	174
6.2.1	Total cholesterol	186
6.2.2	Free Fatty Acids	187
6.2.3	Phospholipids	188
6.2.4	Triglycerides	189
8.2.1.1	Quality and yield of RNA isolated from liver tissue	217
8.2.1.2	Quality and yield of RNA isolated from bone marrow cells	218
8.2.1.3	Quality and yield of RNA isolated from heart tissue	218
8.2.2.2.1	Showing the calculation of the expression levels of the NF-kB gene in the liver of rats of the different groups	222

8.2.2.2.2	Showing the expression levels of the NF-kB gene in the bone marrow, heart and liver of rats of the different groups	222
8.2.2.3.1	Showing the expression levels of the TNF- α gene in the rats of the different groups	223
8.2.2.4.1	Showing the expression levels of the COX-2 gene in the rats of the different groups	225
8.2.2.5.1	Showing the expression levels of the Caspase 8 gene in the rats of the different groups	227
8.2.2.6.1	Showing the expression levels of the Caspase- 3 gene in the rats of the different groups	229
9.2.1	Serum insulin levels on 21 st day in different groups of rats	244
9.2.2	Body weight of different groups of rats during the experimental period	245
9.2.3	Fasting Blood Sugar Level	246
9.2.4	MDA level in the different tissues of the rats of different groups on 21 st day	247
10.2.1	Change in body weight of rats treated with bone marrow stem cells	267
10.2.2	Blood glucose level and serum insulin level in bone marrow cells injected rats	268

LIST OF FIGURES

<i>Figure No.</i>	<i>Title</i>	<i>Page No.</i>
3.2.1.A	HOMA IR index	105
3.2.1.B	Effect of DK on oral glucose tolerance (OGT)	106
3.2.2	Effect of DK on Haemoglobin Concentration	111
3.2.3	Effect of DK on Glycosylated haemoglobin levels	112
3.2.4	Effect of DK on histopathology of liver in normal and STZ induced diabetic rats	115
3.2.5	Effect of DK on histopathology of pancreas in normal and STZ induced diabetic rats	117
4.1.1	Molecular structure of tannins	127
4.1.2	Molecular structure of the flavone backbone (2-phenyl-1,4-benzopyrone).	128
4.1.3	Structure of polyphenols.	129
4.2.1	Total tannins	130
4.2.2	Total flavonoids	131
4.2.3	Total phenolic content	132
4.2.5.1	Ferric Reducing Antioxidant Power (FRAP)	133
4.2.5.2	2,2-Diphenyl-1-Picryl Hydrazine (DPPH) scavenging activity	134
4.2.5.3	ABTS radical scavenging assay	135
4.2.5.4	Hydroxyl radical scavenging activity	136
4.2.5.5	Hydrogen peroxide-scavenging activity	137
4.2.5.6	Nitric oxide scavenging activity	138
5.2.1.1	Catalase	154
5.2.1.2	Super Oxide Dismutase	156

5.2.1.3	(a) shows the graphical representation of amplification curve of GPx-1 in liver from Real Time PCR analysis, (b) shows the melt peak and (c) represents the melt curve	160
5.2.1.4	Glutathione Reductase	162
5.2.2.1	Effect of DK on nonenzymatic antioxidant vit C in normal and experimental rats	166
5.2.2.2	Vitamin C synthesis at mRNA level	168
5.2.3.1	Effect of DK on nitric oxide levels in liver tissue of normal and experimental rats	171
5.2.3.2	Melt peak of iNOS	172
7.2.1	MDA	203
7.2.2	Expression of PPAR- α at mRNA level	204
7.2.3	Expression of PPAR- δ at mRNA level	205
8.2.2.1	Expression of β actin gene in the liver tissue of rats belonging to the different groups studied by real-time PCR. (A): Shows the graphical representation of amplification curve, (B) shows the melt curve and (C) represents the melt Peak	219
8.2.2.2.1	Expression of NF- κ B gene in the liver tissue of rats belonging to the different groups studied by real-time PCR. (A): Shows the graphical representation of amplification curve, (B) shows the melt curve and (C) represents the melt Peak	221
8.2.2.3.1	Expression of TNF- α gene in the liver tissue of rats belonging to the different groups studied by real-time PCR. (A): Shows the graphical representation of amplification curve (B) shows the melt curve and (C) represents the melt Peak	223
8.2.2.4.1.	Expression of Cox-2 gene in the liver tissue of rats belonging to the different groups studied by real-time PCR. (A): Shows the graphical representation of amplification curve (B) shows the melt curve and (C) represents the melt Peak	224

82.2.5.1	Expression of caspase 8 gene in the liver tissue of rats belonging to the different groups studied by real-time PCR. (A): Shows the graphical representation of amplification curve, (B) shows the melt curve and (C) represents the melt Peak	226
8.2.2.6.1	Expression of caspase 3 gene in the liver tissue of rats belonging to the different groups studied by real-time PCR. (A): Shows the graphical representation of amplification curve (B) shows the melt curve and (C) represents the melt Peak	229
9.2.1	NO levels in the liver of rats of different groups	248
9.2.2	Showing the copy numbers of CD34 ⁺ gene at mRNA level in rats	250
9.2.2.A	Showing the copy numbers of CD34 ⁺ gene at mRNA level in SE2 administrated rats	250
9.2.2.B	Showing the copy numbers of CD34 ⁺ gene at mRNA level in DK administrated rats	250
10.2.1	Viability of bone marrow cells	262
10.2.2	Determining the presence of bone marrow cells in pancreas by PCR	264
10.2.2.A	Gel electrophorogram showing the PCR bands of X chromosome, Y chromosome and 1Kb ladder	264
10.2.2.B	Showing the intensity peak of the corresponding PCR bands for Y chromosome protein in Figure A obtained by using "ImageJ" software (NIH)	264
10.2.3	Showing the change in the number of different blood cells in response to the injection of bone marrow stem cells (BMSCs)	265

LIST OF ABBREVIATIONS

AA	- Arachidonic Acid
ABTS	- 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)
AFA	- Aphanizomenon flos aquae
AFA	- Aphanizomenon Flos Aquae
AGEs	- Advanced Glycation End products
ASCs	- Adult Stem Cells
BMDSCs	- Bone Marrow Derived Mesenchymal Stem Cells
BMSC	- Bone Marrow stromal Stem Cells
CAT	- Catalase
CDKA	- Control + DK-Dose A
CDKB	- Control + DK-Dose B
CDKC	- Control+ DK-Dose C
CE	- Catechin Equivalentents
COX-2	- Cyclooxygenase-2
CSE2A	- Control + SE2 Dose A
CSE2B	- Control + SE2 Dose B
CSE2C	- Control + SE2 Dose C
CVD	- Cardiovascular Disease
DBMSC	- Diabetic rats received BMSC
DC	- Diabetic Control
DDKA	- Diabetic + DK Dose A
DDKB	- Diabetic + DK Dose B
DDKC	- Diabetic + DK Dose C
DK	- Dhanwantaram Kashaya
DKD	- Diabetic Kidney Disease
DM	- Diabetes Mellitus
DNA	- Deoxyribonucleic acid

DPPH	- 2,2-Diphenyl-1-picryl Hydrazine
DSE2A	- Diabetic + SE2 Dose A
DSE2B	- Diabetic + SE2 Dose B
DSE2C	- Diabetic + SE2 Dose C
EC-SOD	- Extra Cellular SOD
EGF	- Epidermal Growth Factor
eNOS	- endothelial Nitric Oxide Synthase
EPCs	- Endothelial Progenitor Cells
FADD	- Fas-Associated Death Domain
FAO	- Fatty Acid Oxidation
FBS	- Fasting Blood Sugar
Fe ²⁺ -TPTZ	- Ferrous Tripyridyltriazine
Fe ³⁺ -TPTZ	- Ferric Tripyridyltriazine
FFA	- Free Fatty Acids
FFAR	- Free Fatty Acid Receptor
FGF	- Fibroblast Growth Factor
FLICE	- FADD-like IL-1 β -Converting Enzyme
FRAP	- Ferric Reducing Antioxidant Power
GAE	- Gallic Acid Equivalent
GLO	- L-gulonolactone oxidase
GLUT 2	- Glucose Transporter 2
GLUT 4	- Glucose Transporter 4
GPx	- Glutathione Peroxide
GRd	- Glutathione Reductase
GSDME	- Gasdermin E
GSH	- Reduced Glutathione
GSIS	- Glucose Stimulated Insulin Secretion
GSIS	- Glucose-Stimulated Insulin Secretion
GSSG	- Oxidised Glutathione
Gulo	- L-Gulonolactone Oxidase
4-HNE	- 4-hydroxy-2E-nonenal
H ₂ O ₂	- Hydrogen peroxide
Hb	- Haemoglobin

HbA1c	- Glycolated Haemoglobin
HDL	- High Density Lipoprotein
HDL-C	- high-density-lipoprotein
HFD	- High Fat Diet
HO·	- Hydroxyl radical
HO·2	- Hydroperoxyl radical
HOMA	- Homeostasis Model Assessment
HOMA-IR	- Homeostatic Model Assessment of Insulin Resistance
HSCs	- Hematopoietic Stem Cells
IDDM	- Insulin Dependent Diabetes Mellitus
IDF	- International Diabetes Federation
IGFI	- Insulin like Growth Factor 1
IL-1	- Interleukin-1
iNOS	- inducible Nitric Oxide Synthase
IP ₃	- Inositol-1,4,5-triphosphate
IR	- Insulin Resistance
JNK	- Jun N-terminal kinase
LA	- Linoleic Acid
L-Arg	- L-arginine
LDL	- Low Density Lipoprotein
LDL-C	- Low-Density Lipoprotein Cholesterol
LH	- Luteinizing Hormone
LOOH	- Lipid Hydroperoxides
LSL	- L-Selectin Ligand
MAPK	- Mitogen-Activated Protein Kinase activation
MDA	- Malondialdehyde
MS	- Metabolic Syndrome
MSCs	- Mesenchymal Stem Cells
NC	- Normal Control
NF-κB	- Nuclear factor-κB
NIDDM	- Non-Insulin Dependent Diabetes Mellitus

nNOS	- neuronal Nitric Oxide Synthase
NO	- Nitric Oxide
NOS	- Nitric Oxide Synthase
O ₂ ⁻	- Superoxide radical
OGT	- Oral Glucose Tolerance
OGTT	- Oral Glucose Tolerance Test
OH ⁻	- Hydroxyl radical
ox-LDL	- oxidized LDL
PBMCs	- Peripheral Blood Mononuclear Cells
PC	- Phosphatidylcholine
PCR	- Polymerase Chain Reaction
PDGF	- Platelet-Derived Growth Factor
PE	- Phosphatidylethanolamine
PGE ₂	- Prostaglandin E ₂
PGs	- Prostaglandins
PHF	- Poly Herbal Formulation
PI3K	- Phosphoinositide 3-Kinase
PIP ₂	- Phosphatidylinositol-4,5-bisphosphate
PL	- Phospholipids
PLC	- Phospholipase C
PPAR	- Peroxisome Proliferator-Activated Receptor
PPARs	- Peroxisome Proliferator-Activated Receptors
PPAR- α	- Peroxisome proliferator-activated receptor-alpha
PPAR- δ	- Peroxisome proliferator-activated receptor-delta
PPs	- Polyphenols
PS	- Phosphatidylserine
PUFAs	- Polyunsaturated Fatty acids
qPCR	- quantitative PCR
ROS	- Reactive Oxygen Species

SAPK	- Stress-activated kinases
SC	- Stem Cell
SE2	- Stem Enhance 2
SM	- Sphingomyelin
Smac	- Second mitochondria-derived activator of caspases
SOD	- Superoxide Dismutase
SRY	- Sex-determining Region on Y-chromosome
STZ	- streptozotocin
T2DM	- Type 2 Diabetes Mellitus
TAE	- Tannic Acid Equivalents
TC	- Total Cholesterol
TG	- Triglycerides
TNFR 1	- Tumor necrosis factor receptor type I
TNFR2	- Tumor necrosis factor receptor type 2
TNF- α	- Tumor necrosis factor – alpha
TPTZ	- Tripyridyltriazine
VCAMs	- Vascular Cell Adhesion Molecules
Vit C	- Vitamin C
Vit E	- Vitamin E
VLDL	- Very Low Density Lipoprotein
VLDL-C	- Very-Low-Density Lipoprotein Cholesterol
VSMC	- Vascular Smooth Muscle Cells
WBC	- White Blood Cells

Chapter 1

Introduction and Review of Literature

I. Introduction

Diabetes mellitus (DM) is a most common chronic disease, rising dramatically and the most relevant data suggests the global prevalence reaching 642 million affected people by the year 2040 (Shiferaw et al., 2020). It is mainly classified into two, type -I and type -II diabetes. Type -I diabetes mellitus is resulted by the autoimmune destruction of pancreatic- β cells which cause severe insulin deficiency. Type -II diabetes on the other hand is caused by the decreased or absent cellular response to insulin at the receptor level (Mahmoud et al., 2020). Till now there is no effective treatment for lifelong recovery from diabetes. Lack of proper treatments, uncontrolled diet and hyperglycaemia makes it a severe devastating disease and cause blindness, kidney failure, lower limb amputation, other long-term consequences, and eventually leads to death (Salehi et al., 2019). There are plenty of medicinal plants used in Ayurveda for the treatment of diabetes mellitus. These plant products were more affordable, with fewer side effects and more effective in the treatment of diabetes mellitus compared to high-cost synthetic medicines (Kooti et al., 2016).

Indian traditional medicinal system Ayurveda offers several plants for the treatment and management of DM. Which are less toxic and cost effective for treating diseases like diabetes. Dhanwantaram Kashaya (DK) is an Ayurvedic polyherbal formulation prepared by using many

of the plants used for the treatment of diabetes. DK is in the form of a decoction having regeneration property (Joshi and Bhonde., 2014). It is available commercially, contains a combination of more than 40 herbal ingredients and is quoted in the classical text Ashtanga Hrudayam of Ayurveda (Upadhyaya., 2006). The results reported and discussed in this thesis is the observations in the studies on this polyherbal drug, which was aimed to evaluate its potential as a long lasting cure for diabetes.

Glucose is one of the primary energy fuels in our body. Glucose homeostasis is very important for the proper functioning of the body. Concentration of glucose in blood depends upon the production and utilization of it by different tissues (Bilal et al., 2016). Insulin is one of the key anabolic hormones produced by pancreatic β - cells in response to increased blood glucose level. Insulin deficiency decreases the uptake of glucose in liver (Baquer et al., 1998) and lead to its increased level in blood, a condition termed as hyperglycemia. For the evaluation of the effect of DK on glucose metabolism, fasting blood sugar level, HOMA IR index, oral glucose tolerance (OGT), serum insulin level, organ weight, body weight, haemoglobin levels and glycosylated haemoglobin levels, expression of insulin gene and histopathological changes in liver and pancreas were studied.

Phytochemicals are naturally occurring biologically active chemical compounds found in plants contributing to the plant's colour, aroma and flavour (Koche et al., 2016). Plant derived products containing large variety of bioactive components and natural antioxidants has got

the ability to scavenge the free radicals produced in the body with fewer side effects. Medicinal plants used in Ayurveda are rich source of natural antioxidants due to the presence of polyphenols and flavonoids. Plethora of research reports are there revealing the ability of these compounds to scavenge free radicals in our body (Musini et al., 2015). This thesis also reports the observations on the preliminary analysis of phytochemicals and the ability of DK as an *in vitro* free radical scavenger.

Free radicals are highly active, unstable atoms or molecules and have unpaired electrons. In biological system two main classes of free radicals exist, namely oxygen-based radicals or reactive oxygen species (ROS) and nitrogen-based radicals or reactive nitrogen species (RNS) (Li et al., 2015). Oxidative stress is one of the major reasons for the pathogenesis of several chronic diseases like diabetes. Several studies have shown that increased free radical production and decreased antioxidant defence system is also associated with insulin dependent (type 1) and noninsulin dependent (type 2) diabetes (Naziroglu and Butterworth., 2005). Both enzymatic and non-enzymatic antioxidants are essential in biological system for the removal of free radicals and to maintain cellular response under various physiological conditions (Li et al., 2015). This study also evaluated the effect of DK on enzymatic and non-enzymatic antioxidants and concentration of serum total protein.

Lipids are one of the important biomolecules and abnormal lipid metabolism leading to dyslipidemia (Wu and Parhofer., 2014) is a

prominent characteristic of diabetes. Alteration in lipid profiles like Total Cholesterol (TC), Free Fatty Acids (FFA), Phospholipids (PL) and Triglycerides (TG) are one of the important characteristics of diabetes mellitus (Kandasamy and Ashokkumar., 2014). Elevated total cholesterol and triglycerides are well established risk factors of cardiovascular diseases (CVD) (Lee et al., 2017). Managing lipid metabolism is an important aspect of diabetes care hence the levels of major lipids such as TC, FFA, PL and TG in the tissues and serum of experimental (diabetic) animals were evaluated.

ROS induce the oxidation in different biological macromolecules such as proteins, lipids, and nucleic acids and ultimately leads to structural and functional changes in these molecules (Ito et al., 2019). Lipid peroxidation is a process in which oxidants such as reactive oxygen species (ROS) or free radicals attack lipids containing carbon-carbon double bonds especially seen in polyunsaturated fatty acids (PUFAs) (Yin et al., 2011). Malondialdehyde (MDA) is one of the usually used biomarker for lipid peroxidation and hence its levels in different tissues and serum were also analysed. Considering the importance of Peroxisome proliferator-activated receptor (PPAR) in diabetic condition, we also studied the effect of DK on the expression of PPAR α and δ at mRNA level in pancreas.

DM is associated with various health complications many of which are due to the alterations in the expression of different genes. Hence this study highlights the effect of DK on expression of various genes associated with diabetic complications (NF- κ B, TNF- α , Cox-2, Caspase 8 and Caspase 3).

Stem cells are unique, unspecialised population of cells having the potential to regenerate and differentiate into specialised cells under appropriate micro environment and signal (Peng et al., 2018). Mesenchymal stem cells (MSCs) are multipotent stromal cells, play a crucial role in the development of different stem cells in vivo (Krebsbach et al., 1999). Use of stem cells for regenerating pancreatic islets in diabetic condition is being tried as a therapeutic aid. But procedural complications, increased costs and the need of immunosuppression makes it a less preferred method of treating diabetic complications. Use of natural products as tools for the mobilisation of stem cells and their differentiation into pancreatic β -cells is considered as a promising method in the treatment of DM. DK is mainly used for regeneration related disorders and also to improve quality of stem cells (Warrier et al., 2013, Sruthi and Sindhu., 2012). DK increased proliferation rate, decreased the turnover time, delayed senescence and also is able to improve the yield and quality of stem cells in vitro (Joshi and Bhonde., 2014).

This study also aimed to corroborate the effect of DK on the viability of bone marrow cells along with dose response. The ability of DK to mobilise stem cells from bone-marrow or other stem cell niches was analysed by studying the levels of CD34+ gene in peripheral blood cells. Effect of injecting stem cells on the regeneration of islet cells was studied for a basic comparison with that of DK administration. In this respect the we studied the parameters such as number of different blood cells (WBC, Neutrophils, Lymphocytes and Monocyte) body weight, fasting blood glucose level and serum insulin level in diabetic rats administered DK and stem cells.

Ability of various pharmaceuticals and natural products to mobilise stem cells from bone-marrow or other stem cell niches was already reported. Mobilised stem cells could home into damaged tissue and result in desired tissue repair or regeneration. Stem Enhance 2 (SE2) is one of the important natural products extracted from *Aphanizomenon flos aquae* (AFA). AFA is a unicellular prokaryotic microorganism included under the category of blue-green algae and belongs to the phylum cyanobacteria (Nuzzo et al., 2018). SE2 tablet is enriched with the cytoplasmic and cell wall fractions of AFA plant and also contains L-selectin ligand (LSL) (Ismail et al., 2013). This ligand helps the release of stem cells (CD34+ cells) from the bone marrow (Jensen et al., 2007). In this study SE2 is used as an established stem cell mobiliser to compare with the ability of DK to mobilise stem cells in diabetic rats. Different physiological or biochemical parameters affected by DK administration were also studied under SE2 administration.

Overall this study mainly focused on the effect of DK in ameliorating diabetic complications which were compared with the ability of injected stem cells and also to that of a well known stem cell mobiliser, SE2. The study was extensive with regard to the effect of DK except that the study is not addressing the mode of action in detail of DK through the mobilisation of stem cells. Lack of time and shortage of biochemicals compelled to limit the parameters studied. It is noteworthy that the observations made in this study are good enough to reach a conclusion that DK is a promising candidate for developing a successful therapeutic regimen with minimum or no side effects.

1.1: Review of Literature

Diabetes Mellitus is a group of metabolic diseases affecting almost all organs of the body. Diabetes is not only a serious chronic health disease but also a public health problem. Hyperglycemia is one of the important characteristics of it (Jayachandran et al., 2018). It is occurring due to insufficient production of insulin in pancreas or inefficiency of the body cells to effectively use the insulin produced. Such situations cause disturbances in the metabolism of carbohydrate, lipid and protein (Shobana et al., 2009). This chronic endocrine disorder is associated with several complications including neuropathy, nephropathy and retinopathy (Sreekutty and Mini., 2016). In addition children with absolute insulin deficiency are suffering from polyuria, polydipsia, polyphagia, weight loss and blurred vision etc. (Kharroubi and Hisham., 2015). According to International Diabetes Federation (IDF) the prevalence of diabetes was 415 million in 2015 globally and the number was expected to increase to 693million by the year 2045 (Cho et al., 2018). Diabetic patients have an increased risk of developing a number of serious life-threatening health problems and increased mortality (Baena-Diez et al., 2016). The global prevalence of diabetes and impaired glucose tolerance in adults has been increasing over a day by day. Diabetes is the 7th leading cause of death and ranked 3 on the basis of considering its all fatal complications (Bhambhani., 2015). 69.1 million diabetic patients lived in India in 2016 (Karna., 2017) and has now been declared as “Diabetic Capital” of the world (Dimple et al., 2018).

According to the etiology and clinical presentation diabetes was mainly classified in to two types. Insulin dependent diabetes mellitus also known as type –I diabetes and noninsulin dependent diabetes known as type 2 diabetes (Spahr., 1996). Type -1 diabetes is characterized by loss of decreased number of pancreatic β -cell of the islets of langerhans by autoimmune attack (Rother., 2007) and type 2 diabetes is characterized by insulin resistance and combined with relatively reduced insulin secretion. Incidence of type-1 diabetes is increasing in both developed and developing countries. It is the major type of diabetes seen in younger generation, frequently in children and adolescents (Dimple et al., 2018) and type 2 diabetes is more prevalent in adults (Nagalakshmi and Sujatha., 2017). Rapidly increasing rate of Type 1 diabetes suggested the involvement of nongenetic factors in its etiology and reactive oxygen species (ROS) (Stene et al., 2008). ROS affect two different ways in type-I and type -2 diabetes. In type I diabetes ROS initiate β -cell dysfunction by autoimmune reactions and inflammatory cytokines (Cnop et al., 2005). In type 2 diabetes, it activates apoptotic pathway of pancreatic β -cells, impaired insulin synthesis and also be the reason for insulin resistance (Evans et al., 2003, Simmons., 2006).

Oxidative stress is the widely accepted participant in the pathogenesis and progression of type –I diabetic complications (Drews et al., 2010). Oxygen plays a crucial role in metabolism and produce free radical such as superoxide (O_2^-), hydroxyl (OH^-) (Raymond and Segrè., 2006) and non-radical hydrogen peroxide (H_2O_2), which are collectively known as reactive oxygen species (ROS). ROS are necessary for

cellular communication and homeostasis in different organisms and have important roles in cell signaling (Egeaa et al., 2017). ROS modulate various physiological processes and lead to the progression of several diseases including diabetes mellitus (DM). Controlling the production or the removal of the ROS from the body could be a therapeutic strategy for such diseases (Mule and singh., 2018). Maintaining proper functional integrity of cell is important in biological systems and the balance between production and neutralization of ROS is necessary for this. This process is carried out by the help of natural antioxidants and the antioxidant enzymes such as Superoxide dismutase (SOD), Catalase (CAT), Glutathione peroxide (GPx), Glutathione Reductase (GRd) etc. (Lubrano and Silvana., 2015). ROS formed from oxygen and nitrogen cause damages to the complex antioxidant system that exists in mammals (Li et al., 2015). Elevated levels ROS create oxidative stress and responsible for severe metabolic dysfunction and damage the macromolecules in the living system (Lledías et al., 1998).

To control the over production of ROS aerobic cells have developed their own defense system, which includes enzymatic or non enzymatic antioxidant system. In this first priority is given to the Superoxide dismutase (SOD), Catalase (CAT), Glutathione peroxide (GPx), Glutathione reductase (GRd), Reduced glutathione (GSH) and the second group consist of ascorbic acid (Vitamin C) and alpha tocopherol (Vitamin E) (Mataix et al., 1998). Imbalance between pro-oxidants and antioxidants occur due to over production of ROS and

mitochondrial electron transport chain is the main source of generation of ROS (Narayanan., 2010). SOD converts the most dangerous superoxide anion to less toxic hydrogen peroxide and oxygen while the haem –containing ubiquitous enzyme catalase detoxifies hydrogen peroxide converting it into water and oxygen (Selvan., 2008). Three forms of SOD are present in humans namely cytosolic SOD consisting of Cu and Zn, mitochondrial SOD containing Mn and extracellular (EC-SOD) (Majima et al ., 1998). Mitochondrial and cytosolic SOD are essential part of the living system and helps to remove O_2^- radical in our body (Guan et al., 1998). Extra cellular SOD is another important Copper and Zn containing tetrameric glycoprotein. In mammalian cells EC-SOD is regulated by cytokines (Buschfort et al., 1997). The antioxidant enzyme catalase is known as most efficient enzyme in the biological system (Lledías et al., 1988). It protects the cells from the attack of H_2O_2 and plays an important role against oxidative stress (Hunt et al., 1988). The antioxidant enzyme GPx also scavenges H_2O_2 in lysosomes and mitochondria (Yung et al., 2006). In addition to GPx involved in diminution of lipid- peroxides and organic hydroperoxides (Sreekutty and Mini., 2016), another important antioxidant enzyme GRd helps for the recycling of oxidized glutathione back to glutathione (Salvayre et al., 2009). Pancreatic β -cells express low level of antioxidant enzymes, so they are increasingly susceptible to oxidative damage (Dinić., 2016).

Reduced Glutathione is one of the main intracellular nonprotein compounds and has many biological functions such as maintenance of

membrane protein sulfhydryl groups in the reduced form, the oxidation of which can otherwise cause altered cellular structure and function. It is also a cofactor for many enzymes such as glutathione peroxidase etc. (Comporti., 1987, Giugliano et al., 1996). Vitamin E is one of the main lipid-soluble chain-breaking antioxidant that protects biological membranes from lipid peroxidation (Scholz et al., 1997). Vitamin C is another potent dietary antioxidant (Wawrzyniak et al., 2013). It is involved in several metabolic activities, increases blood circulation and essential cofactor for the enzymes prolyl hydroxylase and lysyl hydroxylase that catalyzes the hydroxylation of proline and lysine residues, respectively, during collagen biosynthesis (Kucharz., 1992).

Biochemical and physiological consequences of long-term hyperglycemia includes the generation of advanced glycation end products (AGEs) and reactive oxygen species (ROS), which are involved in the development of several human diseases (Mapanga and Essop., 2016). Concentration of glucose and its duration of exposure determines effect of glucose on β -cell function. Chronic hyperglycemia was reported to generate glucose-derived reactive oxygen species (ROS) which lead to the dysfunction of β -cell. Excess glucose increase glycosylation of proteins and the glycosylated proteins induce endoplasmic reticulum stress and apoptosis (Cohen et al., 2011). Molecular level studies had also revealed the effect of oxidative stress due to hydroxyl radicals generated by autoxidation of glucose on β cells.

Streptozotocin (STZ, 2-deoxy-2-(3-(methyl-3-nitrosoureido)-D-glucopyranose) is synthesized by *Streptomyces achromogenes* (Szkudelski., 2001). Rats made diabetic by STZ injection are the most commonly used animal model for the investigation of antidiabetic activity of plant extracts (Kooti., 2016). It induce experimental type I diabetes in animal models through its cytotoxic effect on pancreatic β -cells by the mechanism associated with generation of ROS (Punitha et al., 2005, Evelson et al., 2005). It causes the deficiency of insulin and act as a diabetogenic agent in experimental models (Szkudelski., 2001). STZ is taken up by β -cells through glucose transporter GLUT 2. Several previous reports have already shown that alkylation of the DNA is the main mechanism behind the action of STZ- induced β -cell death (Delaney et al., 1995, Elsner et al., 2000).

Hypertension is also associated with diabetes and 70 % of diabetic people are suffering from this side effect. It is one of the main reasons for developing cardiovascular disease in diabetic patients (Dhaliwali., 2015). Atherosclerotic cardiovascular diseases are one of the main diseases associated with diabetic patients. Dyslipidemia is another one of the important common disorder seen in diabetic condition, which also causes cardiovascular complications (Bhambhani., 2015).

Till now there is no effective treatment for life-long recovery from Diabetes Mellitus. Stem cells are used as an alternative (Abdulazeez., 2015), but need advanced clinical skills and advanced laboratory manipulations. Restoration of β -cell function through pancreas or islet

transplantation is a possible way to provide lifelong recovery from DM (Jamiolkowski., 2012) but the immune rejection of allosteric stem cells warrants the need of expensive immune suppression.

Indian traditional medicinal system Ayurveda considers the imbalance in thridoshas ie., vata (air or wind energy), pitta (transformative nature of Fire energy), kapha (phlem – water energy) leads to diseases (Dimple et al., 2018). There are plenty of medicinal plants used in Ayurveda for the treatment of diabetes mellitus. These plant products were more affordable, less side effects and more effective in the treatment of diabetes mellitus compared to high cost synthetic medicines (Kooti et al., 2016). Dhanwantaram Kashaya is a Ayurvedic polyherbal formulation prepared by using many of the plants used for the treatment of diabetes.

1.2: Dhanwantaram Kashaya

Medicinal plants are used from ancient time for the treatment and management of various diseases. Dhanwantaram Kashaya is a poly herbal formulation in the form of a decoction having regeneration property (Joshi and Bhonde., 2014). It is quoted in the classical text Ashtanga Hrudayam of Ayurveda (Upadhyaya., 2006). This formulations is extensively used in the management of diseases manifested due to vitiation of vata and vata-rakta (mostly disease of connective tissues, bones, joints and nervous system) (Sruthi and Sindhu., 2012). General dosage of Dhanwantaram Kashaya is 10ml to 15 ml dissolved in 30 to 45ml boiled and cooled water twice a day.

Dhanwantaram Kashaya (DK) is widely used in Ayurvedic medicine as growth stimulant in children and for nerve regeneration. It also improves memory, help post-partum restoration and in the treatment of neuralgia. It is available commercially and contains a combination of more than 40 herbal ingredients. DK is mainly used for regeneration related disorders and also improve quality of stem cells (Warrier et al., 2013) (Sruthi and Sindhu., 2012). DK increased proliferation rate, decreased the turnover time and also delayed senescence and also is able to improve the yield and quality of stem cells in vitro (Joshi and Bhonde., 2014).

Dhanwantharam kashayam is a polyherbal formulation of forty ingredients. The key ingredients that account for its antioxidant property are;

1.3: Ingredients of Dhanwantaram Kashaya (Ashtanga Hrudayam)

Table 1.1: Dhanwantaram Kashaya Ingredients

Sl No	Common Name (colloquial name)	Botanical Name
1	Balamula	<i>Sida cordifolia</i>
2	Yava	<i>Hordeum vulgare</i>
3	Kola	<i>Ziziphus abyssinica</i> ³
4	Kulattha	<i>Macrotyloma uniflorum</i>
5	Kasmari	<i>Gmelina arborea</i>
6	Vilwa	<i>Aegle marmelos</i>
7	Patala	<i>Stereospermum Colais</i>

8	Shyonaka	<i>Oroxylum indicum</i>
9	Agnimantha	<i>Premna corymbosa</i>
10	Shalaparni	<i>Desmodium gangeticum</i>
11	Prinshnaparni	<i>Pseudarthria viscida</i> 6
12	Brihati	<i>Solanum anguivi</i>
13	Nidigdhika	<i>Solanum surattense</i>
14	Gokshura	<i>Tribulus terrestris</i>
15	Dwimeda	<i>Asparagus racemosus</i>
16	Daru	<i>Cedrus deodara</i>
17	Manjishta	<i>Rubia cordifolia</i>
18	Dwikakoli	<i>Withania somnifera</i>
19	Chandana	<i>Santalum album</i>
20	Sariba	<i>Hemidesmus indicus</i>
21	Kushtha	<i>Saussurea costus</i>
22	Tagara	<i>Valeriana Jatamansi</i>
23	Diwjiwaka	<i>Pueraria tuberosa</i>
24	Kalanusari	<i>Trigonella foenum- graecum</i>
25	Shaileya	<i>Parmelia perlata</i>
26	Vacha	<i>Acorus calamus</i>
27	Punarnava	<i>Boerhaavia diffusa</i>
28	Ashwagandha	<i>Withania somnifera</i>
29	Vari	<i>Asparagus racemosus</i>
30	Kshirasukla	<i>Ipomoea mauritiana</i>
31	Yashti	<i>Glycyrrhiza glabra</i>
32	Haritaki	<i>Terminalia chebula</i>
33	Amalaki	<i>Phyllanthus emblica</i>
34	Vibhitaki	<i>Terminalia bellirica</i>
35	Rasa	<i>Commiphora myrrha</i>
36	Satahwa	<i>Anethum graveolens</i>
37	Ela	<i>Elettaria cardamomum</i>
38	Twak	<i>Cinnamomum verum</i>
39	Patra	<i>Cinnamomum tamala</i>

1. Balamula - *Sida cordifolia*

Root of *sida cordifolia* (Family: Malvaceae) commonly known as ‘Balamula’ is one of the valuable drug used in Ayurveda. *Sida cordifolia* is a small erect, downy shrub grows well through the plains of India especially in damp climates. Its roots consist of phytoconstituents such as ephedrine, saponine, choline, pseudoephedrine, betaphenethylamine, vasicine, hypaphorine and related indole alkaloids (Jain et al., 2011). It was reported to have more effect on vata dosha. It is a potential remedy to reduce severity of Parkinsonism (Khurana and Gajbhiye., 2013). Methanolic extract of sida was found elevated the activity of antioxidant enzymes such as SOD, CAT, glutathione S-transferase etc. in HepG-2 cells (Pieme et al., 2010). Several previous studies have already reported its significant anti hyperglycemic and anti hypercholesterolemic activities (Kanth et al.,1999, Kaur et al., 2011).

2. Yava – Barley- *Hordeum vulgare*

Yava (*Hordeum vulgare*) popularly known as ‘Barley’ (Family: Poaceae) used in Ayurveda for various medicinal preparations and dietary treatment of disease like diabetes mellitus, obesity etc. (Rajesh et al., 2015). It is regarded as a most ancient cereal in Atharva Veda (Rajesh et al., 2016). It is an annual, erect, stout, tufted grass and its origin is considered in middle east. Detailed description of yava was seen in different samhita like charaka, sushurta and astanga hridayam. It also have the ability to lower the fasting blood glucose and

glycosylated hemoglobin levels, and decreased plasma total cholesterol, triglycerides, and free fatty acid levels (Li et al., 2003).

3. Kola- *Ziziphus abyssinica*

Ziziphus abyssinica (Family: Rhamnaceae) is commonly known as ‘catch thorn’ in English (Gyasi et al., 2017). It is a small evergreen spiny shrub growing in arid or dry tropical and subtropical regions, with severe heat and slight frost. The whole parts of the plant have been traditionally used for the treatment of various diseases like pneumonia, tonsillitis, newcastle disease, snake bite, burns, wounds, tachycardia, pectoral pain, migraines and as pain-killers (Okello et al., 2010). Several previous studies have already reported the antioxidant activity of the extract of the different parts of the plant (Nyaberi et al., 2010). It also have gastro protective effect and used for the treatment of ulcer (Yau et al., 2017).

4. Kulattha- *Macrotyloma uniflorum*

Macrotyloma uniflorum is (Family: Fabaceae) commonly known as ‘horse gram’. It is one of the leguminous plants and seeds are rich source of carbohydrate, protein, vitamins, minerals fat and soluble fibers (Ranasinghe and Ediriweera., 2017). It is mainly grown in India, Africa, Australia, Burma, Malaysia, Mauritius, and the West Indies (Prasad and Singh., 2015). Horse gram seeds are widely used in Ayurveda and consist of bioactive substances like phytic acid, phenolic acid, fiber, enzymatic and proteinase inhibitors (Duke and Reed., 1981). Several studies have reported the anti-diabetic and anti-hypercholesterolemic activity of seed extract (Gupta et al., 2011). It

improved the activity of antioxidant enzymes such as SOD, CAT etc and increased the concentration of non enzymatic antioxidant glutathione (Panda and Suresh., 2015). Its ability to scavenge the hydroxyl free radicals in our body is also well reported (Marimuthu and Krisnamoorthi., 2013).

5. Kasmari- *Gmelina arborea*

Gmelina arborea (Family: Verbenaceae) occurring naturally throughout India used as a traditional remedy against diabetes mellitus. It lowered fasting blood glucose level, induced β - cell regeneration and increased insulin secretion in STZ induced diabetic model (Attanayake et al., 2016).

6. Vilwa- *Aegle marmelos*

Aegle marmelos (Family: Rutaceae) commonly known as ‘Bael’ is widely used in Indian traditional medicinal system due to its various medicinal properties. It is a native to Northern India but widely seen throughout the India, Sri Lanka, Burma, Bangladesh, Thailand and Indo-China (Brijesh et al., 2009). It is a medium to large sized tree with trifoliolate leaves, short flower and globular fruits (Das and Roy., 2012). It also has anti diabetic, antihyperglycemic (Narender et al., 2007) and antioxidant activities (Ghatule., 2014).

7. Patala- *Stereospermum Colais*

Stereospermum Colais (Family: Bignoniaceae) is one of the ingredients of dasamoola and used in many important Ayurvedic formulations (Rani and Padmakumari., 2012.) It is a large straight

stemmed deciduous tree found throughout in moist regions of India chiefly in deciduous forests (Prema et al., 2013). The roots of this plant are bitter, astringent, acrid, cardio tonic, anti inflammatory, antidiabetic, antibacterial, febrifuge and tonic (Warrier., 1996). The antioxidant and wound healing activity of these plant extract was also reported in previous studies (Bharathi et al., 2010).

8. Shyonaka- *Oroxylum indicum*

Oroxylum indicum (Family–Bignoniaceae) is a traditional medicine in Asia used for prevention and treatment of several diseases such as jaundice, arthritic and rheumatic problems, gastric ulcers, tumors, respiratory diseases, diabetes, diarrhea and dysentery (Dinda et al., 2015). It is a small medium sized deciduous tree commonly called as Indian trumpet tree seen in tropical countries like India, Japan, China, Sri Lanka and Malaysia (Harminder et al., 2011). Flavonoids are one of the main constituents of all parts of the plant (Chen et al., 2003) and have potent free radical scavenging activity (Upaganlawar and Tende., 2007). It is also one of the ingredients of dasamoola.

9. Agnimantha- *Premna corymbosa*

Premna corymbosa (Family: Verbenaceae) is a small sized tree or large shrub with short trunk and numerous branches (Karthikeyan and Deepa., 2011). It is used as a traditional medicine for rheumatic disorder and have anti inflammatory activity (Karthikeyan and Deepa., 2010). It is a major ingredient of several Ayurvedic preparations.

10. Shalaparni- *Desmodium gangeticum*

Desmodium gangeticum (Family: Fabaceae) is commonly known as shalaparni, widely used in several formulations in Ayurveda and treatment of neurological diseases (Joshi and Parle., 2006). It is abundantly seen throughout India and considered as a master of medicinal plants in ayurveda (Vedpal et al., 2016). This Indian medicinal plant widely used in our traditional medicinal system for various diseases like fever, cataract, typhoid, piles, bronchitis, dysentery, asthma and various other inflammatory conditions arising from 'vata' disorder (Rastogi et al., 2011). It is one of the important ingredients of Dasmulakwatha of Ayurveda (Vedpal et al., 2016).

11. Prinshnaparni- *Pseudarthria viscida*

Pseudarthria viscida (Family: Leguminosae) is a perennial shrub commonly called Moovila useful in treat diseases like fever, rheumatism, bronchial asthma, hemorrhoids (Warrier et al., 1996) and diabetes Mellitus (Rajendran et al., 2010). The plant and root extracts possess potential antioxidant activity (Mathew and Sasikumar., 2007). It is one of the essential components of many ayurvedic formulations like Dashamoola, Mahanarayana taila and Dhantara taila (Sangeetha et al., 2014).

12. Brihati- *Solanum anguivi*

Solanum anguivi (Family: Solanaceae) is a rare ethnomedicinal herb found in many places throughout the non arid part of Africa and

commonly known as African eggplant (Elekofehinti et al., 2013). It is also widely distributed in India, China, Thailand etc. This plant is used as a therapeutic agent for various diseases and rich in essential minerals and vitamins (Denton and Nwangburuka., 2011). It is also used for the treatment of diabetes mellitus, atherosclerosis (Elekofehinti et al., 2012) and have antioxidant activity (Elekofehinti et al., 2012). This plant extract exhibit potential free radical scavenging activity, ferric reducing power, metal iron chelating and also have lipid peroxidation activity (Elekofehinti et al., 2013).

13. Nidigdhika - *Solanum surattense*

Solanum surattense (Family: Solanaceae) commonly growing perennial herbaceous weed found throughout India, Sri Lanka, South East Asia, Malaysia and tropical Australia. This plant is traditionally used for curing several diseases such as fever, cough, asthma and diabetes (Ghildiyal and Joshi., 2014). This plant have antioxidant potential (Meena et al., 2010) and antidiabetic activities (Gupta et al., 2005).

14. Gokshura- *Tribulus terrestris*

Tribulus terrestris (Family: Zygophyllaceae) is an annual plant commonly known as Tribulus. It is commonly seen in sub-tropical regions such as India, China, South America, Mexico, Spain, Bulgaria, and Pakistan. This plant has the ability to enhance sexual functions and providing cardiac protection. It also has anti-urolithic, antidiabetic, anti-inflammatory, antitumor and antioxidants effects (Zhu et al., 2017).

15. Dwimeda- *Asparagus racemosus*

Asparagus racemosus (Family: Asparagaceae) is an amazing medicinal plant used in Ayurveda commonly known as “Queen of herbs”. It is seen throughout India, Sri Lanka and the Himalayas. Its root extract have antiulcer, antioxidant, antidiarrhoeal, antidiabetic and immunomodulatory activities. This drug is an effective medicine for the problems related with female reproductive system and used as a rejuvenation tonic for females. Steroidal saponins are the major active constituents of *Asparagus racemosus* and which are present in the roots of this plant. It is one of the most commonly used indigenous medicinal plants in Ayurveda (Alok et al., 2013).

16. Daru- *Cedrus deodara*

Cedrus deodara (Family: Pinaceae) is an evergreen tree commonly seen in Mediterranean region and the western Himalayas. The wood of *Cedrus deodara* used in Ayurveda for the treatment of inflammations, rheumatoid arthritis (Shinde and Phadke., 1999) and the ethanolic extract of wood have significant anti-spasmodic activity (Kar., 1975). The alcoholic extract of stem of this plant showed anti- cancer activity (Singh and Shanmugavel., 2007).

17. Manjishta- *Rubia cordifolia*

Rubia cordifolia (Family: Rubiaceae) is commonly known as Indian Madder. This perennial, climbing herb growing near streams and rivers along the upper ghats in evergreen forests up to 3750m above sea level (Priya and Siril., 2014). It is a rich source of anthraquinones and the

root of this plant has high medicinal value. This plant has ability to remove toxins from the blood. *Rubia cordifolia* is used for the treatment of a number of ailments such as Alzheimer's, diabetes, cancer, acne, inflammation, allergy, enterocolitis, bacterial and viral infection. It also has immunomodulatory, analgesic, diuretic, gastroprotective, hepatoprotective and nephroprotective effects (Bhatt and Kushwah., 2013). It is also used as an effective medicine for non healing diabetic foot ulcer (Ojha et al., 1994). Alcoholic extract of the roots of this plant showed significant hypoglycemic activity in alloxan induced diabetic rats (Patil et al., 2006).

18. Dwikakoli- *Withania somnifera*

Withania somnifera (Family: Solanaceae) commonly known as Ashwagandha is a well known Indian medicinal plant widely used in Ayurveda for many clinical ailments such as diabetes mellitus, nervous and sexual disorders (Singh et al., 2010). This xerophytic plant was found in the drier parts of India, Sri Lanka, Afghanistan, Baluchistan and Sind and is distributed in the Mediterranean regions. It act as a powerful antioxidant and increase the activities of the enzymes such as SOD, CAT and GPx in rats brain (Uddin., 2012).

19. Chandana- *Santalum album*

Santalum album (Family: Santalaceae) commonly known as Indian Sandalwood is one of oldest and precious natural resource with high medicinal value (Kumar et al., 2015). This evergreen hemi parasitic tree is a native of the highlands of southern India mainly Coorg,

Chennai and Mysore (Sindhu et al., 2010). It is mostly found in the drier tropical regions of Indian peninsula, eastern Indonesia and northern Australia. Ayurveda regarded sandalwood as an antiseptic, antipyretic, antiscabietic, diuretic, expectorant, and stimulant. It is prescribed for the treatment of bronchitis, dysuria, urinary infection and gonorrhoea (Pande., 1977). It also have antihyperglycemic and antioxidant activities (Misra and Dey., 2013).

20. Sariba- *Hemidesmus indicus*

Hemidesmus indicus (Family: Asclepiadaceae) commonly known as “Indian Sarsaparilla” (Lalrinpuia et al., 2017). This perennial semi erect plant found in India, Sri Lanka, Pakistan, Iran, Bangladesh and Moluccas (Siddique., 2004). All parts of this plant is used in Ayurveda for various ailments such as dysentery, diarrhoea, syphilis, dyspepsia, leucoderma, diuresis, burning of body, chronic fever, asthma, liver diseases, venereal diseases, leprosy, urinary tract infection, asthma, arthritis, bronchitis, epileptic seizures, high blood pressure, skin diseases, rheumatism, chronic nervous diseases, impotence and immune disorders (Lalrinpuia et al., 2017). Aqueous extract of whole plant of *H indicus* showed significant free radical scavenging activity (Kumar et al., 2013) and the ethanolic extract of this plant increased the level of kidney superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and reduced glutathione (GSH) (Nadana and Namasivayam., 2007). It also has anti diabetic (Banerji et al., 2017) and anti hyperlipidemic activity (Verma et al., 2005).

21. Kushtha- *Saussurea costus*

Saussurea costus (Family: Asteraceae) is a perennial herb distributed across Himalayan region at 2500-3500m altitude (Rao et al., 2013). It consists of several active constituents such as terpenes, anthraquinones, alkaloids and flavonoids. This plant has antidiabetic activity (Upadhyay et al., 1996). It is also used for the treatment of several diseases and possesses various bioactivities such as antihepatotoxic, anti-inflammatory, antimicrobial, antitumor and antiulcer (Zahara et al., 2014).

22. Tagara- *Valeriana Jatamansi*

Valeriana Jatamansi (Family: Caprifoliaceae) is a small perennial herb native to Himalaya and distributed from Afghanistan to southwest China, India, Nepal, Bhutan, and Myanmar. It is one of the high value medicinal plants and contains flavone glycosides, lignans, sesquiterpenoids or sesquiterpenoid glycoside, bakkenolide type sesquiterpenoids, phenolic compounds, terpinoids, etc (Jugran et al., 2019). This plant possesses various medicinal activities such as antibacterial, anticancer, anticoagulant, antifungal, anti-inflammatory, antioxidative, antiprotozoal, hepatoprotective and neuroprotective (Dinda et al., 2009).

23. Diwjivaka- *Pueraria tuberosa*

Pueraria tuberosa (Family: Fabaceae) is commonly known as Vidari is an important and potential medicinal plant in our traditional

medicinal system Ayurveda. It is a deciduous climbing shrub distributed almost throughout India (Amin and Arbar., 2014). It is used as a fertility control agent, aphrodisiac, cardiogenic, diuretic and galactagogue. It also exhibited anti hyperglycemic, anti-hyperlipidemic and anti-fertility activities in male rats. It also has got hepatoprotective and anti-implantation activities in female rats (Rastogi et al., 2013).

24. Kalanusari- *Trigonella foenum- graecum*

Trigonella foenum- graecum (Family: Fabaceae) commonly known as Fenugreek is a short-living annual plant grown in many parts of Asia, Africa, and Europe (Venkata et al., 2017). Its hypoglycemic activity in diabetic subjects including experimental animal models and humans is well known. Soluble dietary fiber derived from its seeds enhanced peripheral insulin action (Hannan et al., 2007). It also has antioxidant activity. Seeds of Fenugreek restore the altered activity of enzymatic antioxidants such as SOD, CAT, GPx and GRd in heart, muscle and brain during diabetes (Baquer et al., 2011).

25. Shaileya- *Parmelia perlata*

Parmelia perlata (Family: Parmeliaceae) commonly known as Stone Flower mainly found in Himachal Pradesh and West Bengal. Methanolic extract of this plant showed significant antioxidant activity and the ethanolic extract have free radical scavenging activity. It also has antidiabetic activity (Lakshmi et al., 2013).

26. Vacha- *Acorus calamus*

Acorus calamus (Family: Acoraceae) commonly known as sweet flag is a wetland perennial monocot plant traditionally used for different ailments such as fever, asthma, bronchitis, cough, and digestive problems (Balakumbahan et al., 2010). It is found in wild or cultivated throughout Himalayas at an altitude ascending up to 6000 feet. It has antioxidant and antidiabetic activity. Oral administration of 200 mg/kg of *Acorus calamus* extract in Male albino rats showed significant restoration of blood glucose level and histopathological studies of the pancreas showed comparable regeneration (Prisilla et al., 2012). It also exhibits free radical scavenging, reducing power and metal chelating property (Subathraa and Poonguzhali., 2012).

27. Punarnava- *Boerhaavia diffusa*

Boerhaavia diffusa (Family: Nyctaginaceae) widely distributed in the tropics, subtropics and temperate regions of the world. It is distributed in India, Australia, China, Egypt, Pakistan, Sudan, Sri Lanka, South Africa, USA and in several countries of the Middle East. In India it is distributed throughout the warmer parts of the country up to an altitude of 2000m in the Himalayan region (Ghosh and Rai., 2018). Ethanolic extract of this plant exhibits significant anti hyperglycemic activities in alloxan-induced as well as streptozotocin-induced diabetic rats. Methanolic extract of *Boerhaavia diffusa* also helps for the regeneration of pancreas. Roots of this plant extract have antioxidant activity (Riaz and Raza., 2014).

28. Kshirasukla- *Ipomoea mauritiana*

Ipomoea mauritiana (Family: Convolvulaceae) is a branched glabrous twinning shrub with lobed, simple leaves and purple, bisexual flowers. It is distributed throughout the tropical parts of the world and in evergreen, deciduous forests of India (Bindu et al., 2018). The tuberous roots are sweet, stimulant, carminative and are used in emaciation, spermatorrhea and enteric fever (Rahmatullah et al., 2010). Previous studies indicated antihyperglycemic potential of the callus extract of this plant (Saiful et al., 2015). It also has antioxidant activity. Different fractions of this plant exhibited radical scavenging potential and high therapeutic action (Pandey., 2004).

29. Yashti- *Glycyrrhiza glabra*

Glycyrrhiza glabra (Family: Leguminosae) commonly known as Licorice and sweet wood. This plant is local to the Mediterranean and certain ranges of Asia. The dried rhizome and base of this plant were utilized therapeutically by the Egyptian, Chinese, Greek, Indian and Roman. It is a hardy perennial shrub, accomplishing tallness up to 2.5m (Kalsi et al., 2016). Ethanolic extract of this plant contains high phenolic component which is responsible for its powerful antioxidant activity, significant free radical scavenging, hydrogen-donating, metal ion chelating, anti-lipid peroxidative and reducing abilities (Visavadiya et al., 2009). Root extract of *Glycyrrhiza glabra* was found to have anti-lipidemic and antihyperglycemic activity (Revers., 1956).

30. Haritaki- *Terminalia chebula*

Terminalia chebula (Family: Combretaceae) is a moderate tree and a common medicinal plant used in Ayurveda. This plant is a native of India and treated as ‘King of Medicine’ in Tibet (Rathinamoorthy and Thilagavathi., 2014). It is one of the co-ingredient in Ayurvedic formula named ‘Triphala’. This plant exhibit many medicinal and pharmacological activities such as antidiabetic, antimicrobial, antioxidant, anti-mutagenic, anti-proliferative, anti-inflammatory, cardioprotective and wound healing. Methanolic and ethanolic extract of this plant have highest free radical scavenging potential. Its fruits also have antioxidant activity reported to reduce the lipid peroxidase enzyme (Ashwini et al., 2011). *Terminalia chebula* plant possess anti-hypoglycemic and anti-diabetic activity and the ethanolic and methanolic extracts has the ability to decrease the levels of glucose (Borgohain et al., 2012).

31. Amalaki- *Phyllanthus emblica*

Phyllanthus emblica (Family: Euphorbiaceae) is one of the important medicinal herb in Ayurveda. It is a small to medium sized deciduous tree, found throughout India, Pakistan, Uzbekistan, Sri Lanka, South East Asia, China and Malaysia. Amla is highly nutritious and is one of the richest sources of vitamin C, amino acids and minerals (Srivasuki., 2012). It contains several chemical constituents like tannins, alkaloids and phenols (Zhang., 2003). Fruits of this plant are sour, astringent, bitter, acrid, sweet, cooling, anodyne, ophthalmic, carminative,

digestive, stomachic, laxative, alterant, aphrodisiac, rejuvenative, diuretic, antipyretic and tonic. Fruits are useful in vitiated conditions of tridosha, diabetes, cough, asthma, bronchitis, cephalalgia, ophthalmopathy, dyspepsia, colic, flatulence, hyperacidity, peptic ulcer, erysipelas, skin diseases, leprosy, haematogenesis, inflammations, anemia, emaciation, hepatopathy, jaundice, strangury, diarrhoea, dysentery, hemorrhages, leucorrhoea, menorrhagia, cardiac disorders, intermittent fevers and greyness of hair (Kumar et al., 2012).

32. Vibhitaki- *Terminalia bellirica*

Terminalia bellirica (Family: combretaceae) found widely throughout the Indian subcontinent, Sri Lanka, South- East Asia, Bangladesh and Nepal. It is a large deciduous tree with broadly elliptic leaves clustered at the ends of branches (Meena et al., 2010). Several phytochemicals are isolated from various parts of the plant which include alkaloid, coumarin, flavones, steroids, lignins, tannins, glycosides, terpenoid, saponin etc (Chikezie et al., 2018). This plant has been reported to possess potent antioxidant action (Fahmy et al., 2015). Administration of methanolic extract of this plant blocks hyperglycemia in diabetic rats via its antioxidant activity (Sabu and Kuttan, 2009). Significant decreases in free radicals, and increased glutathione, superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase activities were also observed in diabetic rats following this extract treatment. Aqueous extract of *Terminalia belerica* fruit also stimulated insulin secretion from a pancreatic β -cells (Kasabri et al., 2010).

33. Rasa- *Commiphora myrrha*

Commiphora myrrha (Family: Burseraceae) is a oleo-gum-resin obtained by incision from the stem of *Commiphora molmol Engler* and from other *Commiphora* species. It is seen in India, Pakistan, Arabia, tropical and southern Africa. It stimulates the production of gastric juices, tones the digestive tract and used to treat diarrhoea, flatulence, dyspepsia, loss of appetite. Also used for treating genital infections, leucorrhoea, thrush, scanty periods, hemorrhoids and arthritis and has expectorant activity and is also used for flu, bronchitis, asthma and sore throat. It stimulates the production of white blood cells, regeneration of skin cells, assists in the healing of wounds (Soni et al., 2013).

34. Satahwa- *Anethum graveolens*

Anethum graveolens (Family: Umbelliferae) is an annual herb growing in the Mediterranean region, central and southern Asia. It is traditionally used for the management and prevention of digestive disease, breath problem, motivation of lactation and also reduction of cholesterol and glucose. There are several reports of different extract of the seed and leaf as well as its essential oil significantly reducing triglycerides, total cholesterol, low-density lipoprotein cholesterol (LDL-C), very-low-density lipoprotein cholesterol (VLDL-C), glucose levels and increased high-density-lipoprotein (HDL-C) level in diabetic models. It also shows antioxidant activity (Jana and Shekhawat., 2010).

35. Ela- *Elettaria cardamomum*

Elettaria cardamomum (Family:Zingiberaceae) commonly known as queen of spices. It is a strong antioxidant plant. The traditional and most world-famous aromatic plant grown and cultivated in some Asian countries such as Sri Lanka, India, Nepal, Indonesia, Guatemala and Tanzania (Garg et al., 2016). It has various biological applications such as antioxidant, anticancer, anti-inflammatory, antibacterial, antifungal, antiproliferative, antidiabetic, and antiviral activities.

36. Twak- *Cinnamomum verum*

Cinnamomum verum (Family: Lauraceae) is a medicinal plant commonly called as “true cinnamon tree” or “Ceylon cinnamon tree”. It is a small tropical tree originated in Sri Lanka, East and Middle Asia (Jayaprakasha and Rao., 2011). Which have the ability to reduce blood pressure, plasma glucose, obesity and ameliorating dyslipidemia (Mollazadeh and Hosseinzadeh., 2016). This plant has antidiabetic, anti-inflammatory, Cardio vascular protective effect and beneficial effects in lipid profile (Hofheins et al., 2006, Couturier et al., 2010 and Shen et al., 2012). Cinnamon verum has an insulin mimetic effect (Khan et al., 1990).

37. Patra- *Cinnamomum tamala*

Cinnamomum tamala (family: Lauraceae) is commonly known as Tejpat. It is a native to India and also seen in Nepal, Bhutan, and China. It is a perennial or small evergreen tree, attaining 8-12 meters

height. This plant is used in Ayurveda for the treatment of various ailments such as anorexia, bladder disorders, dryness of mouth, coryza, diarrhoea, nausea and spermatorhea (Kapoor., 2000). It also has hypoglycemic, hypolipidemic (Kar et al., 2003) and antioxidant activity (Sultana et al., 2010).

Phytochemicals are naturally occurring biologically active chemical compounds found in plants and protect the plant cells from diseases and damages caused by environmental hazards such as pollution, stress, drought, ultraviolet (UV) exposure etc. (Gibson et al., 1998, Mathai., 2000). It also has roles in protection of human health. Dietary phytochemicals are commonly found in fruits, vegetables, legumes, whole grains, nuts, seeds, fungi, herbs, spices etc (Moorachian., 2000). Polyphenols, tannins, and flavonoids are plant-based dietary antioxidants that have beneficial effect on health and aging. Phenolic compounds are the most common water soluble antioxidant compounds in plants. Studies showed that DK has high phenolic content. This justifies the protective and corrective effects produced by the product in vata and vatarakta disorders. The free radical scavenging role of the phenolic compounds is attributed to their unique structure. The phenolic compounds in the water extracts thereby take the role of antioxidants and play a protective role in vata vyadhis where the damage of bones and cartilage takes place (Sruthi and Sindhu., 2012). This study also revealed the richness of DK with flavanoids and tannins.

Lipids are one of the important biomolecules, which are insoluble in water and soluble in several organic solvents. Abnormal lipid metabolism leading to dyslipidemia is a prominent characteristic of diabetes. Alteration in lipid profile like Total Cholesterol (TC), Free Fatty Acids (FFA), Phospholipids (PL) and Triglycerides (TG) is one of the important characteristics of diabetes mellitus (Kandasamy and Ashokkumar., 2014).

Lipid peroxidation is a process in which oxidants such as free radicals or non radical species attack lipids containing carbon-carbon double bond(s), mainly polyunsaturated fatty acids (PUFAs) (Ayala et al., 2014). The lipid peroxidation product malondialdehyde (MDA) level is another reliable marker for increased oxidative stress (Madhuri et al., 2014). MDA is an end- product generated through enzymatic or nonenzymatic process by decomposition of arachidonic acid and larger poly unsaturated fatty acids (PUFAs) (Esterbauer et al., 1991). MDA can be generated in vivo as a side product by enzymatic reactions during the biosynthesis of thromboxane A₂. This MDA has ability to act as a signaling messenger and regulate glucose-stimulated insulin secretion (GSIS) mainly through Wnt pathway. Moderately high MDA levels affect the gene expression and protein production or activity of the key regulators of GSIS. MDA production by non enzymatic process originate under stress conditions and affects biomolecules such as protein and deoxyribonucleic acid (DNA) that leads to the formation of adducts and excessive MDA production is associated with different pathological conditions (Wang et al., 2014). We hypothesized that DK

could be effectively used to set right the complications associated with diabetes. DK is effectively used in the rejuvenation of tissue and body functions. The possible involvement of stem cells mobilized from Bone Marrow or other niches in body is proposed as a possible mode of actions of DK in ameliorating diabetic complications.

Stem Enhance 2 (SE2) is one of the important natural products extracted from *Aphanizomenon- flos aquae* (AFA). AFA is a unicellular prokaryotic microorganism included under the category of blue- green algae and belong to the phylum cyanobacteria (Nuzzo et al., 2018). Stem Enhance tablet is enriched with the cytoplasmic and cell wall fractions of AFA plant and also contains L-selectin ligand (LSL) (Ismail et al., 2013). This ligand helps the release of stem cells (CD34+ cells) from the bone marrow and its effect was detected on bone marrow stem cell mobilization (Jensen et al., 2007).

We also hypothesized that administration of DK could beneficially alter the expression levels of various genes induced in lipid peroxidation, antioxidant response, carbohydrate metabolism etc. It is proposed to study the expression of selected genes by Real time PCR. Real Time polymerase chain reaction (PCR) or quantitative PCR (qPCR) is a PCR based method and mainly used for the sensitive, specific and reproducible quantification of nucleic acids. It is used for various applications such as agriculture, medicine, molecular diagnostics, forensic testing and testing of genetically modified organisms (Bustin et al., 2009). It reliably detects and measure the

products generated during each cycle of the PCR process and products formed are directly proportional to the amount of template prior to the start of the PCR process (Arya et al., 2005). In the present study we evaluated the expression pattern of the following genes by semi-quantitative real time PCR.

1.4: Genes studied

- | | |
|--------------|------------------------------|
| 1. Insulin | 9. PPAR- α |
| 2. Caspase 3 | 10. PPAR- δ |
| 3. iNOS | 11. COX- 2 |
| 4. Caspase 8 | 12. L-gulonolactone- oxidase |
| 5. GPx1 | 13. Beta – actin |
| 6. GRd | 14. β 2- macroglobulin |
| 7. TNF-alpha | 15. Y-Chromosome protein |
| 8. NF-kB | 16. X-Chromosome protein |

1. Beta- actin (β -actin)

Housekeeping genes are a group of typically constitutive genes that are necessary for the maintenance of basic cellular function. β - actin is one of the house keeping gene and commonly used internal reference gene in real time PCR. β -actin is a conserved cytoskeleton structural proteins member and distributed in all eukaryotic cells. It plays a crucial role in cell migration, cell division, wound healing, embryonic development and the immune response (Bunnell et al., 2011, Nowak et al., 2005).

2. Insulin

Type -I diabetes mellitus occurred due to production of insufficient amount of endogenous insulin by autoimmune destruction of pancreatic β -cells (Atkinson et al., 2014). Insulin is synthesized in the form of preproinsulin then it is processed to form proinsulin. This proinsulin is again converted to form mature insulin and c-peptide and stored as secretory granules in pancreatic β - cells (Fu et al., 2013). A change in glucose concentration is the major factor for the regulation of insulin gene expression under normal conditions. Several *in vivo* and *in vitro* studies had already established the relation between chronically elevated levels of glucose and fatty acids with reduced insulin gene expression under normal circumstances (Poitout et al., 2006).

3. Caspase 3

Caspase 3 is an initiation Caspase. Apoptosis of β cells is a major incident leading to diabetes type I and II. Cerasi et al. (2000) observed a 14-fold increase in apoptosis in pancreas of diabetic animals 20 days after the onset of diabetes, whereas the apoptotic rates were very low in normal individuals. Caspases are cysteine requiring aspartyl proteases and key regulators of cell apoptosis. Caspase-3 plays an important role in the activation and development apoptosis in cells (Yun et al., 2013, Nakada et al., 2003). Second mitochondria-derived activator of caspases (Smac), plays a crucial role in promoting caspase activation and facilitating apoptosis induction (Allensworth et al.,

2013). Caspase 3 plays many roles in cell processes such as apoptosis, embryonic development, hematopoietic development and homeostasis (McIlwain et al., 2013, Porter et al., 1999, Zeiss et al., 2004, Lo et al., 2015). It has an essential role in the development of brain in some genetic mouse strains (Leonard et al., 2002). In the hematopoietic system, loss of Caspase 3 leads to impaired differentiation and accelerated proliferation of bone marrow cells (Janzen et al., 2008). Caspase 3 also participated in the negative regulation of β -cell proliferation following antigen stimulation (Woo et al., 2003). Regulating the activation of caspase 3 is important in maintaining cellular health as well as maintenance.

4. Caspase 8

Caspase 8 is another cysteine protease involved in the initiation and the execution of programmed cell death. Activated caspase 8 has a very important role in death receptor-induced apoptosis (Muzio et al., 1996, Juo et al., 1998). Its role as a tumor suppressor is already reported and Caspase 8 is genetically or epigenetically silenced in several tumors (Teitz et al., 2000, Meier et al., 2003) which favors continuous proliferation of tumor cells. In human caspase- 8 is encoded by CASP8 gene (Fianco et al., 2018). Post-translational modifications such as tyrosine phosphorylation and ubiquitination lead to the enzymatic activation of Caspase 8 (Cursi et al., 2006). Caspase 8 has very important role in the proliferation and homeostasis of human immune cells (Fianco et al., 2018). Caspase 8 is also a necessary factor for the activation of NF- κ B in B and T –cells (Su et al., 2005) (Bidère et al.,

2006). Casp 8 is a critical component of the death receptor–mediated apoptotic pathway (Varfolomeev et al., 1998) and this pathway play an important role in the development of type 1 and type 2 diabetes (Mathis et al., 2001, Donath et al., 2005). Casp8 is reported to have an essential role in β -cell apoptosis in streptozotocin (STZ) and high-fat diet (HFD)–induced diabetes models (Liadis et al., 2007).

5. Inducible Nitric Oxide Synthase (iNOS)

Nitric Oxide (NO) is one of the smallest bioactive products of mammalian cells and 3 distinct isoforms of nitric oxide synthase (NOS) have been identified in mammals. They are; neuronal (nNOS), inducible (iNOS) and endothelial (eNOS) (Pautz et al., 2010). Major role of this enzyme was to transform L-arginine (L-Arg) to L-citrulline and NO. This mediates relaxation of the blood vessels in vascular smooth muscle cells (VSMC). Different cellular stimuli including cytokines induce iNOS expression in a wide range of cells and tissues (Soski et al., 2011). iNOS has been implicated in many human diseases associated with inflammation (Fujimoto et al., 2005, LaPointe and Isenovic., 1999). iNOS deficiency prevent high-fat diet-induced Insulin Resistance (IR) in skeletal muscle of mice (Fujimoto et al., 2005). Impaired cardiovascular function in T2DM is associated with over expression of iNOS (Nagareddy et al., 2009).

6. GPx1

Glutathione peroxidase 1 (GPx1) is a ubiquitously expressed selenium-dependent antioxidant enzyme that having crucial role in protecting the

cells against oxidative damage. This enzyme catalysis the reduction of hydrogen peroxide and a wide range of organic peroxides to water to limit its harmful effects (Arthur., 2000). Reduced glutathione is involved in the process. The GPx1 gene is located at chromosome 3p21.3 (Kiss et al., 1997) and belongs to the family selenium-dependent peroxidases (Chu et al., 1993). Enhanced susceptibility to hydrogen peroxide-induced apoptosis in cultured neurons isolated from GPx-1 knockout mice. Several previous studies have already shown this was found to correlated with increased accumulation of intracellular ROS (Cheng et al., 1998). Decreased GPx is associated to the development of various pathological conditions, including cardiovascular diseases (Shuvalova et al., 2010). The cytosolic form GPx-1 is the ubiquitous and the most abundant intracellular isoform (Arthur., 2000). Shear stress in vascular endothelial cells was reported to up regulate GPx expression and activity (Takeshita., 2000). Endothelial dysfunction was reported in heterozygous deficient in GPx-1 (Forgione et al., 2002) and this was found to cause significant structural, vascular and cardiac abnormalities (Forgione et al., 2002). From this it is evident that GPx-1 is a key enzyme in protecting vessels against oxidative stress and atherogenesis. GPx-1 gene is also associated with risk of cardiovascular disease and peripheral vascular disease in diabetic patients (Hamanishi et al., 2004).

7. GRd

Glutathione reductase is a homodimeric flavoprotein (Qiao et al., 2007) which is an enzymatic antioxidant that plays a key role in

providing protection against free radical mediated damages in our body (Iskusnykh et al., 2013). The major function of GRd was to carry out regeneration of oxidized glutathione by using NADPH as reducing equivalents (Pashkov et al., 2005). Mammalian GRd has two isoforms and its activity is mainly present in cytosol and mitochondria. Both isoforms are encoded by a single nuclear gene (George et al., 1976). It has 13 exons in both human and mouse and both sequences code for an N-terminal mitochondrial-targeting sequence (Kelner and Montoya., 2000, Iozef et al., 2000). Qiao et al in 2007 reported the protection offered by the enhanced expression of both cytosolic and mitochondrial GRd to macrophages from oxidized LDL induced mitochondrial dysfunction and reduced atherosclerosis (Qiao et al., 2007). This study evaluated the expression pattern and enzymatic activity of GRd in DK administrated diabetic and normal rats

8. TNF-alpha (TNF- α)

Tumor necrosis factor – alpha (TNF- α) is produced in several kinds of cells such as cardiomyocytes, activated macrophages, endothelial cells, lymphoid cells, mast cells, fibroblasts, neurons and adipose tissue (Kapadia et al., 1995). It is produced as a 212-amino acid-long type II trans-membrane protein arranged in stable homotrimers (Black et al., 1997). TNF- α has various biological functions mainly binding to tumor necrosis factor receptors 1 (p55 receptor) and tumor necrosis factor receptors 2 (p75 receptor) and stimulate complex signaling cascades that control various intracellular functions (Eck and Sprang., 1989). Tumor necrosis factor receptor type I (TNFR1) has been seen in

various tissues and can be completely activated by both the soluble trimeric and membrane-bound forms of TNF- α but tumor necrosis factor receptor type 2 (TNFR2) is restricted to the cells of the immune system and can only react to the membrane-bound form of the TNF homotrimer (Theiss et al., 2005). It is one of the leading pro-inflammatory cytokines inhibiting neurogenesis and has a negative effect on hippocampal adult neurogenesis, but the anti-TNF treatment promotes neurogenesis (Reus et al., 2015). Astrocytes and microglia cells of the central nervous system release TNF- α in reaction to inflammatory or infectious stimuli (Kronfol et al., 2000). TNF- α is important in controlling brain functions (Kaster et al., 2012). Over production of TNF- α was seen in several human diseases including atherosclerosis (Swardfager et al., 2010).

9. Nuclear factor- κ B (NF- κ B)

NF- κ B is found in all cell types and also a nuclear transcription factor. It is involved in cellular responses to stimuli such as cytokines, free radicals, stress, ultraviolet irradiation and bacterial or viral antigens (Patel and Santani., 2009). It is a group of structurally related and evolutionarily conserved proteins and belongs to the Rel family. They are regulated *via* shuttling from the cytoplasm to the nucleus in response to cell stimulation (Birbach et al., 2002). Inhibitors of NF- κ B control the activity of NF- κ B expressed in the cytoplasm of all cell types (Hayden and Ghosh., 2004). Classical and alternative pathways are two major signaling pathways that leads to the activation of NF- κ B

(Patel and Santani., 2009). Autoimmune attack of pancreatic β -cells in type 1 diabetes mellitus is mediated by cytokines, such as interleukin-1 (IL-1) and interferon. In pancreatic β -cells exposure to IL-1, NF- κ B was reported to activate and translocated to the nucleus (Cardozo et al., 2001).

10. Peroxisome proliferator-activated receptor-alpha (PPAR- α)

The peroxisome proliferator-activated receptors (PPARs) are included in the subfamily of ligand activated nuclear receptors/transcription factors that belongs to the superfamily of nuclear receptors (Michalik et al., 2006). The three isotypes found in the PPARs sub family are: PPAR- α (NR1C1), PPAR β/δ (NR1C2) and PPAR γ (NR1C3) encoded by separate genes (Michalik et al., 2006). Human PPAR- α gene consists of eight exons (Sher et al., 1993). PPAR- α is activated by natural ligands, including saturated, monounsaturated and polyunsaturated fatty acids and their metabolites such as leukotriene B4 (LTB4), oxidized phospholipids and lipoprotein lipolytic products (Azhar., 2010, Moraes et al., 2006). The expression of PPAR- α is predominantly seen in high-energy requiring tissues such as skeletal muscle, heart, liver and brown adipose tissue (Kliwer et al., 1994, Braissant et al., 1996, Auboeuf et al., 1997, Abbott., 2009). Its expression is also seen in intestinal mucosa, the adrenal gland, proximal tubules of the kidney and brown adipose tissue and most cell types present in the vasculature including endothelial cells (ECs), smooth muscle cells and monocytes/macrophages (Chen et al., 2003, Touyz and Schiffrin., 2006, Berhold and Krone., 2005).

11. Peroxisome proliferator-activated receptor-delta (PPAR δ)

Peroxisome proliferator-activated receptor-delta (PPAR- δ) belongs to the nuclear receptor superfamily and a ligand-activated transcription factor. It regulates major cellular metabolic functions such as insulin secretion and sensitivity, fatty acid uptake, transport, and β -oxidation etc. It also provides protection against several diseases like insulin resistance or type 2 diabetes, obesity, dyslipidemia and atherosclerosis (Liu et al., 2018). The activation of PPAR- δ in liver helps to decrease hepatic glucose concentration and improved glucose tolerance (Seedorf and Aberle., 2007). The beneficial impact of activation of PPAR- δ in weight reduction and regulating skeletal muscle metabolic rate etc. are revealed by several *in vivo* studies (Reilly and Lee., 2008).

12. COX-2

The isoenzyme COX-2 was discovered in early 1990s and it was entirely different from COX-1 (Fu et al., 1990). COX-2 is widely expressed in several organs such as kidney, brain and reproductive tract (Yamagata et al., 1993, Ferreri et al., 1999, Kniss., 1999). Cox -2 is not commonly seen in all normal tissues but its expression is rapidly induced by stimuli such as proinflammatory cytokines (IL-1b, TNF α), mitogens, oncogenes (phorbol esters), lipopolysaccharides, hormones (luteinizing hormone- LH), growth factors (fibroblast growth factor- FGF, platelet-derived growth factor- PDGF, epidermal growth factor- EGF) and disorders of water-electrolyte hemostasis (Zarghi and Arfaei., 2011). This inducible COX-2 is involved in pathological

processes such as inflammation and various cancer types (Williams and DuBois., 1996, Konturek et al., 2005).

13. L-gulonolactone oxidase (gulo gene)

Gulo gene is mainly seen in the microsomes of liver cells and is the major factor for the biosynthesis of ascorbic acid (Ching et al., 2001). This microsomal enzyme catalyses the aerobic conversion of gulonolactone to L- ascorbate along with the production of H₂O₂ (Chatterjee et al., 1960). The molecular weight of gulo protein is 50.6 kDa (Koshizaka et al., 1988). In 2004 Opara had clearly shown the strong relation between vitamin C and Type II diabetes mellitus (Opara., 2004). He reported the reduction blood glucose level, glycosylation, and capillary fragility by mega doses of vitamin C in type II diabetes. This investigation assessed the expression pattern of gulo gene in DK administrated diabetic and normal control rats.

1.5: Stem cells in diabetes

Stem cells are unique cell types which have the capacity to self-renewal and potential of differentiating into various cell types (Zeeshan et al., 2017). Stem cells are rapidly divided in certain organs such as gut and bone marrow where there is a need for repair and replace the damaged cells or form new type of cells like blood cells. But in organs like heart and pancreas stem cells remain as a resident cell and divided only under certain specific conditions (Abdulazeez., 2015). Mesenchymal stem cells (MSCs) are multipotent stromal cells, play a crucial role in the development of different stem cells *in vivo*

(Krebsbach et al., 1999). It has the ability to enhance the development and function of various cell types and isolated from variety of sources including bone marrow and various human organs (Busser et al., 2015, Moroni and Fornasari., 2013). It also has self- renewal ability and immunomodulatory effects. Because of these reasons MSCs are widely used and ideal for stem cell therapy of various diseases (Rasmusson et al., 2003, Gao et al., 2016, Papazova et al., 2015, Zhang and He et al., 2019).

The current treatments for diabetes include the stimulation of insulin secretion by the remaining β cells or decreasing insulin resistance and/or the replacement of the β cell mass (Weir et al., 1990). Recently the regeneration of the lost functional β cells of pancreas is effectively utilised in diabetes therapy and the damaged β cells are replaced either by cadaveric islet transplantation or by the regeneration of β cells from stem cell niches in body (Aguayo-Mazzucato and Bonner-Weir., 2018). Pancreatic islets transplantation is successfully used for replenishing damaged islets (Ryan et al., 2001, Shapiro et al., 2000), but the need for a large number (in the range of 2 million) of β cells per kg body weight makes this technique less attractive (Keymeulen et al., 2006) as the availability of healthy islets for this application are limited. Stem cell therapy offers an effective strategy that could potentially offer the required number of β cells for the graft. The current global epidemiologic burden of diabetes urges the scientific community to identify key influencers for the regeneration of

endogenous β cell by increasing the successful differentiation of stem cells into functional β cells. This will offer a possibility of ameliorating the diabetic complications through stem cell therapy. DK was effectively used for centuries for the rejuvenation of the body and we assumed that this function is achieved by the rebuilding of lost tissues and functions. We also hypothesised the involvement of stem cell mediated tissue regeneration under DK administration.

1.6: Objectives of the study

This study was designed to investigate the physiological and pharmacological properties of Dhanwantaram Kashaya, an Ayurvedic polyherbal formulation. The study involved *in vitro* as well as *in vivo* components. The objectives of the study were;

1. Study the effect of DK on blood sugar level in diabetic condition.
2. Study the mode of action of the drug through investigating;
 - i. the change in insulin level which is an indicator of the functioning β cells in pancreas.
 - ii. the expression of various genes on administration of DK.
 - iii. the cytological changes in pancreas and liver of DK fed normal and diabetic rats.
 - iv. the biochemical changes in DK administered conditions.

3. Comparative study of the effect of Dhanwantaram Kashaya, SE2 and Bone marrow derived stem cells in ameliorating the disease symptoms of diabetes.
4. Study the dose response to Dhanwantharam Kashayam to identify the optimum dose in diabetic animals.

2.1: Experimental Animals

Male albino rats of wistar strain weighing 180-210 gm were used for this study. The animals were housed individually under hygienic conditions in polypropylene cages and were given standard rat chow (“VRK Nutritional Solutions”, Maharashtra, India) and distilled water *ad libitum*. Temperature of the animal house was maintained at $25\pm 1^{\circ}\text{C}$ with alternate 12 hours light and dark cycles. All procedures involving animals were in accordance with the guidelines on the care of laboratory animals and their use for scientific purpose. Protocols used in this study are approved by the Institutional ethics committee (Registration number 426/2/CPCSEA) dated 7th October, 2015. One group consisted of 6 rats each.

2.2: Induction of Diabetes in rats

Diabetes was induced by a single intraperitoneal injection of freshly prepared Streptozotocin (STZ) (Sisco Research Laboratories, Mumbai, India) at a dose of 40mg/kg, prepared in 0.1 molar citrate buffer, pH 4.5 (Yildirim., 2009). Rats were immediately supplied with 5% glucose in drinking water for the first 24 hours to encounter any initial hypoglycaemia. Blood glucose was measured by using “Dr. Morepen Gluco One Blood glucose monitoring system” and the animals with blood glucose level >300 mg/dl on the third day were classified as diabetic group.

2.3: Animals Groups & Experimental Design

The study was conducted in three sets of rats and the details of the sets are given below.

In set I study DK freshly diluted with sterile water (1:3) was administered orally at a dosage of 1ml (Dose A), 1.5ml (Dose B) and 2ml/ kg (Dose C) respectively twice a day on empty stomach or before food for 21 days. The dose selected was equivalent to the dose recommended for human use. In set II study SE2 was dissolved in distilled water and given orally in three doses 250 mg (Dose A), 270mg (Dose B) and 300mg/kg (Dose C) daily by using gastric gavage. The intermediate dose, 270mg/kg (Dose B) selected was equivalent to the human dose and one lower dose 250 mg (Dose A), and a higher dose 300mg/kg (Dose C) were also used. In set III there were four groups; Normal Control (NC), Diabetic Control (DC), Normal +Bone marrow derived mesenchymal stem cells (BMSC) injected and Diabetic + BMSC injected.

2.3.1: Set I

Animals were divided in to eight groups namely.

Experimental group I: Normal Control (NC)

Experimental group II: Diabetic Control (DC)

Experimental group III: Diabetic + DK Dose A (DDKA)

Experimental group IV: Diabetic + DK Dose B (DDKB)

Experimental group V: Diabetic + DK Dose C (DDKC)

Experimental group VI: Control + DK-Dose A (CDKA)

Experimental group VII: Control + DK-Dose B (CDKB)

Experimental group VIII: Control+ DK-Dose C (CDKC)

2.3.2: Set II

animals were divided in to eight groups namely;

Experimental group I: Normal Control (NC)

Experimental group II: Diabetic Control (DC)

Experimental group III: Diabetic + SE2 Dose A (DSE2A)

Experimental group IV: Diabetic + SE2 Dose B (DSE2B)

Experimental group V: Diabetic + SE2 Dose C (DSE2C)

Experimental group VI: Control + SE2 Dose A (CSE2A)

Experimental group VII: Control + SE2 Dose B (CSE2B)

Experimental group VIII: Control + SE2 Dose C (CSE2C)

On 21st day Set I and II animals were deprived of food overnight and sacrificed. Blood collected in centrifuge tubes. Tissues (Heart, Liver, Pancreas and Kidney) were collected in ice cold containers. Pancreas and liver were also collected in ice cold containers for histopathological analysis.

2.3.3: Set III

Animals were divided in to four groups namely;

Experimental group I: Normal Control (NC)

Experimental group II: Diabetic Control (DC)

Experimental group III: Normal + BMSC injected

Experimental group IV: Diabetic + BMSC injected

On 21st day Fasting Blood Sugar level and Serum Insulin Level was analysed. Also determined the presence of bone marrow cells in pancreas by PCR and Agarose Gel Electrophoresis.

2.4: Dhanwantaram Kashaya (DK)

DK used in this study was purchased from ‘Kottakkal Arya Vaidya Sala’ (Kottakkal, Kerala, India) in the form of a decoction.

2.5: Chemicals

All biochemicals used for the study were obtained from Sigma-Aldrich (Bangalore, India), Sisco Research Laboratories (Mumbai, India), HiMedia (Mumbai, India), Loba Chemie Pvt Ltd (Mumbai, India) and Thermo Scientific (USA). Other chemicals used were of analytic grade.

2.6: Determination of Fasting Blood Sugar Level

Fasting Blood glucose was measured by using “Dr. Morepen Gluco One Blood glucose monitoring system” BG-03 model glucose strips (Morepen Laboratories Limited, Sanjay Gandhi Transport Nagar, Delhi). Blood is drawn from tail vein of the albino rats and used for determination of fasting blood sugar.

2.7: Determination of Serum Insulin Level

Estimation of insulin levels in the serum samples was carried out by using a commercially available Rat Insulin ELISA Kit from Thermo Scientific as per manufacturer's instructions.

2.8: Homeostasis model assessment (HOMA)

HOMA model, which represent both fasting plasma glucose (FPG) and insulin levels, was used as an index of insulin resistance (IR).

For serum insulin determination, blood samples were collected using a glass cannula from the retro-orbital artery of 12h (overnight) fasted rats. Blood samples were collected directly into eppendorf tubes and kept in a cooled room for one hour. Later the tubes were centrifuged at 1000 xg for 10 minutes at room temperature. Insulin in serum was determined by using Rat Insulin ELISA Kit as described above. Glucose levels in arterial blood collected from the tail was determined as described above.

HOMA-IR index was calculated using the following formula:

HOMA = [insulin ($\mu\text{U/ml}$) \times glucose (nM) \div 22.5] as described previously (Matthews et al., 1985).

2.9: Oral glucose tolerance tests (OGTT)

At the end of the study period, oral glucose tolerance test (OGTT) was conducted by the method described elsewhere (Huhn et al., 2016). Rats were devoid of food overnight for 12hr and fasting blood glucose was determined. Each rat was administered with a dose of 2 g/kg body mass of sterile 50% (w/v) D-glucose solution (Sigma, India) via orogastric gavage. Thereafter blood glucose concentrations were measured at T = 30, 60, 90 and 120 min using “Dr. Morepen Gluco One Blood glucose monitoring system” BG-03 model glucose strips (Morepen Laboratories Limited, Delhi). The blood samples were obtained through a sterile pin prick of the distal tail vein.

2.10: Determination of haemoglobin and glycosylated haemoglobin

Haemoglobin (Merck, India) and glycosylated haemoglobin (Glyco-Tek Affinity column kits and reagents from Helena Laboratories (Beaumont, TX) levels were measured in plasma samples by using commercial kits by following the instructions from the manufacturers.

2.11: Histopathology of Liver and Pancreas

Histopathology of liver and pancreas tissues were done by using the methods described by Jamshidzadeh et al, Pearse A, Nurdiana et al (Jamshidzadeh et al., 2008, Pearse A., 1985, Nurdiana et al., 2017).

Rats were sacrificed and dissected immediately for collecting the samples of liver and pancreas. The tissues to be examined (liver and pancreas) were cut into small pieces (2 x 2mm thick) and fixed in 10% neutral formalin for 24 hours. After this the samples were washed and dehydrated in ascending grades of ethanol (60% -100%). After dehydration, tissue sections were cleared in Xylene to aid better impregnation with paraffin wax. Tissues were embedded in paraffin wax. After these sections of 5 µm thickness were cut using a microtome. Ribbons of tissue sections were floated on warm water (50°C) for spreading the tissue without wrinkles. Spread sections were collected on microscopic slides which are pre-treated with Mayer's albumen and allowed to dry. Sections were deparaffinised in xylene and rehydrated in descending series of ethanol. Staining was performed by using haematoxylin and counterstained by aqueous eosin. All stained sections were dehydrated through ascending series of ethanol (40%, 70%, 90%, 100%, respectively) cleared in xylene and mounted with DPX. The visualization of slides was done by using a light microscope at 40x.

2.12: Estimation of total tannins

The total tannins content was determined using the method of Broadhurst and Jones (Broadhurst and Jones.,1978).

Reagents

1. HCl
2. Methanol

3. Vanilin
4. Tannicacid

Procedure

Different concentrations of tannic acid (0.5-2.5ml) were taken in test tubes labelled as standard. 0.5ml of DK was taken in triplicate in test tubes marked as test and the volume of all the tubes were made up to 3ml with methanol. 5 ml of HCl- Vanillin reagent (Equal mixture of 4% HCl in methanol and 2% vanillin in methanol) was added to all the tubes. Tubes were incubated at 30⁰C in water bath for 30 minutes and cooled in room temperature. Absorbance was measured at 495 nm against methanol blank. The total tannin content was expressed in terms of tannic acid equivalent (mg TAE/g).

2.13: Total flavonoid content

Total flavonoid content was estimated according to the method of Barros et al (Barros et al., 2010).

Reagents

1. Deionised water
2. 5% NaNO₂
3. 10% AlCl₃
4. 4% NaOH

Procedure

Briefly, 500 μ l of DK was mixed with 2ml of deionised water and 150 μ l of 5% w/v NaNO_2 solution. After 6 min, 150 μ l of 10% w/v AlCl_3 solution was added and allowed to stand for further 6 min. Subsequently added 2 ml of 4% w/v NaOH solution and deionised water was added to make a final volume of 5 ml. Kept in dark at room temperature for 15 min and the intensity of the pink color developed was measured at 510 nm. Total flavonoid content was calculated using a standard curve with catechin and the flavonoid content was expressed as mg catechin equivalents (CE)/g.

2.14: Total Phenolic Content

Total Phenolic Content was estimated according to the method of Kosar et al (Kosar et al., 2008).

Reagents

1. H_2O
2. Folin-Ciocalteu reagent
3. 20% Na_2CO_3
4. Working Standard: 10mg gallic acid was dissolved in 10ml distilled water.

Procedure

Briefly 2 ml of H₂O and 50 µl of sample were transferred into a test tube, to which 250 µl of undiluted Folin-Ciocalteu reagent was added subsequently. The solution was neutralized by adding 750 µl 20% (w/v) Na₂CO₃ and the volume was made up to 5 ml with deionised H₂O. After 2 h incubation at room temperature in the dark with intermittent shaking, the absorbance was measured at 765 nm. The total phenolic contents were determined from the linear equation of a standard curve prepared with Gallic acid as standard. The levels of total phenolic compounds were expressed as mg/g gallic acid equivalent (GAE) of DK.

2.15: Ferric reducing antioxidant power (FRAP) assay

Ferric reducing antioxidant power assay was made according to the method of Vijayalakshmi and Ruckmani (Vijayalakshmi and Ruckmani., 2016).

Reagents

1. 0.2 M Sodium Phosphate Buffer (pH 6.6)
2. 1% Potassium Ferricyanide [K₃Fe(CN)₆]
3. 10% Trichloroacetic acid (TCA)
4. Deionised water
5. 0.1% Ferric Chloride

6. Working Standard: 10mg ascorbic acid was dissolved in 10ml distilled water.

Procedure

Take different volumes of the DK (10-50 μ l) in different test tubes and the volume were made up to 50 μ l with distilled water. Then added 2.5ml of 0.2M sodium phosphate buffer (pH 6.6) and 2.5ml of 1% potassium ferricyanide [$K_3Fe(CN)_6$] solution. Mixed the reaction mixture well by vortexing and then incubated at 50°C for 20min to reduce ferricyanide into ferrocyanide. After incubation the reaction was stopped by adding 2.5ml of 10% trichloroacetic acid to the mixture. Then centrifuged at 3,000rpm for 10min. Mixed 2.5ml of the supernatant with 2.5ml of deionised water and 0.5ml of 0.1% ferric chloride. Measured the colour intensity at 700nm using a UV-visible Spectrophotometer. Standard ascorbic acid was used as a reference standard and the calibration curve was prepared by plotting the absorbance versus the concentrations of $FeSO_4$. The reducing powers of the samples were calculated using the standard curve of the reference standard and expressed as μ g/ml.

2.16: 2,2-Diphenyl-1-Picryl Hydrazine (DPPH) Scavenging activity

DPPH scavenging activity (%) was determined by the method of Blois (Blois., 1958).

Reagents

1. 0.1mM DPPH in ethanol Solution
2. 95% ethanol

3. Working Standard: 5mg ascorbic acid was dissolved in 10ml ethanol.

Principle

The assay measures the scavenging capacity of antioxidants on DPPH. The odd electron of nitrogen atom in DPPH is reduced by receiving a hydrogen atom from antioxidants to the corresponding hydrazine. This causes a loss of the deep violet color of DPPH. The loss of color is proportional to the antioxidant capacity (donate H atoms) of the substance under test and obeys the Beer Lambert Law. The reduction in the color of the solution by free radicals (DPPH) can be measured at 515nm using a spectrophotometer.

Procedure

Briefly took various volumes of DK in different test tubes and the difference in volume was compensated by adding distilled water. Control was prepared without DK, but volume compensated by adding distilled water. Added 1ml of 0.1 mM DPPH Solution and made up to a final volume of 4ml with 95% ethanol. The mixture was vigorously shaken and incubated in the dark at room temperature for 30 minutes when the colour of the reaction mixture changed from purple to yellow with decreasing absorbance at wavelength 517 nm. The reduction in the color of the solution by free radicals (DPPH) was measured at 517nm against ethanol blank using a spectrophotometer.

Stock ascorbic acid standard solution was prepared by dissolving 10 mg of ascorbic acid in 100 ml ethanol to obtain a solution with a concentration of 100µg/ml. Ascorbic acid concentrations varying between 0-16 µg/ml (0, 0.25, 0.5, 2, 4, 6, 8, 10, 12 and 16) were used for making standard curve for antioxidant activity assay.

The capability of samples to reduce DPPH was determined by using the following equation and expressed as % of inhibition:

% of inhibition =

$$\frac{(\text{Abs of control} - \text{Abs of DK})}{\text{Abs of control}} \times 100$$

2.17: ABTS radical scavenging assay

Free radical scavenging activity of DK was determined by ABTS cation radical scavenging assay Re et al (Re et al., 1999).

Reagents

1. 7.4 mM 2, 2'-azinobis-(3-ethylbenzothiazoneline-6-sulphonic acid)
2. 2.45 mM potassium persulfate
3. 98% ethanol.
4. Working Standard: Trolox

Procedure

ABTS·+ cation radical was produced by adding 7.4 mM ABTS (2, 2'-azinobis-(3-ethylbenzothiazoneline-6-sulphonic acid) in water with 2.45 mM potassium persulfate (1:1) and stored in the dark at room temperature for 12-16 h before use. The solution was diluted to obtain an absorbance of 0.7 -1.5 at 734 nm with 98% of ethanol. Different volumes (5-120 µl) of DK were transferred into different test tubes containing the reagent, and mixed thoroughly and difference in volume of DK added was compensated by adding 98% ethanol. The OD was examined after 90 min using a UV-vis spectrophotometer. OD of the reagent without adding DK, but 120 µl 98% ethanol was taken as the initial absorbance. Likewise antioxidant capacity of trolox was also determined. The capability to scavenge the ABTS radical cation was calculated using the following equation;

% ABTS radical cation scavenging ability=

$$\frac{Ab1 - Ab2}{Ab1} \times 100$$

Where Ab1 is the absorbance of the control (ABTS solution without DK/trolox), and Ab2 is the absorbance in the presence of DK or trolox.

2.18: Phosphomolybdenum assay

The antioxidant activity of DK was determined by phosphomolybdenum method of Prieto et al (Prieto et al., 1999).

Reagents

1. 0.6 M sulphuric acid
2. 28 mM sodium phosphate
3. 4 mM ammonium molybdate

Procedure

Different volumes of DK were taken in different test tubes and volume was made to 100 μ l by adding distilled water. 1 ml of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate) was added to these test tubes. The tube was capped and incubated in a water bath at 95°C for 90 min. After the incubation, samples were cooled to room temperature, and the absorbance of the mixture was measured at 765 nm against a blank. Percent inhibition was calculated by using the formula and IC₅₀ was calculated.

$$\% \text{ inhibition} = \frac{1 - \text{absorbance of DK}}{\text{absorbance of control}} \times 100$$

2.19: Hydroxyl radical scavenging activity

The interaction of DK with hydroxyl radicals was assayed by using the deoxyribose method of Kunchandy and Rao (Kunchandy and Rao., 1990).

Reagents

1. Deoxy ribose
2. FeCl₃
3. EDTA

4. H₂O₂
5. Ascorbic acid
6. KH₂PO₄-KOH buffer (20mM, pH 7.4)
7. TCA
8. TBA
9. Mannitol

Procedure

The competition between deoxyribose and DK for hydroxyl radicals generated from the Fe³⁺/ascorbate/ EDTA/ H₂O₂ system (Fenton reaction) was assessed in this method. The hydroxyl radicals attack deoxyribose and form thiobarbituric acid reactive substance (TBARS) as final product. Various volumes of DK (5-150 µl) was taken in different test tubes and the volumes were made up to 150 µl with distilled H₂O. 900 µl of the reagent containing deoxyribose (28mM), FeCl₃ (0.1mM), EDTA (0.1mM), H₂O₂ (1mM), ascorbic acid (0.1mM), KH₂PO₄-KOH buffer (20mM, pH 7.4) was added to each test tube with DK. The mixture was incubated for 1hr at 37°C. After incubation 0.5 ml of this reaction mixture was added to 1 ml of 2.8% TCA. Colour was developed by adding 1 ml of 1% aqueous thiobarbituric acid (TBA) and incubating the mixture at 90°C for 15 min. The mixture was cooled and the absorbance was measured at 532 nm against a blank containing all reagents except DK. Mannitol, a classical OH· scavenger was used as a positive control. Percentage inhibition was evaluated by comparing the test and blank solutions.

The ability to scavenge the hydroxyl radical was calculated using the equation;

$$\% \text{ Hydroxyl radical scavenging activity} = \frac{1 - \text{absorbance of DK}}{\text{absorbance of control}} \times 100$$

2.20: Hydrogen peroxide-scavenging activity

The ability of DK to scavenge hydrogen peroxide was determined by the method of Ruch et al. (Ruch et al., 1989).

Reagents

1. 50 mM phosphate buffer (pH 7.4)
2. Hydrogen peroxide

Procedure

2 mM hydrogen peroxide solution was prepared in 50 mM phosphate buffer (pH 7.4). Different concentrations of DK (5, 10, 20, 40, 60, 80, 100, 120 and 150 µg) were taken in test tubes and their volumes were made up to 0.4 mL with 50 mM phosphate buffer (pH 7.4). 0.6 ml of hydrogen peroxide solution was added to each test tube and vortexed. After 10min the absorbance of the remaining hydrogen peroxide was measured at 230 nm against a blank containing all reagents except hydrogen peroxide.

Hydrogen peroxide scavenging ability was calculated by the equation:

$$\text{Hydrogen peroxide scavenging activity} = \frac{1 - \text{absorbance of DK}}{\text{absorbance of control}} \times 100$$

2.21: Nitric oxide scavenging activity

NO scavenging activity was measured by the method described by Marcocci et al (Marcocci et al., 1994) with minor modifications.

Reagents

1. Sodium nitroprusside(10mM)
2. PBS (0.25 M, pH 7.4)
3. Phosphate buffer
4. 1% Sulphanilamide
5. 20% Glacial acetic acid
6. 0.1% (w/v) Naphthylethylene diamine dihydrochloride
7. Working Standard: Ascorbic acid

Procedure

Nitric oxide generated from sodium nitroprusside was exposed to the scavenging activity of DK. 1ml of Sodium nitroprusside (10 mM) in PBS (0.25 M, pH 7.4) was incubated at 25°C for 5 h with different concentrations (5-150 μ l) of DK and the difference in volume of DK used was compensated by adding phosphate buffer. A mixture containing all reagents except DK, but with 150 μ l of buffer was used as control. After 5 h 0.5 ml of Griess reagent (equal volumes of 1% sulphanilamide in 20% glacial acetic acid and 0.1% (w/v)

naphthylethylene diamine dihydrochloride) was added. The absorbance of the pink coloured mixture was measured at 546 nm. Ascorbic acid was used as standard. % NO scavenging activity was determined by the equation;

$$\% \text{ NO scavenging} = \left(\frac{A_0 - A_1}{A_0} \right) \times 100$$

where, A₀ and A₁: Absorbance of mixture before and after reaction with Griess reagent.

Calculation of IC₅₀ values

A scatter graph was plotted in excel with concentration on X axis and % activity on Y axis. Equation for slope (Y=mx+c) was generated from the excel software. IC₅₀ value was calculated using the equation, where Y=50, M and C values were obtained from the equation itself.

2.22: Assay of Catalase (EC 1.11.1.6)

Catalase was assayed by the method of Maehley and Chance (Maehley and Chance., 1954).

Reagents

1. 0.01 M phosphate buffer, pH 7: Dissolved 3.55 g of KH₂PO₄ and 7.27 g of Na₂HPO₄ in water and made up to 100 ml.
2. 30% H₂O₂ – phosphate buffer: Diluted 0.16 ml H₂O₂ to 100 ml with phosphate buffer.

Procedure

The tissue was homogenized with 2 ml phosphate buffer at 4°C and centrifuged at 5000 rpm. The supernatant was collected in a test tube and kept in ice. 3 ml H₂O₂ in phosphate buffer and 10 µl of enzyme solution were pipetted into a quartz cuvette and mixed well by inverting the cuvette. Readings were taken after every 10 seconds for about 2 minutes against a control cuvette containing enzyme solution and H₂O₂ free phosphate buffer. The estimation was done spectrophotometrically following the decrease in absorbance at 230 nm.

2.23: Assay of Superoxide Dismutase (SOD, EC 1.15.1.1)

Superoxide dismutase was assayed by the method described by Kakkar et al (Kakkar et al., 1984).

Reagents

1. Sucrose buffer: 0.25 M
2. Sodium pyrophosphate buffer. 0.052 M, pH 8.3
3. Phenazine methosulphate (PMS): 186 µM
4. Nitroblue tetrazolium (NBT): 300 µM
5. NADH: 780 µM
6. Glacial acetic acid
7. n-butanol

Procedure

The tissue was homogenized in 0.25 M sucrose buffer at 4 °C and centrifuged at 5000 rpm. The supernatant was used as the enzyme source. The assay mixture contained 1.2 ml sodium pyrophosphate buffer (0.052 M, pH 8.3), 0.1 ml of 186 µM phenazine methosulphate, 0.3 ml of 300µM nitroblue tetrazolium, 0.2 ml of the enzyme preparation and water into a total volume of 3 ml. The reaction was started by the addition of 0.2 ml of NADH. After incubation at 30 °C for 90 seconds the reaction was stopped by the addition of 1ml glacial acetic acid. The reaction mixture was stirred vigorously and shaken with 4ml of n-butanol. The mixture was allowed to stand for 10 minutes, centrifuged and butanol layer was taken. Colour intensity of the butanol layer was measured at 560 nm against a butanol blank. A system devoid of enzyme served as control.

One unit of enzyme activity is defined as the enzyme concentration required inhibiting the OD of the chromogen production by 50% in one minute under the assay condition and expressed as specific activity in units/mg protein. The assay was done for 90seconds and hence a factor 2/3 was applied for calculating the unit.

2.24: Assay of Glutathione Peroxidase (EC 1.11.1.9)

Glutathione peroxidase activity was determined by the method of Lawrence and Burk., as modified by Agerguard and Jence (Agerguard and Jence, 1982).

Reagents

1. Phosphate buffer: 50mM, pH 7
2. Sodium azide : 1mM
3. EDTA: 1.5 mM
4. Reduced glutathione: 1mM
5. NADPH: 0.2 mM
6. 30% H₂O₂: 0.25mM

Procedure

The reaction mixture contained 2 ml of 50mM phosphate buffer (pH 7), 0.3 ml of 1 mM sodium azide, 0.2 ml of 1.5 mM EDTA, 0.1 ml of 1 mM reduced glutathione, and 0.1 ml of 0.2 mM NADPH and 0.3 ml of water. To this added 0.2 ml of enzyme solution and the mixture was incubated at room temperature for 5 minutes before the initiation of the reaction by the addition of 0.05 ml of 0.25 mM H₂O₂ solution. The absorbance was read at 340nm at 15 sec intervals. Blank reaction with replaced enzyme solution by distilled water was subtracted from each assay.

2.25: Assay of Glutathione Reductase (EC 1.8.1.7)

Glutathione Reductase activity was determined by the procedure of David and Richard (David and Richard., 1983).

Reagents

1. Phosphate buffer: pH 7.2, 0.12 M

2. EDTA: 1.5 mM

3. Oxidized glutathione: 65.3 mM

4. NADPH: 9.6 mM

Procedure

The reaction mixture contained 1.0 ml of 0.12 M phosphate buffer (pH 7.2), 0.1 ml of 1.5 mM EDTA, 0.1 ml of 6.3 mM oxidized glutathione and 0.1 ml of enzyme extract (prepared by homogenizing the tissue in phosphate buffer). To this added 0.05 ml of 9.6 mM NADPH and the absorbance was read at 340 nm at 15 sec intervals for 3 minutes. A mixture of all reagents except oxidized glutathione and containing 0.1 ml of water was used as control. Activity of the enzyme was expressed as μM of NADPH oxidised/minute/mg protein.

2.26: Estimation of Glutathione Content

The glutathione content was determined by the procedure described by Patterson and Lazarow (Patterson and Lazarow; 1955).

Reagents

1. Precipitating solution: 1.67 g of glacial metaphosphoric acid + 0.2 g of EDTA + 30 g NaCl in 100 ml distilled water.

2. Phosphate solution: 0.3 M Na₂HPO₄ in distilled water.

3. 0.04% 5, 5' dithiobis-2-nitrobenzoic acid (DTNB) solution.

Procedure

500 mg of the tissue was homogenized in 4 ml of the precipitating solution. After mixing, the solution was allowed to stand for 5 minutes and filtered. To 2 ml of the filtrate added 3 ml of phosphate solution. To all the tubes 1 ml of DTNB solution was added, mixed well and the optical density was measured at 412 nm. Blank was prepared by substituting the sample with water and following the entire procedure for test.

In the case of blood 0.2ml blood was diluted to 2ml using 1.8ml distilled water. 3ml precipitating solution was added, mixed and allowed to stand for 5 minutes at room temperature and filtered. 2ml filtrate was taken and added 8ml of phosphate solution followed by 1ml DTNB. Mixed well and OD was read at 412 nm.

2.27: Estimation of vitamin C in Tissues

Vitamin C was estimated by the method of Omaye et al (Omaye et al., 1979).

Reagents:

1. Metaphosphoric acid (6%)
2. Acetic acid (2N)

3. 20% TCA
4. DTC Reagent: 3g Dinitro phenyl hydrazine (DNPH), 0.4g thiourea, 0.05g CuSO₄ in 100ml 9N sulphuric acid (H₂SO₄)
5. Sulphuric acid (85%)

Procedure:

500mg tissue was homogenized in 5ml of 6% Metaphosphoric acid containing 2N acetic acid. The contents were centrifuged at 5000 rpm for 20minutes. A mixture is made with 1.5ml 20% TCA and 0.5 ml tissue homogenate and centrifuged at 3500 rpm for 20minutes in a cold centrifuge at 4°C. From this 1ml of supernatant was mixed with 0.5ml of DTC reagent and incubated at 37°C for about 3 hours. This was followed by the addition of 2.5ml of 85% sulphuric acid drop by drop while the mixture is in ice. Incubated at room temperature for about 30min. The absorbance was measured at 530nm. Test values were found out using a standard graph plotted with a standard series of ascorbic acid.

2.28: Estimation of vitamin E

Vitamin E was estimated by the procedure of Desai (Desai., 1984).

Reagents:

- 1.Ethanol
- 2.Dipyridyl reagent
- 3.FeCl₃

4. Petroleum ether

5. α -Tocopherol

6. Butanol

Procedure:

Vit E was extracted by the addition of 1.6ml ethanol and 2ml petroleum ether to 0.5ml plasma and centrifuged. The supernatant was separated and evaporated. To the residue add 0.2ml of 0.2% 2,2-dipyridyl and 0.2ml of 0.5% ferric chloride and kept in dark for 5minutes. The intensity of the red colour obtained on the addition of 4ml of butanol was read at 520nm. The concentration of the test sample was obtained from a standard graph plotted by standard series of α -tocopherol.

2.29: Estimation of Nitric oxide levels in liver tissue

Nitric oxide level was estimated by using Griess Reaction Sun et al (Sun et al., 2003).

Reagents

1. Sulphanilamide
2. N-naphthyl-ethylenediamine

Procedure

In this method nitrite is first treated with a diazotizing reagent, sulphanilamide (SA) in acidic media to form a transient diazonium

salt. This intermediate is then allowed to react with a coupling reagent, N-naphthyl-ethylenediamine (NED), to form a stable azo compound. The intense purple colour of the product allows nitrite assay with high sensitivity and can be used to measure nitrite concentration as low as ~0.5 μM level. The absorbance of this adduct at 540 nm is linearly proportional to the nitrite concentration in the sample.

2.30: Estimation of Protein

Protein was estimated according to the method of Lowry et al (Lowry et al., 1951).

Reagents

1. Alkaline copper reagent: (A) 2% Na_2CO_3 in 0.1N NaOH; (B) 0.5% copper sulphate in 1% Rochelle's salt. A & B were mixed in the ratio 50: 1
2. Diluted Folin's reagent (1:2) with distilled water.
3. Stock: 100mg BSA was dissolved in 100ml normal saline (1mg/ml).
4. Working standard was prepared by diluting 1ml of stock to 10ml (100 $\mu\text{g}/\text{ml}$).

Procedure

0.5 ml of tissue homogenate was mixed with 0.5ml of 10% ice cold TCA, kept in ice for 20 minutes and centrifuged 5000rpm for 10

minutes. The precipitate obtained was dissolved in 1ml of 0.1 N NaOH. This was taken as test. Graded volumes (50-500 μ l) of working standards were pipetted out into a series of test tubes and made up to 1ml using distilled water. 0.1ml of the test was pipetted out into another tube and made up to 1ml. 1ml distilled water was taken as blank. To all the tubes 5ml of alkaline copper reagent was added and incubated at room temperature for 10 minutes. Then 0.5ml Folin's reagent was added and incubated for 30min in the dark. The tubes were mixed, and the absorbance was read at 670nm. A calibration graph was drawn and extrapolated to obtain the test value.

2.31: LIPID PROFILE IN TISSUE AND SERUM

2.31.1: Extraction of Tissues for Lipid Analysis

The lipids in tissues were extracted by the procedure of Radin (Radin., 1981).

Reagents:

1. Hexane
2. Isopropanol

Procedure:

Approximately 1g tissue was homogenized with 18ml of extraction solvent (hexane: isopropanol 3: 2v/v). After 30-60sec of mixing it was centrifuged and transferred the supernatant into a 25ml graduated flask. The insoluble residue was re-suspended in 3ml extraction solvent and

centrifuged for 5 minutes. The supernatant was added to the previous one. Extraction was repeated with another 3ml of extraction solvent and was made up to 25 ml. Aliquots of these extract were used for lipid estimation.

2.31.2: Extraction of serum for lipid estimation

Serum lipids were extracted according to the procedure of Folch et al (Folch et al., 1957).

Reagents:

1. Methanol
2. Chloroform
3. KCl

1ml serum was homogenized and extracted with chloroform: methanol (2:1). The mixture was incubated at 50°C for 15 minutes. It was filtered and the residue washed with chloroform: methanol (2:1) at least 3 times. The filtrates were combined. To the filtrate, 0.7% KCl (20% of the total volume of the extract) was added and mixed. The aqueous upper phase was removed with a Pasteur pipette and the lower layer was washed three times with chloroform: methanol: KCl (2:48:47) solution. The washed lower layer of chloroform was evaporated to dryness and the residue was re dissolved in a known volume of chloroform. Aliquots of the extract were used for the estimation of various lipids.

2.31.3: Estimation of cholesterol

The cholesterol was estimated by the method of Zak et al (Zak et al., 1953).

Reagents

1. Con: H₂SO₄
2. FeCl₃- Acetic acid reagent (0.5gm FeCl₃ in 100ml glacial acetic acid)

Procedure

An aliquot of the 0.2ml lipid extract was pipetted out into a glass stoppered centrifuge tube and was evaporated to dryness. 5 ml of FeCl₃ -Acetic acid reagent was added and stoppered and was shaken well. It was then warmed in a water bath at 60°C for 2 minutes. After cooling to room temperature, it is centrifuged and the supernatant was decanted into another test tube. 3 ml of concentrated sulphuric acid was added to the supernatant and the absorbance was read after 20 min at 560 nm.

2.31.4: Estimation of Free fatty acids

Free fatty acids were estimated according to the procedure of Falholt et al (Falholt et al., 1973).

Reagents

1. Extraction solvent: chloroform: heptane: methanol (5:5:1)

2. Phosphate buffer: pH 6.4 33Mm. 2 volumes of KH_2PO_4 (4.53gm/L) and 1 volume Na_2HPO_4 (5.93g/L) were mixed.
3. Stock copper solution: 500 mM (12.07g $\text{Cu}(\text{NO}_3)_2 \cdot 3\text{H}_2\text{O}$ made up to 100ml)
4. Triethanolamine solution: 1M (10ml triethanolamine in 100ml of water)
5. NaOH: 1M
6. Copper reagent: 10ml of copper solution, 10ml of triethanolamine and 6ml of 1N NaOH were mixed and diluted to 100ml. 33g NaCl was added and the pH adjusted to 8.1.
7. Diphenyl carbazide solution: 4g/L in ethanol. Prepared immediately before use by adding 40mg in 10mL ethanol to 0.1N triethanolamine solution.
8. Standard palmitic acid: (2mM/L) 51.2 mg palmitic acid in the extraction solvent and made up to 100ml. Stored in a tightly stoppered container.
9. Working standard: 5ml stock solution made up to 20ml with extraction solvent to give a solution containing 500 $\mu\text{M/L}$.

Procedure

0.1ml sample was evaporated to dryness at 60-65°C in a water bath. Then 1ml phosphate buffer, 6ml extraction solvent and 2.5 ml copper

reagent were added. Blank contained 1ml phosphate buffer, 6ml extraction solvent and 2.5 ml copper reagent. 0.2 ml of the working standard was pipette out to a test tube and 1ml phosphate buffer, 5.8 ml extraction solvent and 2.5 ml copper reagent were added. Then the solution was transferred to a stoppered tube and shaken vigorously for 90 sec. It was allowed to stand for 15minutes, centrifuged at 4000xg for 5 minutes and the supernatant (1ml) was transferred to a tube containing 0.5ml diphenyl carbazide solution. The contents were mixed carefully, diluted with 5ml of the extraction solvent. The optical density was read after 5minutes at 550nm. Concentration was determined with the help of a standard curve.

2.31.5: Estimation of Phospholipids

Phospholipids were estimated according to the method of Zilversmith and Davis (Zilversmith and Davis., 1950).

Reagents

1. HNO₃ (2N)
2. H₂SO₄(5N)
3. Ammonium molybdate (2.5%)
4. ANSA reagent: a mixture of 1.2g sodium metabisulphite, 1.2gm sodium sulphite and 0.2g 1-amino 2-naphtha-4-sulphonic acid were powdered and dissolved in 100ml distilled water.

5. KH_2PO_4 standard solution

Procedure

An aliquot of the extract was taken in a digestion tube and evaporated to dryness in a warm water bath. Added 1ml of 5N H_2SO_4 and digested for 3 hours. When charring occurred one drop of 2N HNO_3 was added and digested till it become colourless. Cooled and added 1ml of water and kept in a boiling water bath for 5minutes. Pipetted out different volumes of standard and made up to 1ml with water. Blank containing 1ml water was also taken. Following this added 1ml of 5N H_2SO_4 and 1ml of 2.5% ammonium molybdate solution followed by 0.1 ml ANSA reagent to all tubes. Mixed well and added 6.9 ml of water and read the optical density within 10 minutes at 660nm against the blank.

2.31.6: Estimation of Triglycerides

Triglycerides were estimated by the method of Handel and Zilversmith (Handel and Zilversmith., 1957) with the modification that florisil was used to remove phospholipids.

Reagents

1. Chloroform (AR)
2. Florisil
3. Ethanolic KOH (0.4%)
4. H_2SO_4 (0.2N)
5. Sodium metaperiodate (0.05M)

6. Sodium arsenite (0.5M)
7. Chromotropic acid: 2g of chromotropic acid was dissolved in 200 ml distilled water. 600 ml of concentrated sulphuric acid was added slowly to 300ml of chilled distilled water. This chilled and diluted acid was then added to the chromotropic acid solution.
8. Glycerol standard: 9mg/ ml.

Procedure

2g of florisil was taken in a glass stoppered tube and 3 ml of chloroform was added. Known aliquot of the extract was layered on the top of the florisil and mixed. Chloroform was then added to make total volume to 10ml. It was then stoppered, shaken intermittently for about 10 minutes and then filtered through a filter paper. After filtration 1ml of the filtrate was pipette out into each of three tubes. 1mL of working standard of glycerol was pipette out into each of three tubes. The solvent was evaporated at 60- 70⁰C, 0.5 ml of ethanolic KOH was then added to two out of the three tubes (saponified sample) and 0.5ml ethanol was added to the third tube (unsaponified sample). The tubes were then closed and kept at 60-70⁰C for 15 minutes. 1mL water was taken as the blank. 0.5ml of 0.2 N H₂SO₄ was added to each of the tubes and placed in a gently boiling water bath for 15 minutes to remove alcohol. They were then cooled to room temperature. 0.1ml of sodium metaperiodate was added to each tube and kept for 10minutes. 0.1ml sodium arsenite was then added and mixed. A yellow colour of

iodine appeared and vanished within a few minutes. 5ml of chromotropic acid was added to each tube and mixed. The tubes were closed and then heated in a boiling water bath for 30 minutes. They were then cooled, and the absorbance was read at 570 nm.

2.32: Estimation of Malondialdehyde

Malondialdehyde (MDA) was estimated by the Thiobarbituric acid (TBA) assay method as described by Ohkawa et al (Ohkawa et al., 1979).

Reagents

1. TCA- TBA- HCl reagent: 15% (w/v) TCA and 0.375 % (w/v) TBA in 0.25 N HCl.
2. Tris HCl (0.1M) pH 7.5

Procedure

The tissue was homogenized in 0.1 M Tris – HCl buffer, pH 7.5 and allowed to stand for 5 minutes. 1 ml homogenate was combined with 2 ml of TBA-TCA-HCl reagent and mixed thoroughly. The solution was heated for 15 minutes in a boiling water bath. After cooling the flocculent, precipitate was removed by centrifugation at 1000 rpm for 10 minutes. Absorbance of the supernatant was read at 535 nm against the blank that contain no tissue homogenate.

2.33: RNA isolation

2.33.1: Materials required

1. Chloroform
2. Isopropyl alcohol
3. 75% ethanol (in DEPC treated water)
4. RNase free water
5. Cooling centrifuge and rotor capable of reaching up to 12,000xg
6. RNase free polypropylene micro centrifuge tube
7. Water bath or heat block (55-60°C)

For homogenising adherent cells, the growth media from culture dish was removed fully and added TRIzol® reagent (ThermoFisher) directly to the cells in the culture dish (1ml of TRIzol® reagent per 10 cm² of culture dish surface area). Cells were lysed in the culture dish by pipetting the cells up and down several times. Pipette up and down till the fluid lose its colloidal nature and then transferred into a 1.5ml sterile RNase free eppendorf tube. Homogenised samples can be stored at room temperature for several hours and at -60 to -70°C for at least one month. For tissues; 100mg of tissue sample was weighed and transferred into a clean mortar and pestle cooled on ice. Homogenise the tissue into a paste and then added 1ml of TRIzol® reagent (ThermoFisher) directly to the tissues and again homogenising well. Pipetting the grounded tissues up and down several times will ensure

full dissociation of the tissue. Then the homogenate was transferred into a 1.5ml sterile RNase free eppendorf tube.

2.33.2: Phase separation

1. Incubated the homogenised sample for 5 minutes at room temperature to permit the complete dissociation of the nucleoprotein complex.
2. Added 0.2 ml of chloroform per 1 ml of TRIzol® reagent used for homogenisation. Capped the tube securely and shaken the tube vigorously by hand for 15 seconds.
3. Incubated for 2-3 minutes at room temperature.
4. Centrifuged the sample at 12,000xg for 15 minutes at 4°C.

The mixture was separated into a lower red phenol chloroform phase, an interphase and a colourless upper aqueous phase. The aqueous phase will be ~50% of the total volume.

5. Removed the aqueous phase of the sample by angling the tube at 45° and pipetting out the solution into another RNase free eppendorf tube. Care was taken to avoid drawing of any of the interphase or organic layer into the pipette when removing the aqueous phase.
6. Placed the aqueous phase into a new tube and proceeded to the RNA isolation procedure.

Appropriate precautions were taken to avoid RNase contamination when preparing and handling RNA

2.33.3: RNA precipitation

1. Added 0.5 ml of 100% isopropanol to the aqueous phase, per 1ml of TRIzol® reagent used for homogenization.
2. Incubated at room temperature for 10 minutes.
3. Centrifuged at 12,000xg for 10 minutes at 4°C. Bluish white precipitate of RNA was visible.
4. Decanted the isopropanol taking care not to lose the RNA pellet.
5. Proceeded to RNA wash.

2.33.4: RNA wash

1. Washed the pellet, with 1 ml of 75% ethanol per 1ml of TRIzol® reagent used in the initial homogenization.
2. Vortexed the sample briefly then centrifuged the tube at 7500xg for 5 minutes at 4°C. Discarded the wash.
3. Air dried the RNA pellet for 5-10 minutes. Vacuum centrifuge was not used for drying as it may cause the RNA to dry completely and the pellet can lose solubility.
4. Proceeded to RNA re-suspension.

2.33.5: RNA re-suspension

1. Re-suspended the RNA pellet in RNase- free water (20-50 μ l) by passing the solution up and down several times through the pipette tip.
2. Incubated in a heat block set at 55-60°C for 10-15 minutes.
3. Proceeded to downstream application, or store at -70°C.

2.33.6: Preparation of DEPC Water

1. Added 0.1 ml Diethyl pyro carbonate to 100 ml of the solution to be treated and shake vigorously to bring the DEPC into solution.
2. Let the solution incubate for 12 hours at 37°C.
3. Autoclave for 15 minutes to remove any trace of DEPC

2.33.7: Determining yield and purity of RNA

1. Diluted (100x) a sample from the above RNA solution in RNase free water and measured absorbance at 260nm and 280 nm.
2. Used the formula $A_{260} \times \text{dilution} \times 40 = \mu\text{g RNA/ml}$ to determine the concentration.
3. A 260/280 ratio above 1.8 shows pure and high quality RNA.

2.33.8: cDNA synthesis

Placed 1µg of total RNA in a micro centrifuge tube and incubated at 65°C for 5 minutes, and then immediately placed on ice. cDNA synthesis was done using Thermo Scientific Verso cDNA synthesis Kit.

10µL 2x Reverse transcription mix which contained.

1. 4µL Reverse Transcription 5x buffer
2. 1µL dNTP Mixture, 10mM
3. 1µL Random Primers
4. 1µL RT Enhancer
5. 1µL Verso Enzyme Mix, 15 units
6. 1µg Template RNA
7. Nuclease- free water to a final volume of 20µL
8. Mixed well and spun down in a micro centrifuge.
9. Heated the reaction mix at 42°C for 60 minutes.
10. Heated the sample at 95°C for 2 minutes, and then incubated at 0-5°C for 5 minutes. This will inactivate the Reverse transcriptase and prevent it from binding to the cDNA. For second strand cDNA synthesis or agarose gel analysis, first-strand cDNA product was used.

2.33.9: Gene Expression Studies

The possible effect of DK on the expression of different genes were studied using RNA from pancreas, liver, heart and bone marrow cells. The details of the genes studied are shown in table 2.1. Gene expression was assessed by Real Time PCR using the cDNA generated from the isolated RNA.

2.33.10: Primers selected for the genes are following.

Table 2.1: Showing the list of the genes studied along with the sequence of primers and the annealing temperatures used for performing the expression studies primers for X Chromosome gene (NC_005120.4) and Y Chromosome gene (NC_024475.1) were taken from Dhakal and Soares., 2018).

Sl No .	Genes	Sequence (5' to 3')	Annealing Temperature (°C)
1	Cox-2	F-AGACTACGTGCAACACCTGA R-GAGCAAGTCCGTGTTCAAGG	54
2	iNOS	F-GTTTGACCAGAGGACCCAGA R-GTGAGCTGGTAGGTTCCCTGT	54
3	TNF- α	F-CAAACCACCAAGCAGAGGAG R-GAGGCTGACTTTCTCCTGGT	54
4	NF-kB	F-AAGCAGGAAGATGTGGTGGA R-GATAAGGAGTGCTGCCTTGC	54
5	GPx-1	F-GACCGACCCCAAGTACATCA R-GCAGGGCTTCTATATCGGGT	54
6	Beta actin	F-TCTTCCAGCCTTCCTTCCTG R-CACACAGAGTACTTGCGCTC	54
7	Caspases 3	F-CATGCACATCCTCACTCGTG R-CCCACTCCCAGTCATTCCTT	54

8	Caspases 8	F-GGTTACAGCTCTCCTACCCC R-TGTCTTCTCCAACATCCCC	54
9	Insulin	F-TGTCAAACAGCACCTTTGTGG R-GTGCCAAGGTCTGAAGGTCAC	54
10	L-gulonolactone oxidase (Gulo)	F- GTTTCACCTTCAGGAGACATCC R- CTCCAGTAGGTAGAACCCGATG	58
11	CD34	F - GGTAGCTCTCTGCCTGATGAGT R AGATGGCTGGTGTGGTCTTATT	62
12	GRd	F-GGAAGTCAACGGGAAGAAGTTCCTG R-CAATGTAACCGGCACCCACAATAAC Iskusnykh et al.,2013	64
13	PPAR- α	F-TGGAGTCCTGGAAGTGAAGC R-CAACGTCTTGTGTCCTGAGC	54
14	PPAR- δ	F-CCCTTCATCATCCACGACATT R-TGGACTGGCAGCGGTAGAAC (Cohen et al., 2011)	63
15	Beta actin	F-TCTTCCAGCCTTCCTTCCTG R-CACACAGAGTACTTGCGCTC	54
16	β 2-microglobulin	F- GTCGTACCACTGGCATTGTG R- CTCTCAGCTGTGGTGGTGAA	58
17	X Chromosome	F-TTTGTACGACTAGGCCCCAC R-CCGCTGCCAAATTCTTTGG	58
18	Y Chromosome	F-TTGGTGAGATGGCTGATTCC R-CCGCTGCCAAATTCTTTGG	58

All the primers except for GRd, PPAR- δ , X and Y chromosome protein were designed in the lab using primer 3 software available online.

2.34: Real Time PCR Analysis

Real time PCR is widely used for quantification of mRNA levels and is used as a fundamental tool for basic research, molecular medicine and

biotechnology. This technique is used to amplify and simultaneously quantify a specific region of the DNA molecule. In the conventional PCR, electrophoresis is used to assess the product of amplification, while in Real Time PCR; fluorescent molecules are used for the chemical reaction, which allows the quantification of the amplicon. The SYBR Green provided the simplest method for the detection and quantification of the PCR product in Real-Time reactions with high sensitivity. The SYBR Green binds to the double stranded DNA and emits light upon excitation. As the reaction proceeds and the PCR products accumulate, the fluorescence increases proportional to the amount of specific DNA present in the original sample. As the amplification starts, more SYBR Green molecules are associated with the newly synthesized double stranded DNA and the fluorescence is steadily increased at a specific wavelength that is measured after each amplification cycle. The step, at which the fluorescence exceeds the background at the exponential phase of the amplification, the cycle threshold (Ct) or crossing point (CP), is therefore recorded. The SYBR Green is added in the PCR reaction along with other reactants and a laser detector is used to detect the level of fluorescence. The presence of double stranded DNA in the solution is capable of increasing the light emission at about 100 times to the same concentration of SYBR Green. This fluorophore is a non specific agent and reveals any double strand generated in the PCR. As the binding is not specific to any particular gene sequence it is essential to establish the nature of the amplicon. This could be done in two ways; 1. Separate the PCR

product on an agarose gel and identify by size of the product by comparing with a molecular weight ladder run along with the amplicon. 2. By assessing the melting point of the PCR product: analysis of the melting temperature (T_m) help in the identification of the PCR product strand. The melting temperature will be specific to a particular piece of DNA double strand. For this an additional step need to be included in the PCR program. This is done at the end of the PCR program after the completion of the last cycle of amplification.

2.34.1: Standardization of internal control

An ideal internal reference gene must be unaffected of any experimental manipulations. More over its expression level was constant among different tissue types, disease states, cell cycle states and at all stages of development. Beta-actin is well known housekeeping gene that was used to normalize expression levels of the genes of interest.

2.34.2: PCR protocol

Relative quantitative real-time PCR assay using the SYBR Green fluorescent intercalation dye was employed in this study. Each amplification trial was performed in a 96-well PCR plate covered with optical tape in the CFX 96™ Optic Module (BIO RAD, Singapore). The reaction mixture contained 2 μ l of cDNA template, 10 μ l Real Time PCR Smart mix, 1 μ l of forward primer and 1 μ l reverse primer, and nuclease-free water to make up to a final volume of 20 μ l. Contamination by exogenous DNA or RNA was ruled out by the

inclusion of a negative control containing all reagents but no sample cDNA. The reaction mixture was subjected to denaturation at 95°C for 15 min followed by 40 cycles of denaturation at 95°C for 20 s, annealing at 60°C (varied between 54-60°C with different primers) for 30s, and elongation at 72°C for 30s. The fluorescence resulting from the binding (intercalation) of SYBR Green I into the double stranded DNA was recorded at the end of the elongation step of every cycle. The melting temperature of the product was analyzed by the inclusion of a melting curve analysis at the end of the PCR program.

Threshold cycle (Ct) for each well was obtained using the software of the instrument. The β -actin and β 2-microglobulin genes were used as the endogenous control genes to normalise the relative expressions of the genes of interest calculated using $2^{-\Delta\Delta CT}$ method as practiced before (Pillai., 2020).

2.34.3: Primer designing and synthesis

Gene specific primers were designed in Primer-3 software (<http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi>) using the mRNA sequence of the gene from NCBI nucleotide database and got custom synthesise by RFCL Science solutions, Bhopal, India. Primer sequences are given in Table 2.1 (Pillai et al., 2008).

2.35: Isolation and Culture of Rat Bone Marrow Derived Mesenchymal Stem Cells (BMDSCs).

Isolation of BMDSCs was carried out according to Huang S et al (Huang et al., 2015) with minor modifications.

Procedure:

- Rat (Wistar albino) aged 4-8 weeks are terminated by cervical dislocation.
- Whole body sprayed with 75% alcohol and moved onto a sterile dissection board.
- Four claws are dissected at the ankle and carpel joints.
- Incisions made around the connection between hind limbs and trunk, fore limbs and trunk.
- Whole skin is removed.
- Muscles, ligaments and tendons were carefully removed from tibias, femurs and humeri using micro dissecting scissors and scalpel.
- Bones are carefully scrubbed to remove residual soft tissues.
- Transferred to a 100mm sterile culture dish with 10ml complete α -MEM medium (Minimum Essential Medium) on ice.

- All samples were processed within 30 minutes following animal death to ensure high cell viability.
- Soft tissues were completely dissociated from the bones to avoid contamination.
- Wash twice to flush away impurities.
- Two ends of the bones are cut to open marrow cavity.
- The bone marrow was flushed out by using MEM medium in a syringe fitted with 23gauge needle. Needle was inserted into one end of the bone cavity and slowly flushed the marrow out.
- The bone cavities were washed twice again until the bones become pale.
- All the bone pieces were removed from the dish and the fat mass was left in the medium.
- Dissociated the tissue layers using base of a sterile syringe.
- Transferred the cell suspension into a T25 flask and the medium was topped up to 6ml and incubated at 37⁰C in 5% CO₂ incubator for 5 days.

2.36: Cell Culture

Bone marrow cells were grown in Alpha MEM medium (Himedia) with 10% Foetal bovine serum (MP Bio-medicals) 1% Antibiotic-

antimycotic solution (100µg/mL Penicillin and 100µg/mL Streptomycin) (Sigma) 1% Sodium pyruvate (Sigma) and 2% L-glutamine solution (Sigma). It was maintained at 37°C in an incubator maintained with 5% CO₂. The detailed procedure is as follows:

1. Removed all medium from the initial culture with Pasteur pipette. The adhering monolayer of cells was washed once with 1ml phosphate buffer, to remove any residual foetal bovine serum that may inhibit action of trypsin.
2. Added 1ml (T25) and 3ml (T75) of 37°C Trypsin/EDTA solution (0.25% Trypsin and 0.001% EDTA-Himedia) to the culture flask and incubated at 37°C for 1 minute.
3. If the cells were not fully dislodged from the culture flask, tap the sides of the culture flask mildly to aid detachment of the cells.
4. Drawn the cell suspension into a 10ml pipette and released without applying pressure, a few times. This ensured single cell suspension. As soon as cells are detached added media containing serum to inhibit further trypsin activity that might damage cells.
5. Counted the cells using haemocytometer. Plated 0.7×10^6 cells into T25 flask that has been appropriately labelled.
6. Added 5ml of fresh medium to each new culture flask. Incubated in humidified 37°C, 5% CO₂ incubator.

The viability of cells was determined by Trypan blue exclusion method.

2.37: Checking viability of cells and counting

Added 0.2ml of 4% Trypan blue solution to 0.2ml of cell suspension and mixed thoroughly. The cells are counted using haemocytometer. The viable cells appear unstained while the non-viable or dead cells stains blue.

For treatment with Dhawantaram Kashayam (DK), 30% confluent flasks were used. Different concentrations of DK were used to treat the cells and incubated for three-time intervals viz; 24 hours, 48 hours and 72 hours. Flasks with untreated cells acted as control. The concentrations of DK used were 2 μ l, 3 μ l, 5 μ l, 10 μ l, 20 μ l, 40 μ l and 100 μ l (10 μ l of DK was made up to 1ml with medium and from these different concentrations were taken). The following higher Concentrations of DK were also directly used for checking viability of cells and counting (2 μ l, 3 μ l, 5 μ l, 7 μ l, 10 μ l, 12 μ l, 15 μ l).

2.38: MTT assay

The viability of Bone marrow cells treated with DK was determined by MTT assay (Pillai., 2014).

Principle

This is a colorimetric assay that measures the reduction of yellow 3 – (4, 5-dimethyl thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT)

by mitochondrial succinate dehydrogenase. The MTT enters the cell and passes into the mitochondria where it is reduced to an insoluble coloured (dark purple) formazan product. The cells are then solubilised with an organic solvent (eg: DMSO) and then the released, solubilized formazan is measured spectrophotometrically. Since reduction of MTT can only occur in metabolically active cells, the intensity of the colour developed is proportional to the number of viable cells.

Materials and Chemicals Required

1. MTT
2. DMSO
3. Phosphate Buffered Saline (PBS)
4. 96 well culture plates
5. Alpha MEM medium

Procedure

Attached cells were released from the surface by scraping and collected.

1. Diluted the cells in culture medium to 8×10^4 cells per well.
2. Set first row as blank and add 100 μ l medium to the wells of the 96 well plate.
3. Set second row as normal.

4. 100µl of cell suspension (8000 cells per well) was added to each well of the 96 well micro titre plate except blank.
5. Incubated at 37^o C and 5% CO₂ for 6 to 48 hours. The time required will vary (12 hours to overnight is sufficient for most cell types).
6. Remove the medium from the wells. Cells were exposed to various concentration of DK.
7. Incubated for 24 hours at 37^oC and 5% CO₂.
8. After incubation with DK, removed the medium and washed with PBS.
9. Added 90µl medium and 10µl MTT reagent (5mg MTT/ml PBS) and incubated for 4 hours.
10. Observed the cells under a microscope for the development of purple crystals due to the formation of formazan.
11. Carefully removed the medium from the wells. Do not disturb the cells and do not rinse with PBS.
12. Finally, 100µl of 100% DMSO is added to the wells, including controls to solubilise the formazan. Swirl gently, do not shake.
13. Cover the well with tin foil and shake for 15 minutes at 350rpm in Spinix multiwall plate shaker (Tarsons). OD was read in an ELISA reader at 570nm.

2.39: Effect of administration of bone marrow cells to diabetic rats

Bone marrow stromal stem cells (BMSCs) obtained from normal male rats were passaged separately for 6–8 times before use in transplantation studies (Goldmacher., 2013). Rat BMSCs were harvested from their culture and centrifuged at 1000 rpm at 10°C. The pellet was re-suspended in sterile phosphate buffered saline (PBS) and counted after viability test. Cell preparations with more than 95% viability only are used for the injection. 0.5ml of cell suspension (1×10^6 cells/ml suspended in sterile saline) was injected into the tail vein of female rats.

2.40: Blood Cell Analysis.

Changes in circulating blood cell populations after the injection of BMSCs was determined in the blood collected from the tail vein 24 and 48 hr (1.5ml) after BMSC treatment. Blood was collected in 2 ml eppendorf tubes and added 1.5 mg EDTA/ mL of blood. Blood samples were kept at 4°C and analysed microscopically after staining with Wright's stain.

2.41: Determining the presence of BMSCs in the pancreas

Homing of injected BMSCs into pancreas was detected by PCR amplification of Y chromosome protein. Male BMSCs are injected into female rats and hence only pancreas with homed BMSCs from male rats alone will only have Y chromosome and (Clapcote and Roder., 2005). 100 ng genomic DNA isolated from the test samples were amplified in a PCR reaction by using specific primers for Y

chromosomes (Table 2.1) (Dhakal and Soares., 2018). Genomic DNA was isolated using GenElute™ Water RNA/DNA Purification Kit.

2.42: Electrophoresis of PCR product

PCR product was analysed on non-denaturing agarose gel electrophoresis (Ishikawa H., 1977).

2.42.1: Reagents and preparation

1. 1x TAE buffer (10%)
2. Agarose gel 1% (0.3g agarose in 30 ml): Melted 0.3g agarose in TAE buffer using a microwave oven. Care was taken to avoid air entrapping in the agarose while mixing. Letting the agarose solution to boil a few seconds will remove most of the dissolved air. Let it cool down to around 60°C before pouring into the gel tray. 1µl (10µg/ml) of Ethidium Bromide (EtBr) solution was added into the agarose gel before pouring into the gel casting tray. Used appropriate combs so the wells produced will hold enough PCR product for visualization. Let the agarose gel solidify. To speed up the cooling process the gel was slowly dropped into the buffer tank of the electrophoresis apparatus once a solid film of agarose was formed on the surface of the agarose solution.
3. Always used fresh gel buffer as well as clean electrophoresis equipment for DNA analysis.
4. Wore gloves to protect DNA samples from degradation by nucleases and to avoid hand contact with EtBr.

5. Running voltage up to 80 V/cm (80V per each cm space between the electrodes in electrophoretic chamber) were used. Use of high voltage avoided to prevent DNA degradation during electrophoresis.
6. Loaded a known amount (2 μ l) of DNA ladder on the first well of the as a standard for determining the DNA the size of the PCR product.
7. An aliquot (8 μ l) of the PCR product was mixed loading dye before loading on to the gel. The mixture was carefully loaded on to different wells using a fine tipped pipette.
8. The gel and electrophoresis was carried out 5 -6 V/cm until the bromophenol blue (the faster migrating dye) has migrated atleast 3-4cm into the gel or as far as 2/3 the length of the gel. Use 1x TAE buffer as running buffer.
9. After electrophoresis, the gel was visualised and photographed in a gel documentation centre.

2.42.2: DNA loading buffer (6x)

1. 30% (v/v) glycerol
2. 0.25% (w/v) bromophenol blue
3. 0.25% (w/v) xylene cyanol FF

Stored at 4°C

2.43: Statistical analysis

Analysis was carried out in triplicate and data are represented as Mean \pm SD (Standard Deviation) of six parallel measurements. Results were analysed by using the SPSS -21 version software for windows. Statistical significance was calculated by using one - way ANOVA (analysis of variance). Differences with p-values equal to or less than 0.05 were considered as statically significant.

Chapter 3

Effect of Dhanwantaram Kashayam on Glucose Metabolism

3.1: Introduction

Diabetic mellitus is a metabolic disorder with hyperglycemia as indicative trait. Hyperglycemia affects almost all tissues of the body with resultant effects of micro and macrovascular complications such as nephropathy, retinopathy, neuropathy, dyslipidemia and cardiovascular disease (Deshpande et al., 2008, Ghorbani et al., 2010), increased oxidative stress etc. Glucose is one of the primary energy fuels in our body. Concentration of glucose in blood depends upon the production and utilization of it by different tissues (Bilal et al., 2016). Changes in glucose metabolism due to diabetes are reported to induce cell damage through different metabolic pathways which includes an increase in glycation of proteins (Konda et al., 2020). Most of the diabetic complications are caused by the mitochondrial over production of reactive oxygen species (ROS) leading to oxidative stress. High glucose in diabetic condition due to insufficient insulin activity is linked to different metabolic abnormalities. Excessive amounts of ROS oxidise biomolecules, such as, DNA, protein, carbohydrates and lipids after surpassing various endogenous anti-oxidative defensive mechanisms which leads to oxidative stress (Al-Kharashi., 2018). Oxidative stress and the resultant tissue damages lead to diabetes associated complications (Asmat et al., 2016). Elevated levels of glycolated haemoglobin (HbA1c) and reduced total haemoglobin concentration were seen in diabetic rats (Kahlon and

Pathak., 2011). The excess amount of glucose present in the blood during severe diabetes reacts with haemoglobin to form glycosylated haemoglobin (Koenig et al., 1976) which alter the affinity for oxygen and lead to tissue hypoxia (Harris et al., 1996). Oxidative stress along with anoxia complicates diabetic conditions (Gerber and Rutter., 2017).

Glucagon and insulin are potent regulators of glucose metabolism. Insulin deficiency decreases the uptake of glucose in liver (Baquer et al., 1998) and lead to its increased level in blood, a condition termed as hyperglycemia. Hyperglycemia is one of the important characteristics of diabetes mellitus and it creates several biochemical and morphological changes in our body due to increased glycosylation resulting in altered metabolism of carbohydrate, protein and lipid (Bilal et al., 2016).

Insulin is one of the key anabolic hormones produced by pancreatic β -cells in response to increased blood glucose level. It is a small protein consisting of two polypeptide chains and 51 amino acids. In the body the insulin performs multifarious functions which includes lowering blood glucose concentrations, affecting glucose metabolism and storage of ingested nutrients, promotes glucose uptake by cells, suppresses postprandial glucagon secretion, promotes protein and fat synthesis, promote use of glucose as an energy source. Other actions of insulin include promotion of triglyceride storage in fat cells, the stimulation of fat synthesis and promotion of protein synthesis in the liver and muscle, and proliferation of cell growth (Cryer., 1992). Glucagon is one of the important catabolic hormones secreted by pancreatic α - cells consists of 29 amino acids and opposing the effect

of insulin (Unger., 1971). Glucagon stimulates the breakdown of stored liver glycogen, promotes hepatic gluconeogenesis and hepatic ketogenesis.

Interaction of pancreas employed through insulin and other pancreatic hormones with other organs in the body and reciprocal interactions are important in the normal functioning of the body (Röder et al., 2016). Other organs especially brain and liver interacts with the pancreas to modulate insulin secretion. Parasympathetic (Rossi et al., 2005, Yoshimatsu et al., 1984) and sympathetic (Borden et al., 2013) nerve fibers from the autonomic nervous system innervate with pancreas and at the same time, insulin receptors are widely distributed within the brain (Hopkins and Williams., 1997). Damage or impaired functions of brain regions are reported to affect pancreatic hormone secretions (Rohner et al., 1977). Insulin released in response to a meal enters the brain via the blood–brain–barrier to decrease food intake (Woods et al., 1984). Both peripheral and central insulin signaling are impaired in obese or diabetic states (Gelling et al., 2006).

Continuous regulated supply of glucose is essential for maintaining normal functioning of all body organs including brain. Under normal conditions, the human body efficiently control blood glucose level which avoids hyper or hypoglycemia and maintain sufficient glucose delivery to the brain and the other organs. Insulin therapy is successfully practiced for decades, but its inability in mimicking the pharmacology of endogenous insulin remains unresolved. This exposes diabetic patients, in particular those aiming for strict glycemic control to continuous risk of hypoglycemia (Pedersen-Bjergaard et al., 2004,

Ostenson et al., 2014). Long term as well as profoundly severe hypoglycemia also causes neuronal death (Ennis et al., 2008).

Insulin resistance (IR) lead to various metabolic abnormalities that are associated with metabolic syndrome (MS), type 2 diabetes mellitus (T2DM) and cardiovascular disease (CVD) (Singh et al., 2013). IR refers to the decreased sensitivity or responsiveness to the metabolic actions of insulin like insulin-mediated glucose utilisation and the inhibition of the glucose production in liver. There are various direct and indirect tests for assessing glucose tolerance which includes glucose tolerance test, homeostasis model assessment (HOMA) of insulin resistance (IR) etc (Matthews et al, 1985). The oral glucose tolerance test (OGTT) is a common method of evaluating glucose tolerance status which provides information about insulin secretion and assessment of insulin resistance through surrogate indices, such as the homeostatic model assessment of insulin resistance (HOMA-IR). OGTT interpretations need to perform complicated calculations and hence is not easy to implement in routine clinical practice and epidemiological studies, but at the same time the shape of the glucose curve during an OGTT is effectively used as a marker to evaluate insulin secretion and resistance. Various studies have shown correlation of the shapes of OGTT glucose curve with glucose tolerance status, insulin sensitivity and pancreatic β -cell function (Wang et al., 2018)

3.1.1: Effect of insulin on protein synthesis

In normal body approximately 15% of body weight is contributed by proteins (Bier., 1989) and approximately 30–45% of this is in skeletal

muscles. Insulin play a crucial role in regulating skeletal muscle protein turnover (Prod'homme et al., 2004), and maintaining a balance between the rates of muscle protein synthesis and its breakdown. Maintaining a balance between the synthesis and breakdown have an important role in maintaining muscle size, quality and function and also the body size and shape (McNurlan and Garlick PJ., 1989). Insulin deficiency decrease muscle quality as well as quantity and lead to poor muscle strength and function observed during diabetes. Additionally, skeletal muscle is an important site for glucose utilisation and hence the reduction in muscle mass might adversely affect glucose metabolism (Mavros et al., 2013).

3.2: Results

Table 3.2.1: Fasting blood sugar level

Sl. No	Groups	0 th Day	7 th Day	14 th Day	21 st Day
1	NC	120.20±3.51**	115.81±4.11**	109.83±3.55**	115.83±10.57**
2	DC	463.77±13.55 ^a	515.14±18.29 ^a	533.11±18.93 ^a	542.23±49.48 ^a
3	DDKA	482.6±17.56 ^{a**}	445.09±19.36 ^{a*}	408.15±18.04 ^{a*}	353.96±41.59 ^{a**}
4	DDKB	477.80±13.96 ^a	391.35±13.90 ^{a**}	307.49±10.92 ^{a**}	253.18±23.11 ^{a**}
5	DDKC	446.74±13.05 ^a	373.38±13.53 ^{a**}	241.60±8.58 ^{a**}	182.45±16.65 ^{b**}
6	CDKA	139.23±4.07**	133.78±4.75**	129.78±4.61 ^{a**}	131.20±11.97**
7	CDKB	140.23±4.10 ^{b**}	123.79±4.40**	134.78±4.79 ^{a**}	129.15±11.79**
8	CDKC	135.23±3.95**	127.79±4.54**	116.81±4.15**	114.80±10.48**

Fasting blood sugar level was expressed in mg/dl. Values are expressed as mean ± SD of six rats. 'a' indicates values are significantly different from Normal Control rats with p<0.001. 'b' indicates values are significantly different from Normal Control rats with p<0.05. '**' indicates values are significantly different from Diabetic Control rats with p<0.001. '*' indicates values are significantly different from Diabetic Control rats with p<0.05.

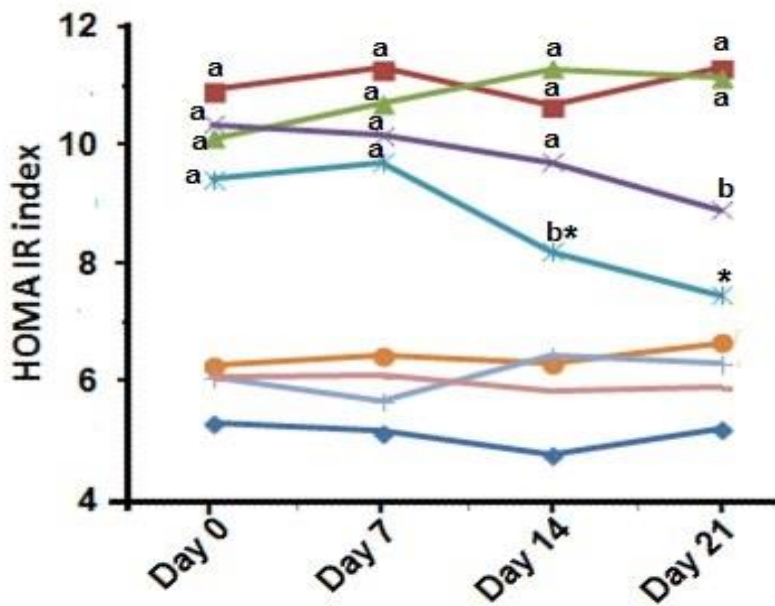
Blood sugar level increased significantly in DC rats ($P < 0.001$) as compared to NC rats. DK administration was found to decrease the blood sugar level in both diabetic and normal rats. This decrease was highly significant in DK fed Diabetic rats ($P < 0.001$ and $P < 0.05$). Compared to DC rats all DK fed rats were having a significantly decreased blood sugar ($P < 0.001$). Fasting blood sugar (FBS) of normal control rats over the course of 4 weeks remained more or less same in the normal range. Diabetic control rats showed steep increase in FBS level (285% on day 3) and at the end of the experimental period FBS level was increased to 542.23 mg/dl (351%). Treatment with DK extract decreased FBS level in both diabetic and normal control rats. Dose A fed diabetic rats the decrease was 24% and dose B fed diabetic rats the decrease in FBS level was 47%. In dose C fed diabetic rats DK significantly decreased FBS level and at the end of the experimental period FBS level was 182.45 mg/dl with a decrease of 59% ($p < 0.05$). In normal rats DK administration decreased FBS level though not significant.

3.2.1: Effect of DK on HOMA –IR index

Homeostatic model assessment of insulin resistance of normal and experimental rats is given in figure 3.2.1:A. HOMA-IR index was significantly higher in diabetic rats compared with normal control rats and remained more or less constant during the study period. Supplementation of dose B and C of DK to diabetic rats significantly ($p < 0.05$) decreased the HOMA-IR index throughout the experimental period in comparison to the untreated diabetic rats. This decrease is

due to the increase in the insulin level and resultant decrease in blood glucose level. In dose A fed rats the HOMA-IR index remained similar to diabetic rats, but on day 21 it decreased slightly. Administration of DK to normal rats did not make any significant changes.

Figure 3.2.1.A: HOMA IR index



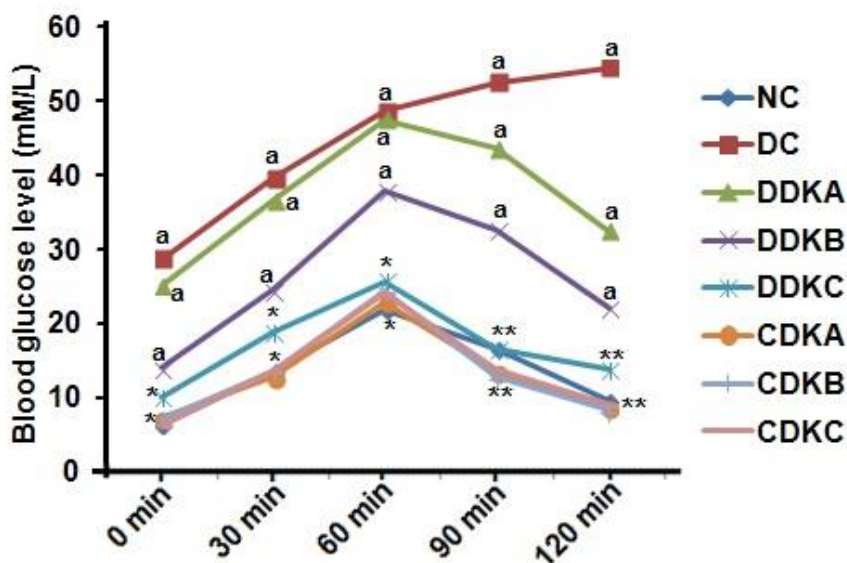
Values are expressed as mean \pm SD of six rats. ‘a’ indicates values are significantly different from Normal Control rats with $p < 0.001$. ‘b’ indicates values are significantly different from Normal Control rats with $p < 0.05$. ‘***’ indicates values are significantly different from Diabetic Control rats with $p < 0.001$. ‘*’ indicates values are significantly different from Diabetic Control rats with $p < 0.05$.

3.2.2: Effect of DK on oral glucose tolerance (OGT)

The results of oral glucose tolerance test (OGTT) of the control and experimental rats are shown in figure 3.2.1:B. OGTT revealed that, in normal control rats, maximum elevation in blood glucose level was at

60 min after glucose load and declined to near basal level at 120 min, whereas, in diabetic rats, the peak increase in blood glucose level was noticed even after 60 min and remained high even at 120 min. Interestingly, supplementation of DK to diabetic rats elicited a significant decrease in blood glucose level at 90 min and beyond when compared with untreated diabetic rats. The effect of DK on glucose tolerance was dose dependent and dose C brought the glucose tolerance to the normal range. In DK fed normal rats, oral glucose tolerance values remained in the normal range.

Figure 3.2.1.B: Effect of DK on oral glucose tolerance (OGT)



Values are expressed as mean \pm SD of six rats. ‘a’ indicates values are significantly different from Normal Control rats with $p < 0.001$. ‘b’ indicates values are significantly different from Normal Control rats with $p < 0.05$. ‘***’ indicates values are significantly different from Diabetic Control rats with $p < 0.001$. ‘*’ indicates values are significantly different from Diabetic Control rats with $p < 0.05$.

3.2.3: Serum insulin level

Table 3.2.2: Effect of DK on serum insulin ELISA.

Sl. No	Groups	Serum
1	NC	18.28 ± 0.654 ^{**}
2	DC	8.45 ± 0.48 ^a
3	DDKA	12.77 ± 1.66 [*]
4	DDKB	14.27 ± 2.12 [*]
5	DDKC	16.58 ± 2.52 [*]
6	CDKA	20.59 ± 1.02 ^{**}
7	CDKB	19.85 ± 1.28 ^{**}
8	CDKC	20.85 ± 2.03 ^{**}

Table 3.2.2. Serum insulin level was expressed in $\mu\text{U/ml}$. Values are expressed as mean \pm SD of six rats. 'a' indicates values are significantly different from Normal Control rats with $p < 0.001$. 'b' indicates values are significantly different from Normal Control rats with $p < 0.05$. '**' indicates values are significantly different from Diabetic Control rats with $p < 0.001$. '*' indicates values are significantly different from Diabetic Control rats with $p < 0.05$.

Streptozotocin causes toxicity in beta cells of pancreatic islets. The destruction of pancreatic islets caused by streptozotocin, as expected, resulted in a significant decrease in the serum insulin levels in the diabetic rats in comparison to normal control rats. In diabetic rats, it dropped by 53.77% (from 18.28 $\mu\text{U/ml}$ to 8.45 $\mu\text{U/ml}$). Administration of DK increased its levels in both diabetic and normal rats. However, the increases induced by DK displayed a group-related pattern. In diabetic rats, the increases induced by low, moderate, and high doses were 51.12, 68.87 and 96.21% respectively. The increases were significant, ($p < 0.05$) and some degree of dose dependence was also exhibited. On the other hand, in non-diabetic rats those respectively were 12.6, 8.5 and 14.05% only.

Table 3.2.3: Organ weight

Sl.No	Groups	Liver	Kidney	Heart
1	NC	4.53±0.14**	1.10±0.04**	0.56±0.02
2	DC	6.89±0.21 ^a	1.70±0.06 ^a	0.56±0.02
3	DDKA	6.14±0.19 ^{a**}	1.70±0.06 ^a	0.61±0.02 [*]
4	DDKB	6.25±0.19 ^{a**}	1.58±0.06 ^{a*}	0.60±0.02 [*]
5	DDKC	5.86±0.18 ^{a**}	1.36±0.05 ^{a**}	0.63±0.02 ^{a**}
6	CDKA	4.87±0.15 ^{b**}	1.24±0.04 ^{a**}	0.58±0.02
7	CDKB	4.58±0.14**	1.24±0.04 ^{a**}	0.60±0.02 ^{b*}
8	CDKC	4.82±0.15**	1.25±0.04 ^{a**}	0.53±0.02

Organ weight was expressed in gm. Values are expressed as mean ± SD of six rats. 'a' indicates values are significantly different from Normal Control rats with p<0.001. 'b' indicates values are significantly different from Normal Control rats with p<0.05. '**' indicates values are significantly different from Diabetic Control rats with p<0.001. '*' indicates values are significantly different from Diabetic Control rats with p<0.05.

Liver and kidney weights were significantly (P<0.001) increased in diabetic rats as compared to normal control rats. Though there was an increase the weight increase in heart was not significant. Compared to diabetic control rats the liver and kidney weights were significantly decreased in all DK fed diabetic rats (P<0.05 and P<0.001), but remained significantly higher than normal control rats. In the case of liver, dose A fed normal rats organ weight is significantly increased compared to normal control rats (P<0.05) but not much difference was seen between dose B and C fed normal rats. In kidney there is not much difference seen between diabetic control and dose A fed diabetic rats. But dose B and C fed diabetic rats showed significant decrease

compared to diabetic rats ($P<0.05$ and $P<0.001$). Compared to normal control rats kidney weight is increased in all DK fed normal rats and compared to diabetic control kidney weight significantly decreased in these rats. An altered pattern of result was obtained in the case of heart weight which did not report any significant change.

Table 3.2.4: Body weight

Sl. No	Groups	0 th Day	7 th Day	14 th Day	21 st Day
1	NC	209.65±7.44**	212.49±9.09**	211.79±7.20**	216.64±7.69**
2	DC	199.67±7.09 ^a	157.45±6.74 ^a	149.75±5.09 ^a	138.77±4.93 ^a
3	DDKA	185.94±6.60 ^{a**}	187.70±8.03 ^{a**}	184.13±6.26 ^{a**}	186.44±6.62 ^{a**}
4	DDKB	212.98±7.56 ^{b**}	191.91±8.21 ^{b**}	187.87±6.38 ^{a**}	188.94±6.67 ^{a**}
5	DDKC	183.69±6.52 ^{a**}	181.99±7.79 ^{a**}	180.90±6.15 ^{a**}	181.95±6.46 ^{a**}
6	CDKA	179.70±6.38 ^{a**}	185.72±7.95 ^{a**}	185.13±6.29 ^{a**}	183.35±6.51 ^{a**}
7	CDKB	191.68±6.81**	205.55±8.79**	207.81±7.06**	213.14±7.57**
8	CDKC	201.66±7.16**	207.53±8.88**	208.05±7.07**	210.47±7.47**

Body weight was expressed in gm. Values are expressed as mean ± SD of six rats. 'a' indicates values are significantly different from Normal Control rats with $p<0.001$. 'b' indicates values are significantly different from Normal Control rats with $p<0.05$. '**' indicates values are significantly different from Diabetic Control rats with $p<0.001$. '*' indicates values are significantly different from Diabetic Control rats with $p<0.05$.

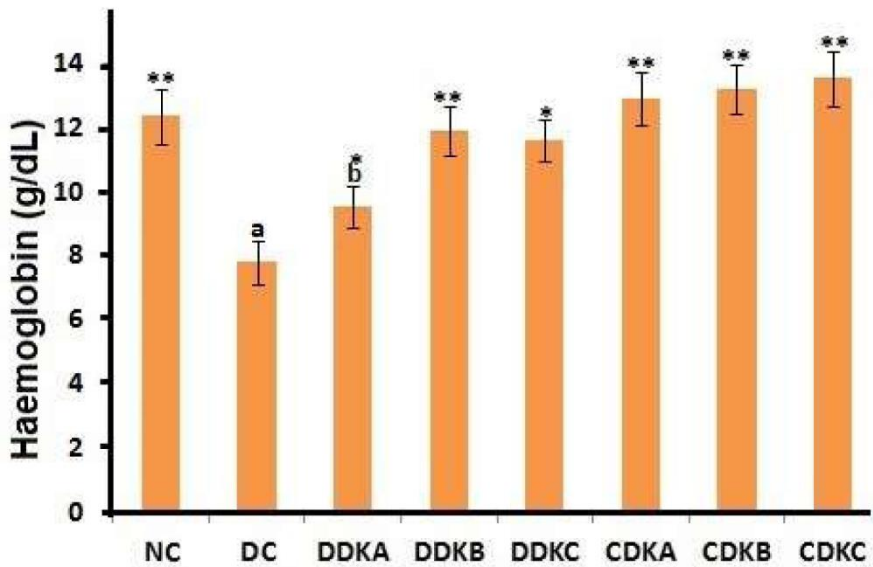
There was no significant increase in the weight of NC rats during the experimental period, but in diabetic rats, body weight gradually decreased throughout the entire experimental period with a total accumulated decrease of 31% on the 21st day. In the case of dose A and dose B fed diabetic rats body weight fluctuated narrowly during the experimental period resulting in an insignificant increase on the

end of the experiment. In dose C fed diabetic rats body weight remained almost same with minor fluctuations. Dose A and C did not produce any significant difference in weight, dose B fed rats had a comparatively higher gain in body weight.

3.2.4: Haemoglobin levels and Glycosylated haemoglobin levels

Decreased haemoglobin (Fig 3.2.2) and increase in glycosylated haemoglobin (Fig 3.2.3) levels were observed in diabetic control rats. Administration of DK reversed these conditions to a great extent. These changes were dose dependent and statistically significant ($p < 0.01$ and 0.05). In diabetic rats the haemoglobin content was decreased by 63% and glycosylated haemoglobin level was increased by 84%. Administration of DK improved the levels of both Hb and HbA1c with haemoglobin level increasing in a dose dependent manner. In dose C fed rats, the haemoglobin level reached near normal (93% of normal value). Decrease in the glycosylated haemoglobin level due to DK was also dose dependent with dose C producing the highest decrease. In dose C fed rats the glycosylated haemoglobin level reached close to normal level (125% of normal value).

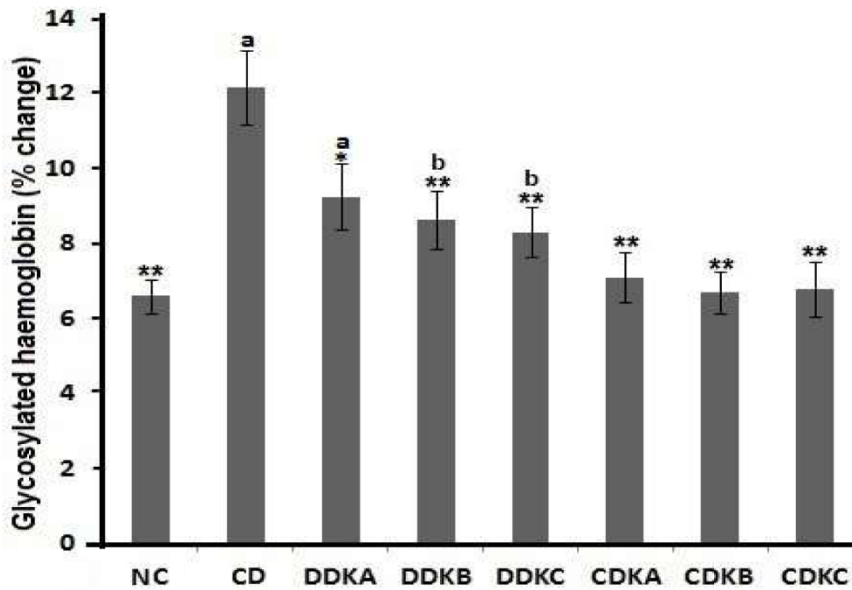
Figure 3.2.2 Effect of DK on Haemoglobin Concentration



Values are expressed as mean \pm SD of six rats. 'a' indicates values are significantly different from Normal Control rats with $p < 0.001$. 'b' indicates values are significantly different from Normal Control rats with $p < 0.05$. '**' indicates values are significantly different from Diabetic Control rats with $p < 0.001$. '*' indicates values are significantly different from Diabetic Control rats with $p < 0.05$.

3.2.5: Glycosylated haemoglobin levels

Figure 3.2.3: Effect of DK on Glycosylated haemoglobin levels



Values are expressed as mean \pm SD of six rats. 'a' indicates values are significantly different from Normal Control rats with $p < 0.001$. 'b' indicates values are significantly different from Normal Control rats with $p < 0.05$. '**' indicates values are significantly different from Diabetic Control rats with $p < 0.001$. '*' indicates values are significantly different from Diabetic Control rats with $p < 0.05$.

3.2.6: Expression of insulin gene

Table 3.2.5: Effect of DK on expression of insulin gene

Sl.No	Groups	Insulin gene
1	NC	1 ^{**}
2	DC	0.22 ^a
3	DDKA	2 ^{a**}
4	DDKB	2.6 ^{a**}
5	DDKC	4 ^{a**}
6	CDKA	1.1 ^{**}
7	CDKB	1.1 ^{**}
8	CDKC	1.13 ^{**}

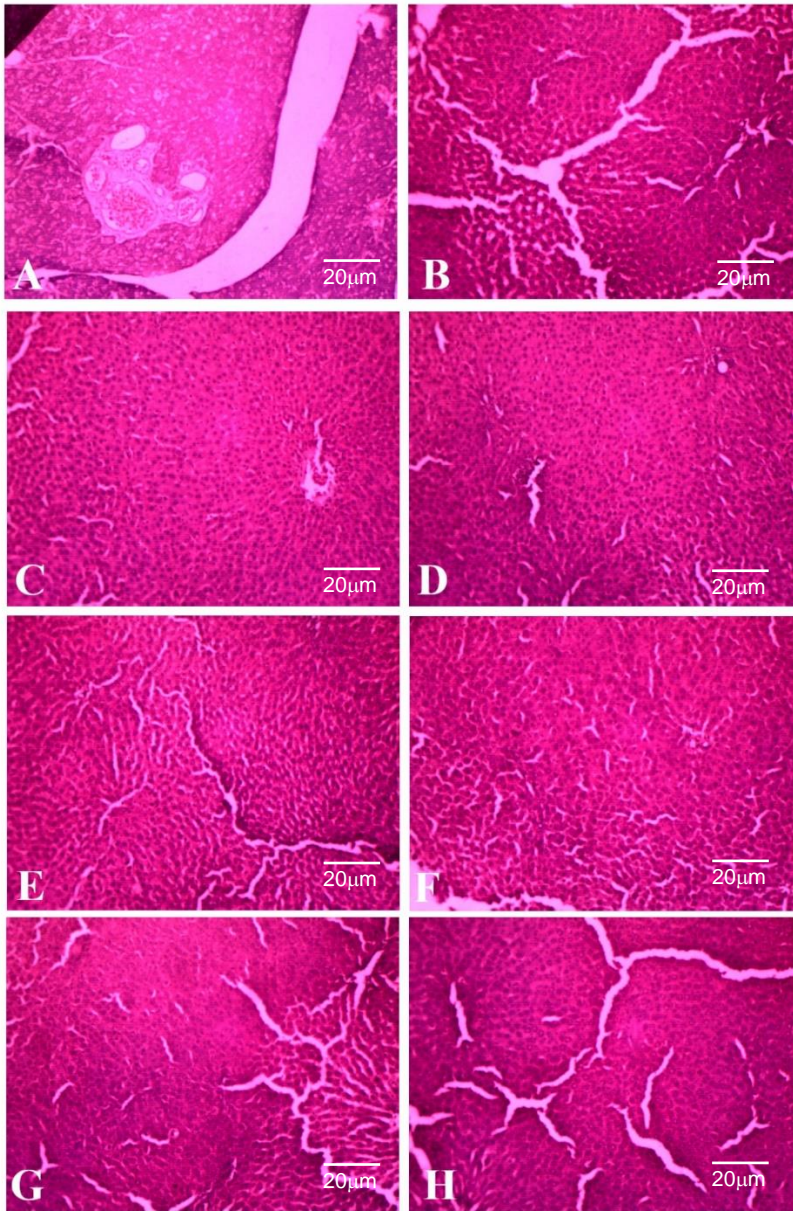
Table 3.2.5: Showing the expression levels of the insulin gene in the rats of the pancreas of the different groups expressed as fold difference in copy number of mRNA of insulin gene relative to the nontreated control. Values are expressed as mean \pm SD of six rats. 'a' indicates values are significantly different from Normal Control rats with $p < 0.001$. 'b' indicates values are significantly different from Normal Control rats with $p < 0.05$. '**' indicates values are significantly different from Diabetic Control rats with $p < 0.001$. '*' indicates values are significantly different from Diabetic Control rats with $p < 0.05$.

Since STZ kills pancreatic islets cells, a profound decrease in the levels of the insulin gene transcripts were observed in diabetic rats. In diabetic rats, its levels dropped by about 78% of normal rats. DK administration enhanced the insulin gene expression in both diabetic as well as in normal rats. In diabetic rats, lowest amount of DK (Dose A) induced expression of insulin gene by nine fold over the untreated diabetic rat group. Highest dose of DK used in the study (Dose C) was able to induce insulin gene expression to the highest levels in both normal and diabetic rats. A group related insulin gene induction was

seen. The highest increase due to DK was for the dose C which was 1700 percent in the diabetic rats and only 13 percent in the normal rats in comparison to the corresponding controls. This observation of increased gene expression reflected the serum levels of insulin. Serum insulin also showed the same pattern of increase in insulin transcripts and protein. It is logical to infer that the increased expression of the insulin gene must have resulted in the increased serum insulin levels. Also, it is logical to infer that the increase in expression might be due to the observed increase in number of β cells in the islets in DK fed rats.

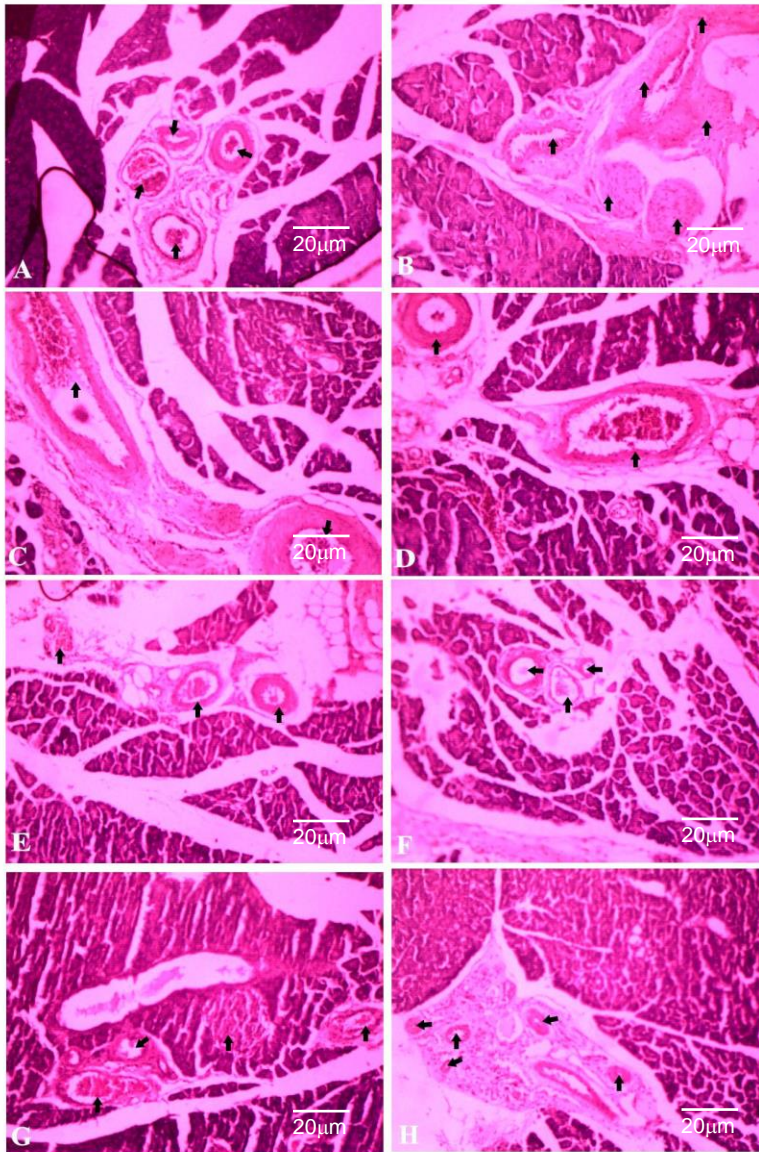
3.2.7: Histopathology of liver and pancreas

Figure: 3.2.4: Effect of DK on histopathology of liver in normal and STZ induced diabetic rats: Histopathology of the normal liver cells (Fig 3.2.4:A) revealed that normal polygonal structure of tightly packed hepatocytes with basophilic central rounded nuclei and separated by the hepatic sinusoids. Diabetic control rats (Fig 3.2.4:B) showed abnormal swelling of hepatocytes together with micro vesicular and macro vesicular changes within cytoplasm. Remaining slides (Fig 3.2.4:C- Fig 3.2.4:H) had normal hepatocyte structure and cytoplasm as compared to DC rats. This gives clear evidence of cellular regeneration as compared to the diabetic control rats. 40X magnification was used for histopathological analysis.



H & E stained microscopic photomicrographs of the liver tissue of different groups were studied. A: Liver tissue of Normal Control rats, B: Liver tissue of Diabetic Control rats, C: Diabetic + Dose A, D: Diabetic + Dose B, E: Diabetic + Dose C, F: Normal + Dose A, G: Normal + Dose B, H: Normal + Dose C.

Figure 3.2.5: Effect of DK on histopathology of pancreas in normal and STZ induced diabetic rats: H & E stained microscopic photomicrographs of the pancreas of different groups studied. A: Pancreas of normal control rats exhibiting histological features of normal islets. B: Diabetic control: Islets of untreated diabetic rats showing shrunken islets with drastic decrease in cell numbers. C: Diabetic rats administered Dhanwantaram Kashaya dose A: showing apparent increase in islet size and number of cells than diabetic pancreas. D: Diabetic rats administered Dhanwantaram Kashaya dose B: showing well formed islets with increased number of cells than dose A fed diabetic rats. E: Diabetic rats administered Dhanwantaram Kashaya dose C: showing well formed islets. Islet size and number are near to normal rats with normal cellular arrangements. F-H: Normal rats fed Dhanwantaram Kashaya dose A, B and C respectively showing islets with histological features of normal rats. 40X magnification was used for histopathological analysis.



Histological studies of pancreas showed degenerating islets in diabetic rats (Fig 3.2.5:B). Signs of regeneration of the islets were observed following the administration of even the lowest dose of DK used during the study (Fig 3.2.5: C-E). Administration of DK increased the density of cells and width of the pancreatic islets in normal rats (Fig 3.2.5: A) as well as in diabetic rats (Fig 3.2.5: C-E). This might be due to the increased number of β -cells which is possibly rooted in the promotion of proliferation of surviving pancreatic islet specific stem cells by DK.

3.3: Discussion

Diabetes mellitus is a universal health problem and affecting large population of the world. Hyperglycemia is one of the main consequences of diabetes mellitus and also altered carbohydrate, protein or lipid metabolism. STZ is the extensively used for inducing diabetes in experimental animal models. This has cytotoxic effects on pancreatic β -cells resulting in decreased insulin secretion and eventually leads to the condition called hyperglycemia (Singh et al., 2002, Kim et al., 2006, Szkudelski., 2001). Hyperglycemia provide the suitable environment for the formation of ROS and excessive production of ROS can cause oxidative stress and exacerbate damage to pancreatic β -cells in our body (Robertson et al., 2003, Pazdro and Burgess., 2010).

Diabetes is a major public health challenge in modern world. Persistent hyperglycaemia results

in secondary complications such as hypertension, coronary artery disease, neuropathy, nephropathy, foot ulcers, sexual dysfunction etc (Duckworth., 2001, Jain and Saraf., 2010). Irrespective of the extensive efforts to find a cure to DM, no permanent cure for this disease is available. The standard form of treatment is through the modifications of diet and lifestyle combined with drug therapy with hypoglycaemics, such as sulphonylureas, α -glucosidase inhibitors and thiazolidenediones.

Administration of exogenous insulin becomes necessary when blood glucose levels cannot be controlled by controlling diet, exercise or by hypoglycaemic. The inconvenience and the pain of taking these treatments are pushing the scientists all over the world to identify a permanent cure or a management program through dietary supplements (Yeung et al., 2018). Healthy diet rich in plant derived materials are found to delay the complications associated with DM or even prevent DM development. DK is a health tonic reported to induce tissue repair and regeneration. In this study we investigated the effect of DK on relieving diabetic complications and found it as a promising health tonic in the management of diabetes. DK administered rats were relieved of hyperglycemia. This might be due to the increase in the production insulin or its effectiveness in the body. OGTT also revealed better management of glucose turnover. HOMA-IR index analysis revealed the positive change in insulin mediated glucose homeostasis. These positive changes show the improved management of hyper glycaemia in DK fed diabetic rats.

Total fluid and food intake and urine production significantly increased in diabetic rats. However the body weights of the diabetic rats were decreased by 20-30%. A significant inhibition in diuresis was observed in all the DK fed diabetic groups. Similarly, weight loss was reverted to a great extent in all the DK fed diabetic groups. These positive changes show the improved management of hyper glycaemia in DK fed diabetic rats. Diabetes mellitus is normally monitored by measuring fasting blood sugar (Rajkumar et al., 2005). Main objective of the present study was to evaluate the effect of DK, a poly herbal

ayurvedic drug with significant antioxidant activity on STZ induced diabetic rats. This experiment revealed that supplementation of DK reduced the blood glucose level and increased insulin level in diabetic and normal rats. DK is rich with phytochemicals such as phenols (Sruthi and Sindhu., 2012), flavonoids and tannins. Phenolic compounds are highly potent water-soluble antioxidants in plants (Machiex et al., 1990) which have the ability to scavenge the free radicals in our body (Shahidi and Wanasundara., 1992). Present study clearly shown that increased level of phytochemicals in DK extract enhance endogenous and exogenous antioxidant system and reduced the oxidative stress.

DK administered rats were relieved of hyperglycemia. This might be due to the increase in the production insulin or its effectiveness in the body. OGTT also revealed better management of glucose turnover. OGTT is effectively used to study the antihyperglycemic activity of hypoglycemic agents (Ali et al., 2020). In the present study, STZ-induced diabetic rats showed an elevation in serum glucose levels at all points of OGTT as compared with normal rats; these data are in concordance with other previous studies (Wang et al., 2018). HOMA-IR index analysis revealed the positive change in insulin mediated glucose homeostasis. These positive changes show the improved management of hyper glycaemia in DK fed diabetic rats.

An increase in the Hb level and a decrease in the level of glycosylated haemoglobin were observed in DK fed diabetic rats. The increased levels of HbA1c in diabetic state could be a consequence of abnormal

carbohydrate metabolism. Increased production of ROS, oxidative stress, and destruction of tissue involved in both plasma and tissue glycoprotein activities would have contributed this (Konda et al., 2020). Results of this study disclose the significantly decreased glycosylated haemoglobin levels in DK fed rats. Decreased hyperglycaemia could be the reason for this positive effect (Konda et al., 2020). Increase in the Hb level and decreased glycosylation of Hb improve the oxygen status of the tissues and the metabolic process will come back to normal and would have resulted in the improved health as observe in the DK fed diabetic rats.

DK is a versatile product and a formulation of 40 different plant ingredients (Sruthi and Sindhu., 2012). Most of the plants used in the preparation of DK are known to have hypoglycaemic activity and regeneration capacity (Kaur et al., 2011, Joshi and Bhonde., 2014). In this study histopathology of pancreatic sections clearly shown the regeneration capacity of DK. Increased pancreatic β -cells may be the reason for the increased insulin level observed in diabetic and normal control rats. Insulin level was significantly increased in both DK fed diabetic and normal control rats but interestingly in this study increase was significant ($p < 0.005$) and in a dose dependent manner in DK fed diabetic rats. Suba et al in (2004) had reported a similar result in diabetic rats fed with Barlerialupulina extract (Suba et al., 2004). They suggested stimulation of the residual pancreatic mechanism, partial regeneration or protection of pancreatic cells, and potentiating insulin secretion from protected β -cells of the islets of Langerhans as the

possible mechanisms behind the decreased fasting blood glucose level in diabetic and normal rats.

Body weight and organ weight of the diabetic and normal rats were also analysed. As we observed a decrease in body weight and an increase in organ weight was already reported in diabetic rats as compared to normal control rats (Sharma et al., 2015, Sreekutty and Mini., 2016). In the case of liver and kidney weight was significantly increased in diabetic rats compared to normal control rats. But in the case of heart altered pattern of results was obtained. Increased weight of liver may be due to increased accumulation of triglycerides or increased influx of fatty acids into the liver induced by hypoinsulinemia (Habibuddin et al., 2008, Lee et al., 2008). Marked elevation in weight of the kidney may be due to fatty infiltration, enlargement of tubular cell lining, large hemorrhagic area and lymphocyte infiltration (Rajkumar et al., 2005). Administration of DK significantly decreased liver and kidney weight in diabetic rats. In the case of liver there is not much difference seen in normal and DK administrated normal rats. But in the case of kidney organ weight was slightly increased in DK administrated rats compared to normal control rats.

Histopathology of pancreas and liver clearly showed that DK have protective effect on diabetic and normal control rats. The normal control rats showed normal lobular architecture of the pancreas. Slide showed a degeneration of islets in diabetic control rats and the administration of DK was found to regenerate the islets in dose A, dose B and dose C fed diabetic rats. The width and number of the islet cells are also increased in dose B and dose C fed normal control rats. In the

case of liver normal histological slides showed well-arranged hepatocyte cells. Major destruction of hepatocytes and severe necrosis was observed in diabetic control rats. Remaining slides from diabetic rats fed different doses of DK showed the curative effect of DK extract on liver tissue and all of the hepatocyte cells were healthy and normal.

A recent study about DK revealed it as a growth enhancer and has the ability to improve the cell proliferation rate, as well as the quality of Mesenchymal Stem Cells (MSCs) (Warrier et al., 2013). We have already reported an increase in the level of vitamin C in DK administered rats (Renganathan et al., 2020). Vitamin C has been reported to be involved in the reprogramming of pluripotent stem cells (Monfort and Wutz., 2013). Mekala et. al., (2013) have demonstrated the ability of vitamin C in regulating the proliferation as well as in differentiation of stem cells (Mekela et al., 2013). In the light of the above reports, we think that this increased vitamin C levels might have a role in the induced regeneration of islets observed in DK fed diabetic rats. There is also a possibility that the observed increase in the number of pancreatic islet β -cells in DK treated diabetic rats may be due to natural mobilization of bone marrow derived stem cells, however, a role of DK in mobilizing stem cells from bone marrow has to be established. We previously have reported the excellent antioxidant properties of DK (Renganathan et al., 2020). This antioxidant property of DK might be protective for the existing cells as well as for the cells regenerated from the stem cells. The possibility that the increased level of antioxidants in DK fed rats also could have contributed to this increase in cell number. As mentioned above DK is a decoction of the ingredients of 40 plants which are reported to have growth enhancing,

antioxidant, antidiabetic, and rejuvenating effects (Upadhyaya., 2006), (Sruthi and Sindhu., 2012). Diabetic rats are accepted as excellent models of diabetes (Islam and Loots., 2009), and here in this study we have seen beneficial impacts of DK in ameliorating diabetic complications. One or more of the components of these plant origins could have performed the major antidiabetic roles. In order to confirm this, further investigations are needed which could be used for developing a possible more effective therapeutic modality that might have none or comparatively fewer undesired side effects.

3.4: Conclusion

In conclusion, the fasting blood sugar level and histopathological findings suggested that supplementation of DK to diabetic rats causes beneficial effects. It is an excellent hypoglycemic agent and helps regeneration of pancreatic β -cells in damaged and normal pancreas. Histopathology of pancreas and liver also revealed the curative effect of DK not only on pancreatic β -cells but also on hepatocytes. It maintained body weight in diabetic rats and significantly decreased organ weight in diabetic rats. From the observations, it is concluded that DK possess preventive and curative effect on STZ induced diabetic rats. Hence this herbal product could be developed as a promising natural and safe remedy for the prevention or delay of diabetic complications. The exact mechanism behind it is clearly unknown. Further studies are needed to understand the mode of action of the DK in our body.

Phytochemical composition and *in vitro* antioxidant power of Dhanwantaram Kashaya

4.1: Introduction

Phytochemicals are naturally occurring biologically active chemical compounds found in plants contributing to the plant's colour, aroma and flavor (Koche et al., 2016) and protect the plant cells from diseases and damages caused by environmental hazards such as pollution, stress, drought, UV exposure etc. (Gibson et al., 1998, Mathai., 2000). Plants are able to produce large variety of bioactive components. Many phytochemicals have the ability to act as a natural antioxidants and have the power to fight against free radical damages caused by reactive oxygen species (ROS) (Suffredini et al., 2004, Boots et al., 2008). Antioxidant properties of the phytochemicals like vitamins A, C, E, and phenolic compounds such as flavonoids, tannins, and lignins found in plants have already reported (Suffredini et al., 2004). Consumption of fruits and vegetables rich with beta carotene, ascorbic acid and phenolics like phytochemicals play dynamic roles in delaying ageing, reducing inflammation, and preventing certain cancers (Duthie et al., 1996). Phytochemical compounds have several mechanisms of action in our body including antioxidant effects, modulation of hormone metabolism, anti-bacterial and antiviral effects, modulation of enzyme actions, stimulation of the immune system, interference with DNA

replication and preventing the adhesion of pathogens to human cell walls (Ngoci et al., 2011).

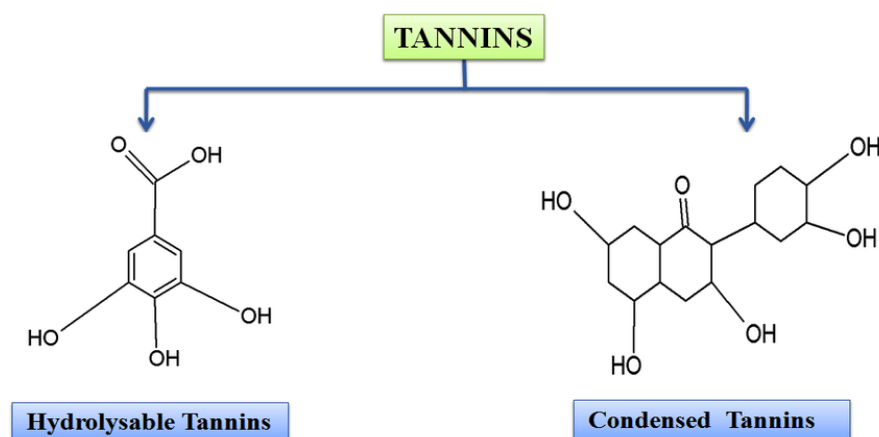
Oxidative stress is one of the major reasons for the pathogenesis of several chronic diseases like diabetes. Reactive oxygen species and free radicals also contribute to progression and development of several diseases like asthma, diabetes etc. Phytochemical compounds have several mechanisms of action in our body including antioxidant effects (Ngoci et al., 2011). Dhanwantaram Kashaya (DK) is a poly herbal formulation in the form of a decoction and the herbs used for its preparation are having regeneration property (Joshi and Bhonde., 2014). It also has excellent antioxidant activities (Sruthi and Sindhu., 2012).

Herbal drugs from Ayurveda have already proved to be important leads for drug development (Rayudu and Raju., 2014). DK is used in Ayurveda for many ailments and improving general health without any noticeable side effects, but as in the case of many other ayurvedic drugs, no objective, verifiable data exist to support many such claims (Panigrahi et al., 2019). As DK is well known as a rejuvenating drug as well as having a suggested ability of preventing tissue degeneration in Ayurveda, in the present study, we investigated on the phytochemical components with antioxidant capacity of DK. We mainly focused on the possible phytochemical components like total tannins, total flavonoids and total phenolic content. We also evaluated it's *in vitro* antioxidant power as well as free radical scavenging activity through the assays like Ferric Reducing Antioxidant Power (FRAP), 2'-

Diphenyl-1-Picryl Hydrazine (DPPH) scavenging activity, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging assay, Phosphomolybdenum assay, Hydroxyl radical scavenging activity, Hydrogen peroxide-scavenging activity and Nitric oxide scavenging activity with a hope to delineate its mode of action.

Tannins are one of the bitter plant polyphenols and astringent that have the capacity to precipitate or shrink proteins. This plant derived natural antioxidants have several actions in our body including free radical scavenging activity, chelation of transition metals, inhibition of prooxidative enzymes and lipid peroxidation (Navarro et al., 2003). Because of these properties tannins modulate oxidative stress, prevent degenerative diseases and also inhibit tumor growth by inducing apoptosis (Scalbert et al., 2005).

Figure 4.1.1: Molecular structure of tannins.

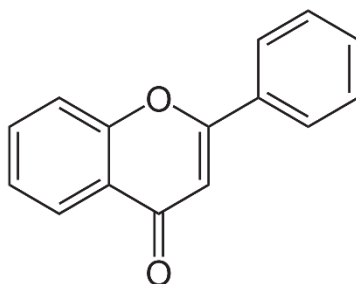


(Source: https://www.researchgate.net/figure/Figure1-Types-of-tannins-and-their-basic-structures-Tannins-in-therapeuticsResearchers_fig1_277029056 (Accessed on:19-09-2020))

Tannins are commonly found in fruits such as grapes, persimmon, berries and tea, chocolate, legume forages, legume trees like *Acacia* spp, *Sesbania* spp, in grasses like sorghum, corn etc. Tannins are chemically classified in to two major groups as hydrolysable tannins and condensed tannins. Hydrolysable tannins react with water forming compounds like gallic acid. Tannins are reported to decrease the frequency of chronic diseases and having the ability to fight against diseases like AIDS and cancer (Koche et al., 2016).

Flavonoids are most studied largest group of plant phenols (Dai and Mumper., 2010) and occur in vegetables, fruits and beverages like tea, coffee and fruit drinks (Pridham., 1960). Flavonoids are phenolic natured water-soluble compounds which are structural derivatives of flavones. It contains conjugated aromatic systems and often bound to sugars as glycosides (Harborne., 1973). Quercetin is one of the important flavonoids that reduces oxidative stress by preventing oxidation of low-density lipoprotein (Ngoci et al., 2011).

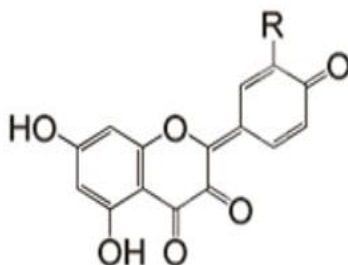
Figure 4.1.2: Molecular structure of the flavone backbone (2-phenyl-1,4-benzopyrone).



(Source: <https://en.wikipedia.org/wiki/Flavonoid> Accessed on 19-09-2020)

Phenolic compounds are widely distributed chemical compounds and largest category of phytochemicals in the plant kingdom (Walton et al., 2003). It contains hydroxyl groups which is directly bonded to an aromatic hydrocarbon group. Polyphenols are one of the most important groups of dietary phenolics. They are important defense compounds that beneficial to humans. Its antioxidant property protects against free radical-mediated disease processes (Koche et al., 2016).

Figure 4.1.3: Structure of polyphenols.



(Source: <https://www.omicsonline.org/open-access/cardioprotecting-effect-of-natural-bioactive-compound-polyphenol-by-inhibiting-ldl-oxidation-with-the-scavenging-of-reactive-oxygen-2155-9880-1000453.php?aid=75741>. Accessed on 19-09-2020)

Polyphenols (PPs) are reported to abrogate diabetes complications (Momtaz et al., 2019). Their ability to improve glucose homeostasis in different body tissues like liver, adipocytes, pancreas, muscles etc. are also well known (Sattarinezhad et al., 2018, Lazavi et al., 2018, Mahmoud et al., 2017). This positive control over glucose homeostasis is achieved by increasing glucose metabolism and by improving the physiology and integrity of β cells of pancreas. Improved β - cell function increase insulin production and help to control blood glucose level in diabetic patients. It has positive influence on regulating insulin

resistance also (Cai and Lin., 2009). Another effect of PPs in glucose metabolism is by affecting the insulin receptors in muscles along with promoting glucokinase activity in liver. This also helps in reducing glucose absorption in intestine (Cao et al., 2018). PPs also interfere with glucose turnover by promoting glucose uptake in glucose disposing tissues (Hardie et al., 2012).

4.2: RESULTS

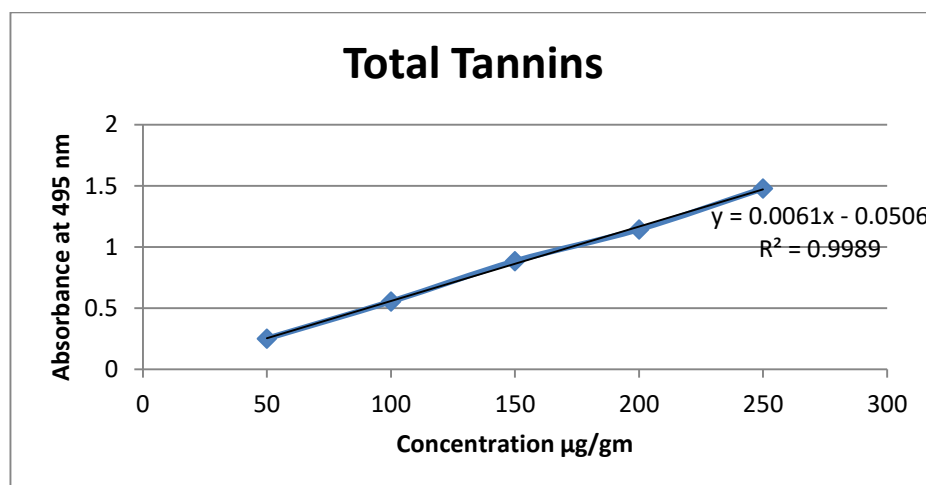
4.2.1: Phytochemicals

4.2.2 Total Tannins

Tannic acid was used as a standard and the total tannin content was expressed as tannic acid equivalents (TAE). Absorbance was measured using a spectrophotometer at 495 nm.

Total tannin in DK is 0.22 ± 0.015 mg TAE/g.

Figure 4.2.1: Total tannins



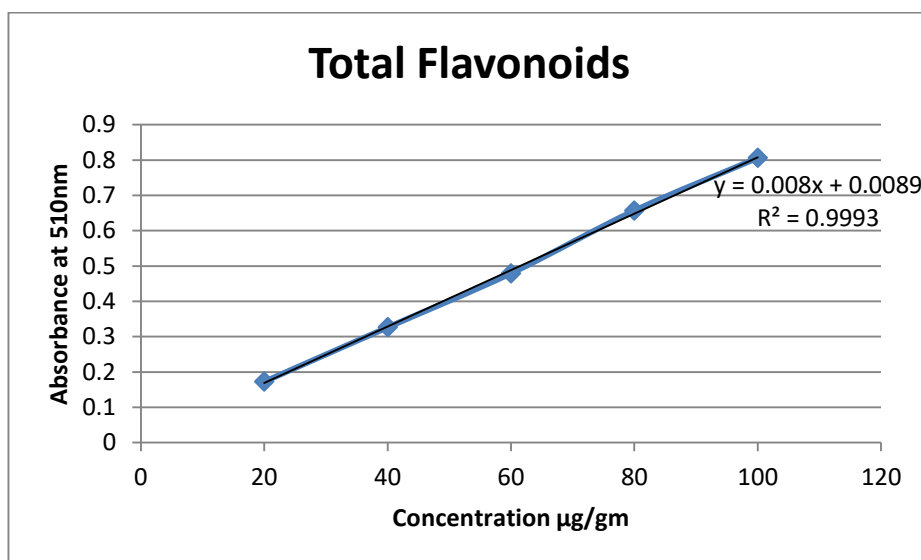
Graphical representation of the standard curve plotted using tannic acid as standard for the quantification of tannins in DK. Concentration of the sample was calculated from the slope of standard curve.

4.2.3: Total Flavonoids

Concentration of flavonoids, was calculated with the help of a calibration curve using catechin as standard. The flavonoid concentration is expressed as catechin equivalents in mg per gm of DK.

Total flavonoids content of the DK is 1.23 ± 0.043 mg CE/g.

Figure 4.2.2: Total Flavonoids



Graphical representation of the standard curve plotted using catechin as standard for the quantification of flavonoid in DK Concentration of sample was calculated from the slope of standard curve.

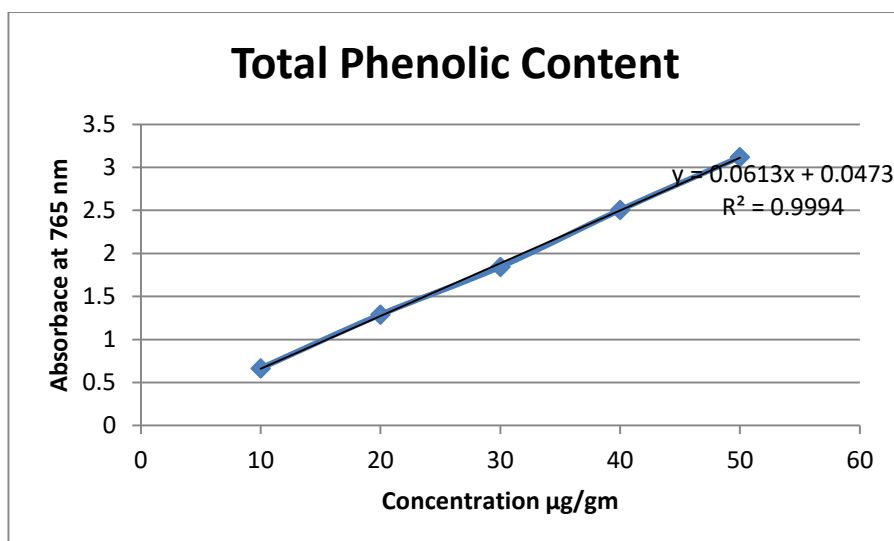
4.2.4: Total Phenolic Content

The total phenolics content of DK was estimated using Folin-Ciocalteu reagent. The absorbance was measured at 765 nm using a spectrophotometer. The distilled water was used as a blank. All assays

were carried out in triplicate. A calibration curve of gallic acid was constructed and linearity was obtained in the range of 10-50 $\mu\text{g}/\text{gm}$. The total phenolics content in the DK was expressed as mg of gallic acid equivalent (mg of GAE/gm extract) by using the standard curve.

The total poly phenolic content in DK is 10.05 ± 0.94 mg GAE/g.

Figure 4.2.3: Phenolic Content



Graph showing the standard curve plotted using gallic acid as standard for the quantification of phenol in the DK. Concentration of the sample was calculated from the slope of the standard curve.

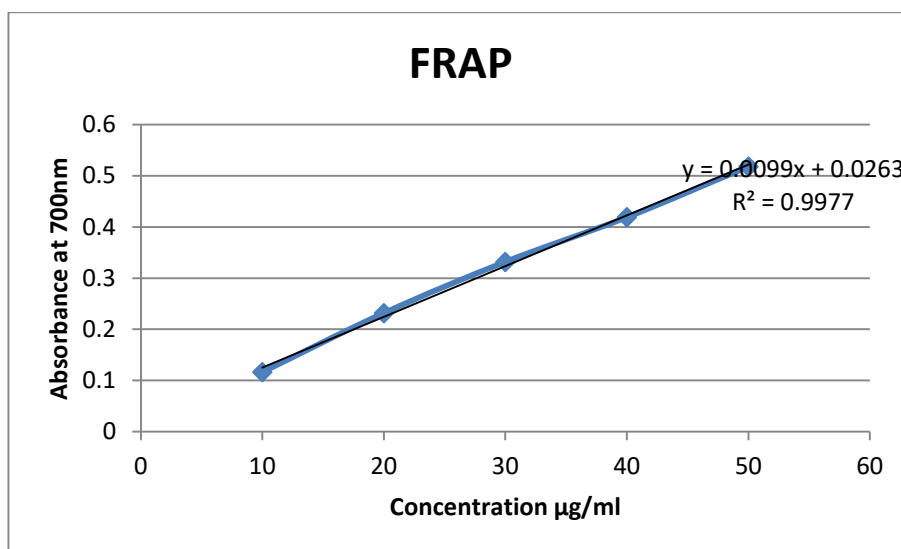
4.2.5: Antioxidant Power Assays

4.2.5.1: Ferric Reducing Antioxidant Power (FRAP)

FRAP assay measures the reaction of an antioxidant with a ferric tripyridyltriazine (Fe^{3+} -TPTZ) complex and producing a colored ferrous tripyridyltriazine (Fe^{2+} -TPTZ), the intensity of which shows the

reducing potential of an antioxidant. FRAP values of the DK was $36.29 \pm 0.90 \mu\text{g/ml}$.

Figure 4.2.5.1: FRAP



Graph showing the standard curve of FRAP plotted with ascorbic acid as standard for finding the reducing power of DK.

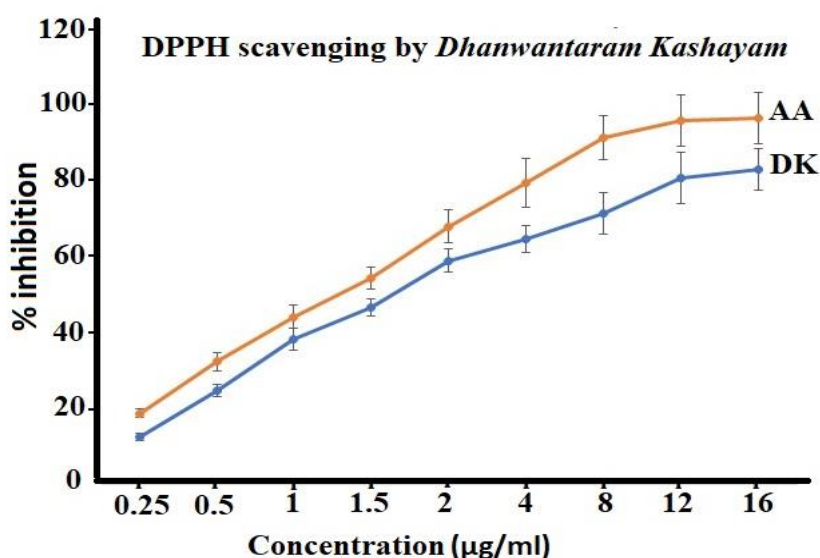
4.2.5.2: 2,2-Diphenyl-1-picryl Hydrazine (DPPH) scavenging activity

Free radicals produced in the body are partly associated with the etiology of diabetes. Determining the radical scavenging effect of the drug is important in determining its effect on ameliorating the diabetic complications. Antioxidant activities of DK were analyzed by measuring DPPH radical scavenging activities. This assay is widely used in evaluating antioxidant activities in a short time compared with other methods.

The changes in the absorbance of the DK extract were measured at 515 nm using spectrophotometer. A lower absorbance value indicates the higher radical scavenging activity. Results were compared with the standard ascorbic acid. Lower IC₅₀ value indicates greater antioxidant activity.

The capability of the sample to inhibit DPPH was found out from the equation given in materials and method.

Figure 4.2.5.2: DPPH Scavenging Assay

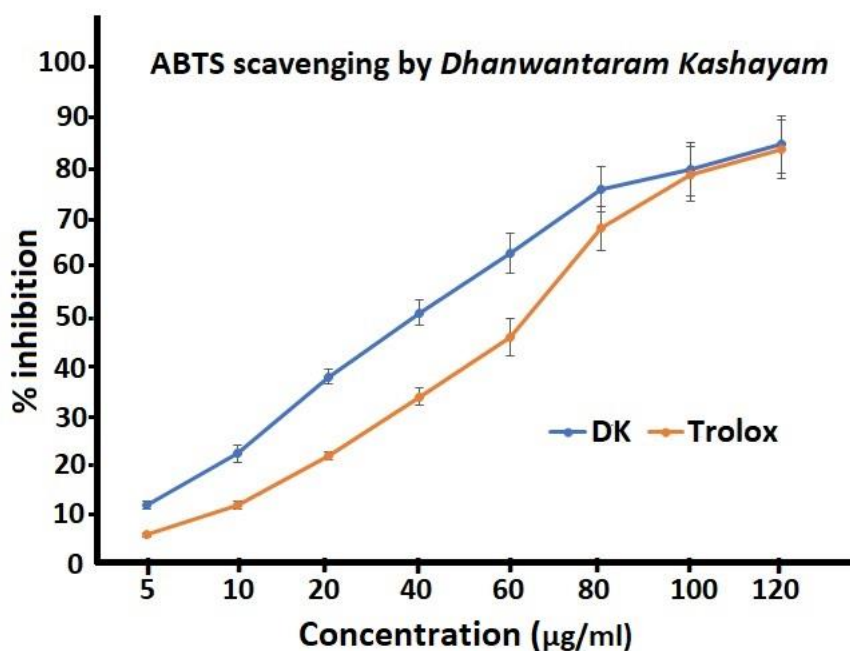


Graph showing the DPPH radical scavenging activity of ascorbic acid, which is used as standard for quantitating the scavenging activity of DK and also showing the DPPH radical scavenging activity of DK. IC₅₀ value (the concentration with scavenging activity of 50%) is calculated and standardized in comparison with that of ascorbic acid.

DK demonstrated strong percentage inhibition and DPPH radical scavenging activity (Figure 4.2.5.2), but not as strong as ascorbic acid. IC₅₀ value of DK was 2.04 ± 0.11 $\mu\text{g/ml}$ which was found to be double of ascorbic acid which was 1.03 ± 0.051 $\mu\text{g/ml}$. The result from the antioxidant assay showed that DK can scavenge the radical to a great extent.

4.2.5.3: ABTS radical scavenging assay

Figure 4.2.5.3: ABTS radical scavenging assay



Graph showing the ABTS radical scavenging activity of trolox, which is used as standard for quantitating the scavenging activity of DK.

The capacity of DK to scavenge the $\text{ABTS}^{\cdot+}$ radical cation was assessed and the antioxidant capacities were expressed by IC₅₀ value

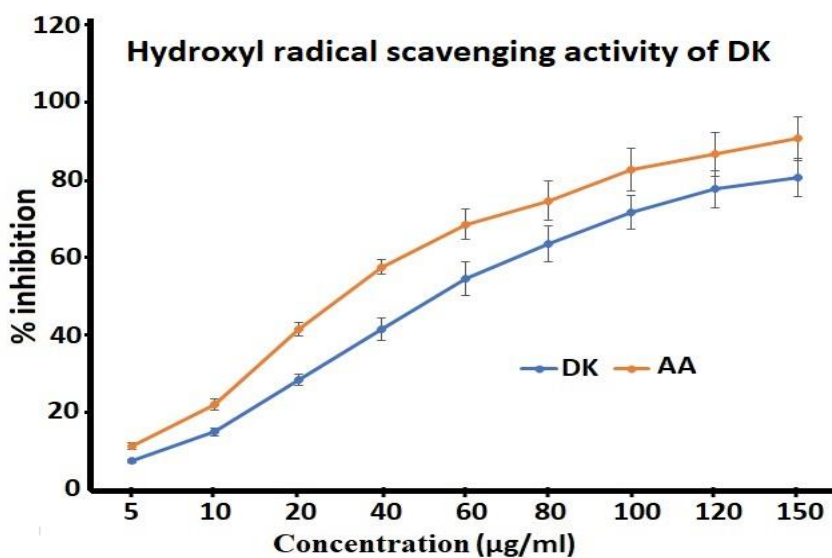
which indicated the concentration of DK needed to scavenge 50% of ABTS^{•+} radical. As shown in Figure 4.2.5.3, the IC₅₀ value of the ABTS^{•+} radical scavenging activity was $38.46 \pm 2.75 \mu\text{g/ml}$. Comparing these values with standard (Trolox IC₅₀ = $66.66 \pm 1.51 \mu\text{g/ml}$), it is obvious that DK is more effective in scavenging the ABTS^{•+} radical cation than trolox.

4.2.5.4: Phosphomolybdenum assay

Antioxidant potency of DK was also assayed by the formation of green phosphomolybdenum complex which revealed strong effects of DK on reducing Molybdenum radical with an IC₅₀ value of $50.4 \pm 2.63 \mu\text{g/ml}$.

4.2.5.5: Hydroxyl radical scavenging activity

Figure 4.2.5.4: Hydroxyl radical scavenging activity

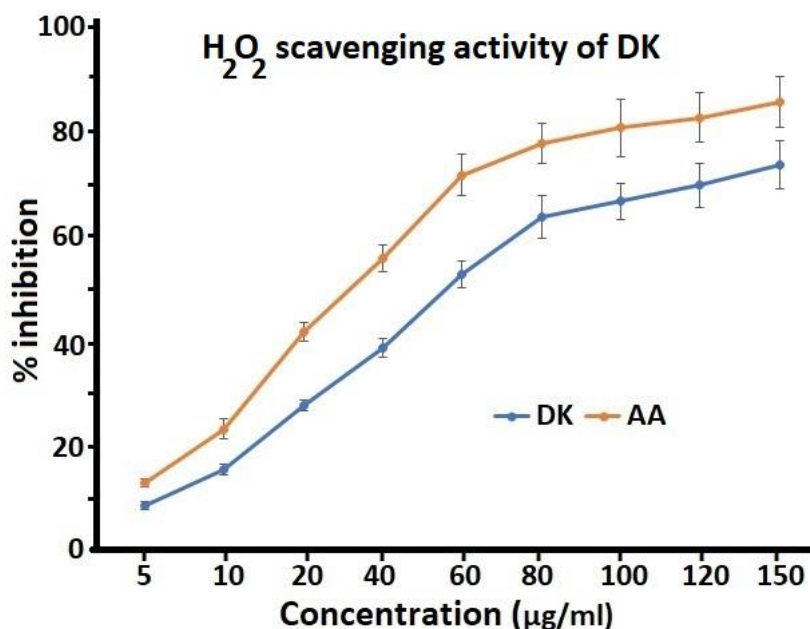


Graph showing the hydroxyl radical scavenging activity of ascorbic acid, which is used as standard for quantitating the scavenging activity of DK.

Hydroxyl radical is the most reactive among the oxygen radicals which are reported to induce severe damage to biomolecules such as DNA, proteins and lipids and also causes lipid peroxidation which is the root cause of many tissue damages, cancer and cell death. Hence removal of this free radical is important in protecting life. Hydroxyl radical scavenging assay also showed DK as having notable effect. The results showed concentration dependent inhibition of DK against hydroxyl radical-induced degradation of deoxyribose (Figure 4.2.5.4).

4.2.5.6: Hydrogen peroxide-scavenging activity

Figure 4.2.5.5: Hydrogen peroxide-scavenging activity

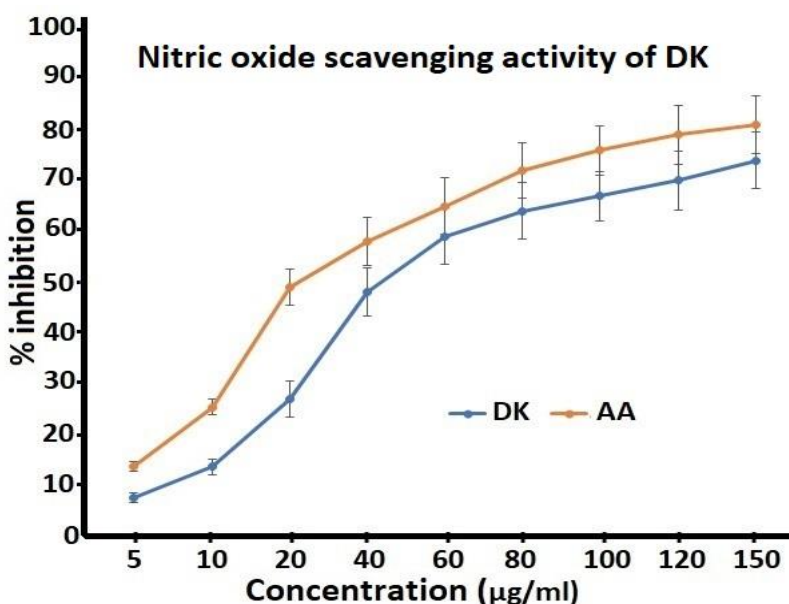


Graph showing the hydrogen peroxide-scavenging activity of ascorbic acid, which is used as standard for quantitating the scavenging activity of DK.

Hydrogen peroxide is nonreactive and unstable, but at high concentrations it is toxic to living cells. Certain cellular metabolic processes change H₂O₂ into hydroxyl radicals; which comes under the harmful group free radicals. Effective removal of H₂O₂ is important to maintain cellular health and normal functions. Therefore, the H₂O₂ scavenging effects of DK was evaluated (Figure 4.2.5.5). DK was found to have strong scavenging capacity with an IC₅₀ of 57.9 ± 3.15 µg/ml, IC₅₀ value of DK was higher than ascorbic acid which was 35.8 ± 2.76. The H₂O₂ scavenging ability of DK was dose dependent which increased with increasing dose.

4.2.5.7: Nitric oxide scavenging activity

Figure 4.2.5.6: Nitric oxide scavenging activity



Graph showing the nitric oxide scavenging activity of ascorbic acid, which is used as standard for quantitating the scavenging activity of DK.

Dose-dependent increase in nitric-oxide radical scavenging activity was observed at studied concentrations of DK with an IC₅₀ value of 57.25 ± 3.7 $\mu\text{g/ml}$ (Figure 4.2.5.6). The result shows that DK is a potent scavenger of NO radicals.

4.3: Discussion

Different phytochemicals such as tannins, flavonoids and total phenolic content present in DK were estimated. We also evaluated its *in vitro* antioxidant power as well as free radical scavenging activity through the assays like Ferric Reducing Antioxidant Power (FRAP) and 2,2-Diphenyl-1-Picryl Hydrazine (DPPH) scavenging activity, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging assay, Phosphomolybdenum assay, Hydroxyl radical scavenging activity, Hydrogen peroxide-scavenging activity and Nitric oxide scavenging activity with a hope to delineate its mode of action.

Phytochemicals are secondary metabolites present in plants which protect plants from diseases and responsible for plant's aroma, flavor and colour. Role in normalising blood pressure and blood clotting and also in improving arterial elasticity by phytochemicals were extensively studied. It also reduced the risk of coronary heart diseases by preventing the oxidation of low-density lipoprotein (LDL) cholesterol (Mathai., 2000). Phytochemicals have the ability to work as antioxidants and also activate enzymes that detoxify carcinogens. Beneficial role of phytochemicals in modulating hormone synthesis (Petric et al., 2015) and tissue regeneration (Kumar and Khanum., 2012) are well studied. Phytochemicals also promoted the prevention

and delay of diabetic symptoms and widely used for treatment of diabetes (Saxena et al., 2013).

The present study mainly investigated the concentration of Phytochemicals like total tannins, total flavonoids and total phenolic content in DK.

Total tannins in the extract of DK was 0.22 ± 0.015 mg/g. Plant tannins is one of the major antioxidant polyphenol groups which had attracted a lot of attention in recent years due to their multifunctional properties which are beneficial to human health (Kumari and Jain., 2012). Tannins can be classified into two broad groups - hydrolysable tannins and condensed tannins. Condensed tannins are widely distributed in plant kingdom than hydrolysable tannins. Tannins was reported to decrease post-prandial rise in blood glucose (Gin et al., 1999) and this might be due to their ability to inhibit α -amylase and α -glucosidase (Asgar., 2013).

Tannins enhance glucose uptake and inhibit adipogenesis and act as potential drugs for the treatment of non-insulin dependent diabetes mellitus (NIDDM) (Kumari and Jain., 2012). Tannins have the capacity to act as free radical scavengers and ability to activate antioxidant enzymes. Tannins enhance glucose uptake through Phosphoinositide 3-Kinase (PI3K) and p38 Mitogen-Activated Protein Kinase activation (MAPK) and GLUT-4 translocation. The main mechanisms behind the reduction in the blood glucose level were the decrease in the intestinal glucose absorption (Shimizu et al., 2000), reduction in food intake (Kao et al., 2000), induction of β cell

regeneration (Kim et al., 2003) and a direct action upon adipose cells to enhance insulin activity (Anderson and Polansky., 2002). Several previous reports already had described tannins as anti-hyperglycemic agents in diabetic rats (Pinent et al., 2004).

Tannins modulate a wide variety of genes by modifying enzymatic and transcriptional activities which make them useful in the treatment of disorders such as diabetes or dyslipidemia (Kumari and Jain, 2012). In addition to these properties, tannins are considered to be cardio-protective, anti-inflammatory, anti-carcinogenic and anti-mutagenic as they have free radical scavenging and antioxidant properties.

They are also known to inhibit lipid peroxidation and lipoxygenases *in vitro*. Their ability to scavenge hydroxyl, superoxide and peroxy radicals helps to restore the oxidative balance of the body (Georgiev et al., 2014) which is quite important during diabetic condition. Some tannins are even better than ascorbic acid (Barreca et al., 2017). Even though tannins are reported to have profound physiological effect, there is limitations in their absorption in the digestive tract which decides their effect on our body. Many of the tannins pass through the digestive tract without being absorbed which are subjected to the digestion by the gut microflora into different metabolites such as phenyl valero lactone, phenylacetic and phenyl propionic acids (Zhang et al., 2016). However, difference of opinions exists regarding their fate in the gut. Therefore, the absorption, bioavailability and metabolism of tannins must be carefully evaluated before ascertaining

their physiological role. That part was not addressed in this study as it did not come under the purview of the aims and objectives, but this is not averting us to suggest that the beneficial effect of DK is also contributed by its rich tannin content.

Flavonoids are a group of hydroxylated phenolic substances known to act as potent free radical scavengers and have the ability to chelate metals (Arts and Hollman., 2005). In biological systems it also reduces α -tocopherol radicals (Hirano et al., 2001) and inhibit oxidases (Lima et al., 2014). Bahadoran et al in (2013) reported that consumption of flavonoids or flavonoid-rich foods may reduce the risk of diabetes (Bahadoran et al., 2013). Total flavonoids content of the DK is 1.23 ± 0.043 mg/g and the total poly phenol content is 10.05 ± 0.94 mg/g.

Flavonoids are large family of phytochemical compounds that are further divided in to several subclasses namely Anthocyanidins, Flavanols, Flavanones, Flavonols and Isoflavones (Testa et al., 2016). Several in vitro animal models and human studies have already shown the essentiality of flavonoids for the modulation of carbohydrate and lipid metabolism. Based on the light of these reports it was clearly shown that flavonoids have important role in attenuating hyperglycemia, insulin resistance, dyslipidemia and adipose tissue metabolism. It also takes part in alleviation of oxidative stress and stress-sensitive signaling pathways (Bahadoran et al., 2013, Johnston et al., 2005, Jung et al., 2004). Phenolic compounds can be used to inhibit the absorption of amylase in the treatment of diseases with

abnormal carbohydrate metabolism, such as diabetes (Sales et al., 2012).

Certain flavonoids like procyanidins has antihyperglycemic effect which is suggested to be due to the insulin mimetic activity on insulin-sensitive cells. This hypoglycemic effect added to their antioxidant activity explain their ability to improve diabetic situations (Pinent et al 2004).

Poly phenols are one of the major antioxidants and plays a very important role in plant species. It includes the protection of plants from the attack of herbivores and several pathogens, and also regulate cell growth and cell division (Wallace and Fry., 1994, Binns et al., 1987). The major mechanisms behind the antioxidant power of phenolic compounds were donating hydrogen atoms to free radicals and scavenging other reactive species such as OH^{\bullet} , NO_2^{\bullet} , N_2O_3 , ONOOH and HOCl (Kumar et al., 2014).

Dietary polyphenols could be beneficial in reducing obesity as they are demonstrated to reduce viability of adipocytes and proliferation of preadipocytes, suppress adipocyte differentiation and triglyceride accumulation, stimulate lipolysis and fatty acid β -oxidation. Furthermore, polyphenols are reported to modulate various signaling pathways like AMP kinase, PPAR- γ , peroxisome proliferator activator receptor gamma activator 1-alpha, NF- κ B etc and antioxidant and anti-inflammatory responses (Mihaylova et al., 2020). Increased consumption of polyphenols-enriched foods was reported to reduce the risk of DM (Panagiotakos et al., 2009) and individuals with the highest

intake of flavonoids had a 9% lower risk of DM (Liu et al., 2014). One mechanism of action of polyphenols in diabetic condition is by improving insulin sensitivity (de Bock et al., 2013, Paquette et al., 2017) and also by protecting pancreatic β -cells against glucose toxicity, oxidative damage and cell apoptosis. They were also found to inhibit glycation products. Our study also supports this report as we have observed a decreased glycated haemoglobin level in diabetic rats fed with DK (Bahadoran et al., 2013, Swaminathan et al., 2014). Supplementation of grape polyphenols combined with exercise was found to increase muscle lipid oxidation and decreased glycogen utilization, which in turn enhanced endurance (Lambert et al., 2018).

Phytochemicals increase antioxidant enzymes like Catalase, GPx, GRd etc which regulate blood glucose level and increase the insulin production in our body (Rizvi and Mishra., 2013). Many plants are used in ayurveda to treat diabetes mellitus and DK is a versatile product of 40 herbal ingredients. These plants containing natural antioxidants especially tannins, flavonoids etc. have the ability to maintain pancreatic β -cells performance and decrease glucose level in the blood (Kooti et al., 2016). Flavonoids, tannins, phenolic compounds, and alkaloids are the most common herbal active ingredients used for treating diabetes (Rashid., 2014).

Results of this study had revealed the richness of DK in various beneficial phytochemicals. The beneficial impacts of phytochemicals are determined by the composition as well as their chemical nature. Each class of phytochemicals contains different compounds in

different proportions, and they vary in the antioxidant as well as other physiological effects. Because of this it is important to measure their physiological effects rather than their concentration. Hence we have studied some of their biochemical effects.

FRAP assay determines the reducing activity of antioxidants against the oxidative effects of reactive oxygen species. In this assay antioxidants present reduce Fe^{3+} to Fe^{2+} in the presence of tripyridyltriazine (TPTZ) and an intense blue Fe^{2+} -TPTZ complex was formed with an absorbance maximum at 593 nm (Benzie and Strain., 1996). Increasing absorbance indicates an increase in reductive ability of antioxidants in the DK extract. Most herbal ingredients of DK have strong antioxidant activities (Sruthi and Sindhu., 2012). In the present study the reducing power of DK was estimated to be $36.29 \pm 0.90 \mu\text{g/ml}$ and the result suggests the excellent antioxidant power of DK.

DPPH radical scavenging activity is one of the commonly used methods for determining the antioxidant activity of plant extracts. Present study analysed the antioxidant activity of DK by using this method and IC₅₀ value of the DK was found to be $2.04 \pm 0.11 \mu\text{g/ml}$. The addition of various concentration of DK (5 -120 $\mu\text{g/ml}$) to an ethanolic solution of DPPH turned its colour from deep violet to light yellow. The main principle behind this assay is the reduction of DPPH by antioxidants. Phytochemical analysis of DK revealed the richness of DK in phenols, flavonoids and tannins. Presence of these components may have resulted the reduction of DPPH (Boly et al., 2016) and lower

IC50 value of DK. Here we have observed a very low IC50 value which is 2.04 ± 0.11 $\mu\text{g/ml}$ which points to the effective functional forms of the various components of DK.

The potential of DK to scavenge free radical was also assessed by ABTS radical inhibition assay, and DK was found to have an IC50 value of 38.46 ± 2.75 $\mu\text{g/ml}$, showing a strong activity. The antioxidant activities measured by ABTS or DPPH assay could be correlated to the concentration and chemical structures of the antioxidants present (Oszmianski et al., 2007). DK was found to have a reasonably good concentration of total tannins, a high molecular weight phenolic compound. They are found to have high ability to quench free radicals (Hagerman et al., 1998). Phytochemical components of DK justify the good ABTS scavenging activity of DK.

The phosphomolybdate assay is another routine test used to evaluate the total antioxidant capacity (Prieto et al., 1999). We observed an IC50 value of 50.4 ± 2.63 $\mu\text{g/ml}$ which is indicative of significant antioxidant power of the drug. This also points to the possibility that the strong antioxidant activity of DK might be due to the presence of phenolics compounds present (Pourreza., 2013).

The results of this study also revealed the strong scavenging activity of DK on hydroxyl radical, hydrogen peroxide and nitric oxide as well as its strong metal reducing power which might have resulted by the presence of bioactive flavonoids.

It is known that hydrogen and electron transfer from antioxidant analytes to DPPH⁻, ABTS⁺ and Mo(VI) complex occur in the DPPH, ABTS⁺ and phosphomolybdenum assay methods. The transfers occur at different redox potentials in these assays and also depend on the structure of the antioxidant. DPPH⁻ and ABTS⁺ scavenging assays detect antioxidants such as flavonoids and polyphenols, whereas the phosphomolybdenum method usually detects antioxidants such as ascorbic acid, some phenolics, α -tocopherol, and carotenoids (Pourreza., 2013). All the assays performed with DK established its strong antioxidant activity and the presence of the above-mentioned phytochemicals.

Muhammad et al in (2016) reported that *Moringa oleifera* has potential to treat diabetic mellitus and also helps for the regeneration of pancreatic β - cells in the islets. This review suggested that this plant improved non enzymatic antioxidant like glutathione and decreased MDA in diabetic condition. He also reported that the presence of phytochemicals such as alkaloids, flavonoids, glycosides, tannins and steroids is responsible for its hypoglycaemic effects (Muhammad et al., 2016). Present study had shown that DK is rich with phytochemicals such as tannins, flavonoids and total phenolic content. So, DK may prevent/suppress the changes caused by free radicals and other components producing oxidative stress in STZ induced diabetic rats. This might reduce the damage to beta cells or induce the regeneration of beta cells which ultimately relieve diabetic symptoms through increased availability of insulin.

The effect of plant derived drugs depends on their components as well as the extent of their absorption and metabolism. Accordingly the

absorption of dietary phytochemicals is a crucial factor for their bioavailability, which in turn is imperative for their biological activity. The absorption and metabolism are mainly affected by the chemical structure of the phytochemicals itself along with the factors related to interpersonal variability like the differences in enzymatic activities, and systemic factors, for example age, gender and pathologies.

Phytochemicals which are not absorbed in the gut are metabolized by the gut microflora and the resulting metabolites can either enter the portal vein reaching the liver where they undergo further metabolism or are excreted. *In vivo*, *in vitro* and *in silico* studies had shown that the polyphenol structure and efflux systems influence their brain bioavailability (Youdim et al., 2004, Figueira et al., 2017). The speed of excretion of the compounds or their metabolites are excreted in urine contribute to a lower bioavailability (Spencer et al., 2004). Absorption, metabolism and excretion of the various phytochemicals present in DK are not studied in this work, which needs to be study to get a complete picture of the effect of DK.

4.4: Conclusion

The present study revealed that the DK is rich with total tannins, total flavonoids and total phenolic content. DK is also found to have strong antioxidant and free radical scavenging activity. The strong antioxidant and free radical scavenging activity of DK suggested by the presence of these phytochemicals may be the reason for its beneficial impact in biological system as reported in Ayurvedic system of medicine.

Antioxidants and Serum total protein

5.1: Introduction

Antioxidants are the substances which delays oxidation of carbohydrate, protein, lipid and DNA at low concentrations. Based upon the free radical defense property antioxidants are mainly classified in to three. The first line of defense antioxidants includes enzymatic antioxidants like SOD, CAT, GPx and GRd. The second line of defense antioxidants includes nonenzymatic antioxidants like GSH, vitamin C and vitamin E. The third line of defense antioxidants contains complex group of enzymes such as lipase, protease, DNA repair enzymes, transferases, methionine sulphoxide reductase etc. (Sindhi et al., 2013). Several studies have shown that increased free radical production and decreased antioxidant defense system is also associated with insulin dependent (type 1) and noninsulin dependent (type 2) diabetes (Naziroglu and Butterworth., 2005). As a result, production and elimination of free radicals or balance between pro-oxidant and antioxidant system is totally disrupted in diabetes. Various factors are responsible for increment of free radicals in diabetes mellitus. One of the most reliable reasons for enhanced production of ROS is auto oxidation of glucose. Antioxidants play a vital role in reduction of oxidative process and protect the human body from the attack of ROS (Sindhi et al., 2013).

CAT, SOD, GPx and GRd are antioxidant enzymes present nearly in all living organisms. Catalase plays an important role against oxidative damage caused by diabetes mellitus (Chelikani et al., 2004). Hydrogen peroxide is a potent highly reactive small molecule produced naturally during the energy metabolism. Catalase enzyme is a key regulator of hydrogen peroxide metabolism. Excessive concentration of hydrogen peroxide may cause significant damage to DNA, RNA, lipid and proteins (Takemoto et al., 2009). Catalase protects pancreatic β - cells from damages caused by hydrogen peroxide and its deficiency was associated with diabetes mellitus (Tiedge et al., 1998).

SOD is another important antioxidant enzyme that protects cells from oxidative damage. There are mainly three forms of SOD seen in all mammalian tissues. They are Cu-Zn-SOD or SOD 1 is localized in cytosol, Mn-SOD or SOD 2 in mitochondria, and EC-SOD or SOD 3 in extracellular space (Oury et al., 1996, Zelko et al., 2002). Over expression of SOD helps to overcome oxidative stress, increase antioxidant enzymes, reduce ROS and prevent diabetes mellitus (Wang et al., 2011). The extra cellular matrix of pancreas containing EC-SOD is the major extracellular scavenger of superoxide radicals (Fattman et al., 2003).

GPx is one of the important antioxidant enzymes and its main function is to reduce hydroperoxides to corresponding alcohols (Toppo et al., 2009). It also has the ability to scavenge reactive oxygen species and involved in several biological functions such as detoxification, regulation of inflammation, apoptosis etc. (Brigelius-Flohe and

Maiorino., 2013). GRd is another endogenous antioxidant enzyme, which catalyses regeneration of GSH from oxidised glutathione (GSSG) by using NADPH as a reducing factor (Hoffmann et al., 2017).

GSH is an intracellular thiol rich tripeptide seen in almost all mammalian tissues and highest concentration is seen in liver tissue. This nonenzymatic antioxidant also have ability to fight against oxidative stress (Lu., 2013). GSH is the most abundant nonprotein thiol, which is considered as a biomarker of redox imbalance at cellular level (Rizvi and Chakravarty., 2011). The main functions of GSH are to maintain SH groups of proteins in reduced state, detoxify foreign radicals, act as coenzyme in several enzymatic reactions, participate in amino acid transport and protect the tissues from oxidative attack (Tsai et al., 2012).

Vitamin C (Ascorbic acid) is essential for normal metabolism and involved in an array of cellular functions (Subramanian et al., 2018). It is a potent water-soluble antioxidant in our body and act as a cofactor for several enzymatic reactions in our body (Packer and Fuchs., 1997). It also has an important role in the metabolism of micronutrients such as folate and iron (Straaten et al., 2014). Humans and many other mammals have no ability to synthesis vitamin C de novo, they obtain the vitamin C (Vit C) from dietary sources via intestinal absorption (Subramanian et al., 2018). Vit C is synthesized in rat liver from glucose (Loewus et al., 1959). Increase glucose level was found to stimulate vit C synthesis (Fu et al., 2020). To further investigate the

molecular mechanism underlying the altered levels of Vit C, the expression of L-gulonolactone oxidase (gulo) gene, a gene involved in the synthesis of Vit C at mRNA level was also studied.

Vitamin E (Vit E) is one of the lipid soluble antioxidant, which is involved in scavenging free radicals in our body. All vitamin E forms are potent antioxidants as they scavenge lipid peroxy radicals by donating hydrogen from the phenolic group on the chromanol ring (Jiang., 2014). Vitamin E, due to its antioxidant properties has been proposed to have a role in preventing or treating numerous health conditions. It also participates in stabilization of the cell membranes and plays an important role in maintaining membrane permeability (Bjorneboe et al., 1990, Navarro et al., 1999). It is an essential component of lipophilic chain breaking process (Bruno et al., 2006). Vit E sets off a chain reaction of lipid peroxidation caused by highly-reactive species. Vitamin E halts this chain reaction in lipid peroxidation, and thereby acts as a chain breaking inhibitor of lipid peroxidation (Magdy et al., 2016).

Nitric oxide (NO) is a lipid soluble free radical, and also a gaseous molecule secreted by endothelium (Shiekh et al., 2011). It acts as a soluble messenger in several tissues and has short half- life (Murad., 2011). NO is produced from L-arginine and oxygen by the action of the enzyme nitric oxide synthase (NOS). It has wide variety of functions in our biological system and have three isoforms, they are neuronal NOS (nNOS or NOS-1), endothelial NOS (eNOS or NOS-2),

and inducible NOS (iNOS or NOS-3) (Becerril S et al., 2019). In the present investigation nitric oxide levels in liver tissue was also studied.

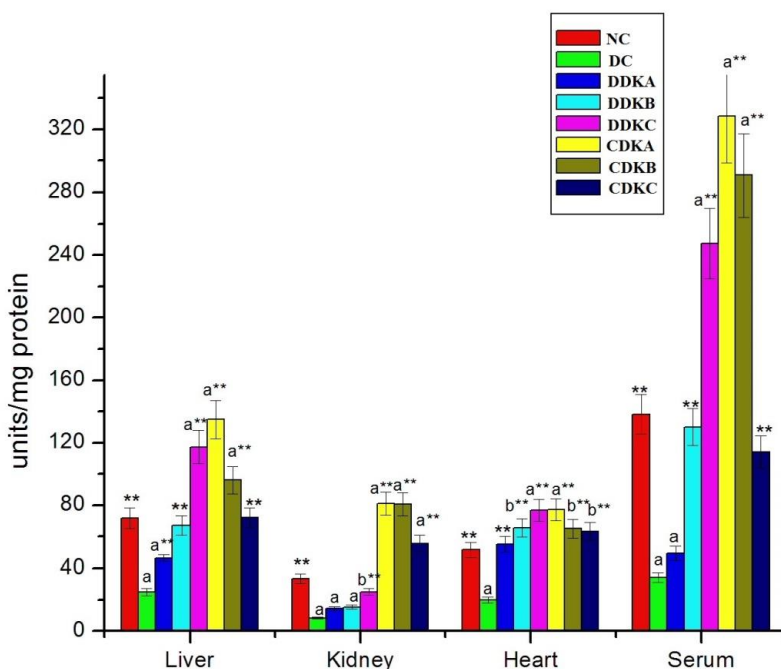
Proteins are considered as important biomolecule in our living system. They play a vital role in cell structure and function and involved in several biological processes in our body. Proteins are a potential target of ROS. They attack proteins by various methods and modify its structure and function. Proteins undergo oxidation and form carbonyls which are reported as the potent biomarker of oxidative stress (Suzuki and Miyata., 1999). Several previous studies have already suggested that increased level of protein carbonyls is seen in different cells and plasma of the diabetic patients (Pandey et al., 2010, Suzuki and Miyata., 1999). Incomplete metabolism of proteins will result in the accumulation of damaged proteins (Grim et al 2011). Protein damage has been reported to cause different degenerative diseases. Protein hydroperoxide formation may lead to lipid oxidation and result in the formation of hydroperoxides of amino acid, peptide and proteins that may contribute to the initiation of lipid oxidation (Davies., 2016).

5.2: Results

5.2.1: Enzymatic Antioxidants

5.2.1.1: Catalase

Figure 5.2.1.1: Effect of DK on the activity of the enzymatic antioxidant Catalase in the normal and experimental rats

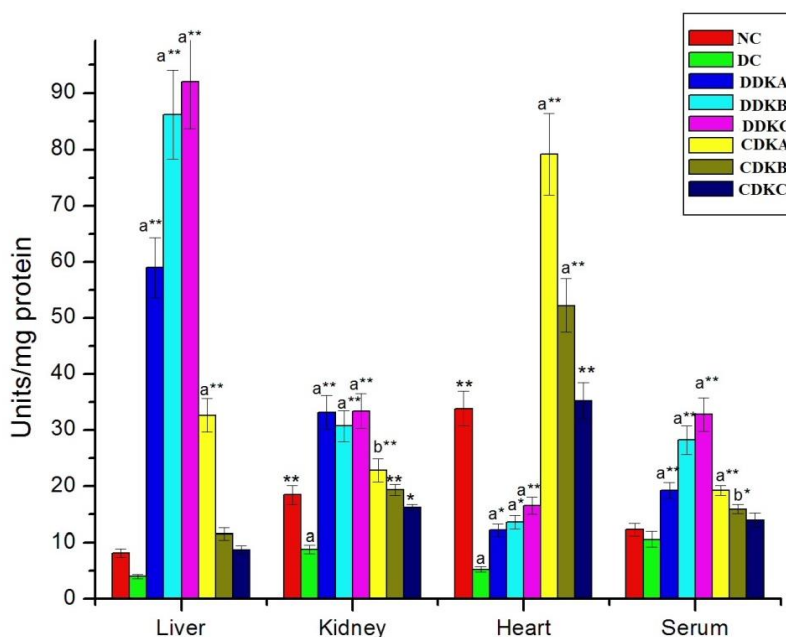


Values are expressed as mean \pm SD of six rats. ‘a’ indicates values are significantly different from Normal Control rats with $p < 0.001$. ‘b’ indicates values are significantly different from Normal Control rats with $p < 0.05$. ‘**’ indicates values are significantly different from Diabetic Control rats with $p < 0.001$. ‘*’ indicates values are significantly different from Diabetic Control rats with $p < 0.05$. Catalase expressed as 10^{-3} units/mg protein. Units - μ mole of hydrogen peroxide consumed per minute.

CAT is an enzyme which is responsible for conversion of hydrogen peroxide into oxygen and water. Levels of CAT in liver, kidney, heart and serum of normal and diabetic rats were studied. Activities of CAT were ranging in a tissue specific manner. Its levels in normal rats were 73 ± 2 , 25 ± 1.8 , 57 ± 3.5 and 113 ± 11 U/mg protein in liver, kidney, heart and serum respectively. Not surprisingly, the activity of CAT decreased in diabetic rats when compared to that in normal control rats which was in a good agreement with previous reports (Lenzen et al., 1996). The percentage decrease in the levels of CAT was highest in serum and it dropped more than 75% in serum. To study the effectiveness of DK, different doses of diluted decoction were given to rats by gavage. Administration of DK led to a significant increase ($p < 0.001$ and $P < 0.05$) in the activity of CAT in all tissues and serum of both diabetic and normal rats. Though there was some correlation between the dose of the DK and increase in CAT activity, but the rate of increase varied in different tissues and serum of normal rats and diabetic rats. There was no significant difference in the CAT levels in liver and serum of diabetic rats receiving intermediate dose (DDKB) and non-diabetic rats receiving highest dose (CDKC) rats in comparison with normal rats. CAT levels in the kidneys of group 3 and 4 were very close to diabetic rats. Administration of DK increased catalase activity in all the tissues. In diabetic rats the highest percentage increase was observed in highest dose fed ones.

5.2.1.2: Super Oxide Dismutase (SOD)

Figure 5.2.1.2: Effect of DK on enzymatic antioxidant, SOD activity in the normal and experimental rats.



Values are expressed as mean \pm SD of six rats. ‘a’ indicates values are significantly different from Normal Control rats with $p < 0.001$. ‘b’ indicates values are significantly different from Normal Control rats with $p < 0.05$. ‘**’ indicates values are significantly different from Diabetic Control rats with $p < 0.001$. ‘*’ indicates values are significantly different from Diabetic Control rats with $p < 0.05$. Units - Enzyme concentration required to inhibit the chromogen production by 50% in one min.

SOD is an enzyme that alternately catalyzes the dismutation of the superoxide (O_2^-) radicals into either ordinary molecular oxygen (O_2) or hydrogen peroxide (H_2O_2). There were tissue specific variations in the levels of SOD as seen in case of catalase. In normal rats SOD activities were 8.2 ± 0.74 , 18.4 ± 1.68 , 33.82 ± 3.08 and 12.3 ± 1.12 U/mg

protein in liver, kidney, heart and serum respectively (Fig. 5.2.1.2). Activity of SOD decreased drastically in diabetic rats which were 4.01 ± 0.037 , 8.81 ± 0.8 , 5.25 ± 0.47 and 10.59 ± 1.332 U/mg protein in liver, kidney, heart and serum respectively. Administration of DK increased SOD activity in all the rats in all the tissues studied. In diabetic rats increase in SOD reflected the increasing dose of DK in all tissues except in kidney. In kidney all the DK doses caused a similar increase and the increase caused by different doses was significantly different from diabetic control and normal control ($p < 0.001$ and $P < 0.05$). 5-10 times increase in SOD activity was observed in the liver of diabetic rats fed DK with the highest dose producing the highest increase. In normal rats low dose of DK (dose-A) produced the highest increase in SOD activity. Higher doses also caused an increase in SOD activity, but the increase was not as high as in dose A fed rats. The change in SOD activity in the liver of group DC, CDKB and CDKC are not significant compared to NC. In the case of heart there was no significant difference between normal and group CDKC rats. DK administration increased the SOD level. No significant difference in SOD activity in the serum of groups DC and CDKC compared to NC. In normal rats the lowest dose of DK caused the highest increase in SOD activity in all the tissues studied. The rate of increase in SOD activity decreased with increasing dose of DK.

5.2.1.3: Glutathione Peroxidase (GPx)

Table 5.2.1.1: Effect of DK on enzymatic antioxidant Glutathione Peroxide activity

Glutathione peroxidase reduces oxygen free radical containing molecules into corresponding alcohols. It also converts hydrogen peroxide into water. The levels of its activities varied due to DK administration in different tissues but those were not as pronounced as that in cases of CAT and SOD. In normal rats, its activities were recorded to be 9.52 ± 0.86 , 10.4 ± 0.52 , 3.49 ± 0.319 and 7.01 ± 0.64 U/mg protein in liver, kidney, heart and serum respectively (Table - 5.2.1.1).

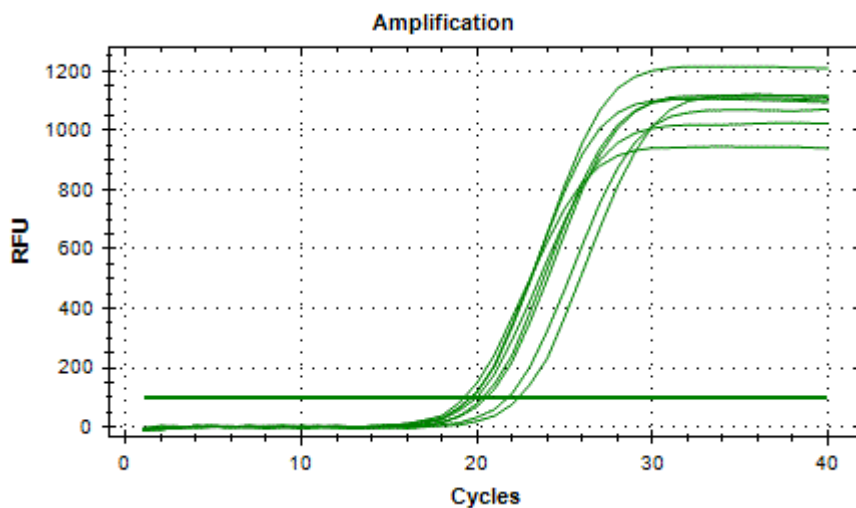
Sl: No	Groups	Liver	Kidney	Heart	Serum
1	NC	$9.52 \pm 0.87^{**}$	$10.41 \pm 0.53^{**}$	$3.50 \pm 0.32^{**}$	7.02 ± 0.64
2	DC	0.27 ± 0.03^a	1.41 ± 0.07^a	0.089 ± 0.010^a	$5.31 \pm 0.49^{a**}$
3	DDKA	$8.49 \pm 0.78^{**}$	$10.38 \pm 0.52^{**}$	0.402 ± 0.367^a	5.41 ± 0.50^a
4	DDKB	$7.95 \pm 0.72^{**}$	$9.92 \pm 0.50^{**}$	0.72 ± 0.07^b	5.79 ± 0.53^b
5	DDKC	$13.85 \pm 1.26^{a**}$	$10.61 \pm 0.54^{**}$	1.36 ± 0.12^b	$6.47 \pm 0.59^*$
6	CDKA	$21.75 \pm 1.99^{a**}$	$15.68 \pm 0.79^{a**}$	$26.54 \pm 2.42^{a**}$	$7.13 \pm 0.65^{**}$
7	CDKB	$15.63 \pm 1.43^{a**}$	$12.78 \pm 0.65^{a**}$	$19.39 \pm 1.77^{a**}$	$6.450 \pm 0.589^*$
8	CDKC	$9.72 \pm 0.89^{**}$	$10.87 \pm 0.55^{**}$	$15.70 \pm 1.43^{a**}$	$6.36 \pm 0.58^*$

Values are expressed as mean \pm SD of six rats. ‘a’ indicates values are significantly different from Normal Control rats with $p < 0.001$. ‘b’ indicates values are significantly different from Normal Control rats with $p < 0.05$. ‘**’ indicates values are significantly different from Diabetic Control rats with $p < 0.001$. ‘*’ indicates values are significantly different from Diabetic Control rats with $p < 0.05$. GPx expressed as units/mg protein. units - μ mole NADPH oxidized/min.

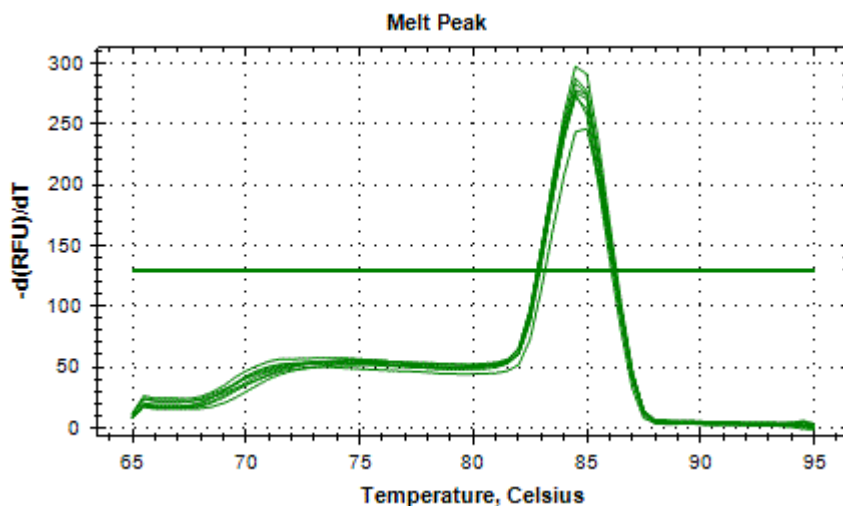
In diabetic rats, GPx activity dropped to 2.8%, 10% and 3.8% in liver, kidney and heart but to about 75% in serum. Here also there was an

increase in the enzyme activity in diabetic rats because of administration of DK and the increase in glutathione peroxidase was more in non-diabetic rats than in diabetic animals. In normal rats the DK administration increased the enzyme activity most effectively by the lowest dose used in the study (dose A) ($P < 0.001$). In normal rats, higher doses of DK produced varying effects in different tissues. In liver and kidney intermediate dose (dose B) also resulted a significant increase, but, interestingly, the highest dose used (dose C) did not produce any noticeable change in activity of this enzyme. In heart of normal rats all the doses produced significant increase ($p < 0.001$), and the pattern of increase was similar to that observed in liver and kidney i.e., dose A producing highest effect and the level of increase was lesser in dose B and least in dose C fed rats. In serum also DK administration increased the enzyme activity, but to a lower level compared to that in other tissues studied. No significant difference was observed in serum of DK fed normal rats.

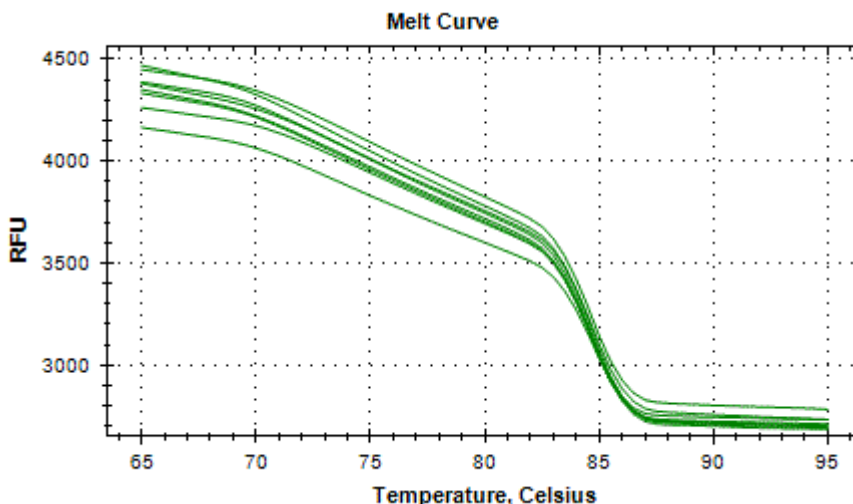
Figure 5.2.1.3: (a) Graphical representation of amplification curve of GPx-1 in liver from Real Time PCR analysis, (b) shows the melt peak and (c) represents the melt curve.



(a) Graphical representation of amplification analysis of GPx-1



(b) Graphical representation showing melt peak of GPx-1



(c) Graphical representation showing melt curve of GPx-1

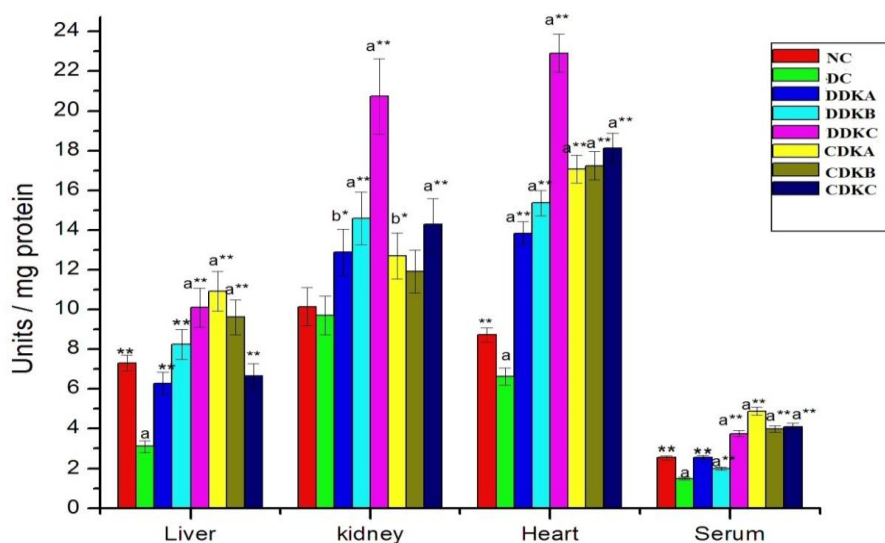
Table 5.2.1.2: Expression of GPx-1 at mRNA level in liver.

Sl:No	Groups	Fold increase in copy number
1	NC	1 ^{**}
2	DC	0.48 ^b
3	DDKA	0.62 ^b
4	DDKB	0.75 [*]
5	DDKC	0.91 ^{**}
6	CDKA	1.05 ^{**}
7	CDKB	1.07 ^{**}
8	CDKC	1.20 ^{**}

Showing the expression levels of the GPx-1 gene in the rats of the different groups expressed as fold difference in copy number of mRNA of GPx-1 gene relative to the nontreated control. 'a' indicates values are significantly different from Normal Control rats with $p < 0.001$. 'b' indicates values are significantly different from Normal Control rats with $p < 0.05$. '**' indicates values are significantly different from Diabetic Control rats with $p < 0.001$. '*' indicates values are significantly different from Diabetic Control rats with $p < 0.05$.

Expression of the gene glutathione peroxidase was studied using real time PCR. The results of the gene expression analysis supported the decreased enzyme activity observed in diabetic rats and also the up regulation of the expression in DK administered rats. In diabetic rats the gene expression was down regulated to 52% of the normal rats. Administration of the DK enhanced the expression in comparison to the diabetic rats in a dose dependent manner with dose A producing an up regulation of 29% followed by 56% by dose B and 89% by dose C. The enzyme activity reached near normal by the administration of DK at dose C.

Figure 5.2.1.4: Effect of DK on enzymatic antioxidant Glutathione Reductase activity in normal and experimental rats.



Values are expressed as mean \pm SD of six rats. ‘a’ indicates values are significantly different from Normal Control rats with $p < 0.001$. ‘b’ indicates values are significantly different from Normal Control rats with $p < 0.05$. ‘***’ indicates values are significantly different from Diabetic Control rats with $p < 0.001$. ‘*’ indicates values are significantly different from Diabetic Control rats with $p < 0.05$. units - μ mole NADPH oxidized/min.

There was a tissue specific variation in activity of GRd also. Enzyme activities were recorded to be 7.30 ± 0.39 , 10.14 ± 0.92 , 8.7 ± 0.36 , and 2.55 ± 0.106 U/mg protein in liver, kidney, heart and serum respectively (Fig 5.2.1.4). GRd activity significantly ($p < 0.001$) declined in all tissues except in kidneys of diabetic rats when compared to normal controls. However, the decrease was not as pronounced as seen in case of other enzymes. This decrease in GRd activity was also reversed by DK administration. Even the lowest dose used produced a significant increase ($p < 0.001$). The increase in GRd activity with DK administration reflected some dose and tissue dependence. In normal rats DK administration increased the GRd activity, but the pattern of increase was different from that observed in DK fed diabetic rats where the increase in activity was proportional to the amount of medication gavaged in rats. In the liver of DK fed normal rats, the lowest dose produced the highest increase in enzyme activity. In heart all the doses produced almost similar increase. Increase in activity observed in kidney and heart of DK fed normal rats with dose C were the highest, but the difference between the doses are not significant.

5.2.1.4: Expression of glutathione Reductase (GRd)

Levels of expression of the gene GRd was analysed by real time PCR to determine whether the altered levels of GRd enzyme activity observed in diabetic rats are due to the change in the production of the enzyme protein. The purity of the amplified product was tested by analysing the melting point. As expected, the melting point of the amplified product was 84.3 (graph not shown) which shows the

amplification of a single product of the expected size. The general pattern of the gene expression was in agreement with the activity pattern of the enzyme in different groups. In the liver of diabetic rats the level of expression was decreased by 72 percent. Administration of DK increased the level of expression of the gene in a dose dependent manner. Dose A produced an increase of 100 percent from that of diabetic rats whereas dose B produced an increase of 125% followed by dose C by 178 percent. The increase in the expression levels due to DK administration in diabetic rats were not enough to fully neutralise the decrease caused by the diabetic condition. Even for the dose C fed diabetic rats the enzyme activity was 22% less than the normal rats.

Table 5.2.1.3: Expression of GRd at mRNA level in liver.

Sl:No	Groups	Fold increase in copy number
1	NC	1**
2	DC	0.28 ^b
3	DDKA	0.56 ^b
4	DDKB	0.63 [*]
5	DDKC	0.78 ^{**}
6	CDKA	0.96 ^{**}
7	CDKB	0.99 ^{**}
8	CDKC	1.05 ^{**}

Showing the expression levels of the glutathione reductase gene in the rats of the different groups expressed as fold difference in copy number of mRNA of GRd gene relative to the nontreated control. ‘a’ indicates values are significantly different from Normal Control rats with $p<0.001$. ‘b’ indicates values are significantly different from Normal Control rats with $p<0.05$. ‘**’ indicates values are significantly different from Diabetic Control rats with $p<0.001$. ‘*’ indicates values are significantly different from Diabetic Control rats with $p<0.05$.

5.2.2: Non Enzymatic Antioxidants

5.2.2.1: Reduced Glutathione (GSH)

Table 5.2.2.1: Effect of DK on nonenzymatic antioxidant GSH in normal and experimental rats.

Sl:No	Groups	Liver	Kidney	Heart	Blood
1.	NC	20.07±1.83**	8.99±0.82*	4.31±0.40	0.43±0.04**
2.	DC	11.31±1.03 ^a	6.62±0.60 ^b	3.67±0.34	0.27±0.02 ^a
3.	DDKA	22.35±2.04**	10.53±0.96**	4.83±0.44*	0.47±0.04**
4.	DDKB	24.50±2.24**	14.63±1.34 ^{a**}	6.96±0.64 ^{a**}	0.50±0.45**
5.	DDKC	26.25±2.40 ^{a**}	13.28±1.21 ^{a**}	6.66±0.61 ^{a**}	0.59±0.54 ^{a**}
6.	CDKA	18.86±1.72**	9.82 ±0.90**	6.07±0.55 ^{a**}	0.55±0.05 ^{b**}
7.	CDKB	24.01±2.47 ^{a**}	10.11±0.92 ^a	6.17±0.56 ^{a**}	0.67±0.62 ^{a**}
8.	CDKC	23.52±2.15**	11.21±1.02 ^{a*}	7.29±0.67 ^{a**}	0.59±0.05 ^{a**}

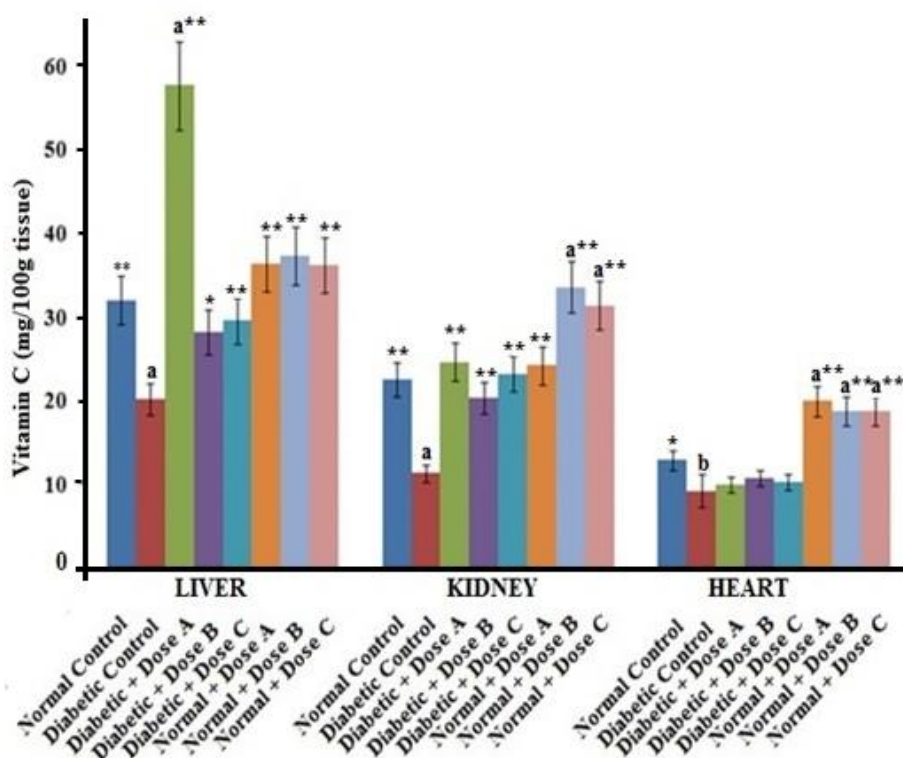
Values are expressed as mean ± SD of six rats. ‘a’ indicates values are significantly different from Normal Control rats with p<0.001. ‘b’ indicates values are significantly different from Normal Control rats with p<0.05. ‘**’ indicates values are significantly different from Diabetic Control rats with p<0.001. ‘*’ indicates values are significantly different from Diabetic Control rats with p<0.05. GSH expressed as mM/100gm tissue and mM/dl.

The level of GSH also decreased significantly (P<0.001 and P<0.05) in all the tissues of DC rats compared to NC rats. Administration of DK significantly increased GSH level in both DC and NC rats in a dose dependent manner in all tissues and blood (P<0.001 and P<0.05). In diabetic rats fed dose B and C, the GSH level went even above the normal value in all tissues. The extent of increase in GSH levels due to DK administration in dose B and C in diabetic rats was between 81 to 132% with the highest difference in the liver of dose C fed diabetic

rats. Similar trends were observed in normal rats fed DK, but the increase is not that drastic as observed in diabetic rats.

5.2.2.2: Vitamin C

Figure 5.2.2.1: Effect of DK on nonenzymatic antioxidant vit C in normal and experimental rats

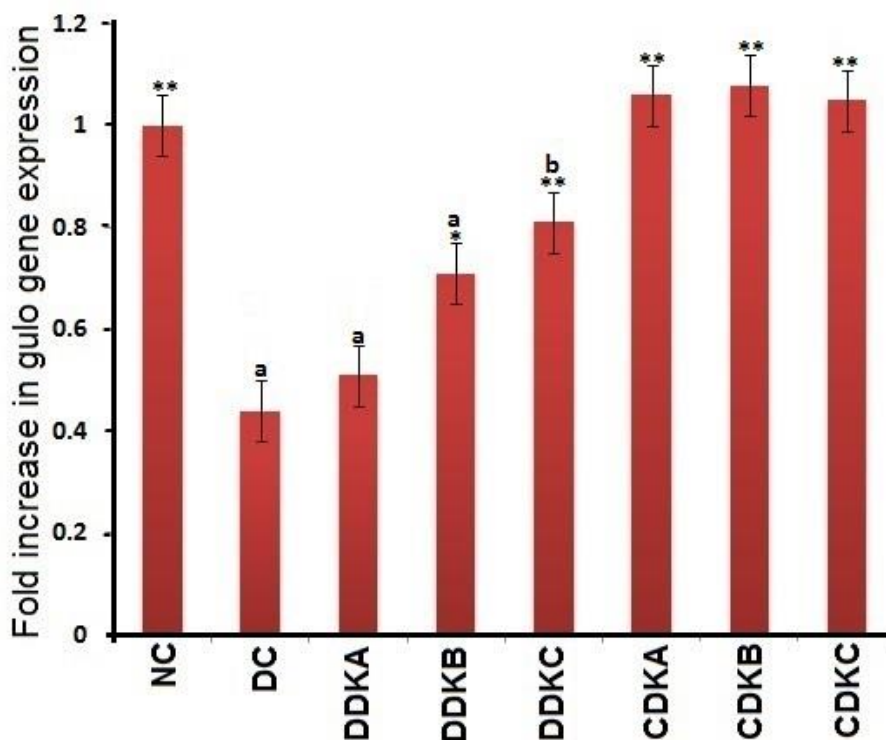


Values are expressed as mean \pm SD of six rats. ‘a’ indicates values are significantly different from Normal Control rats with $p < 0.001$. ‘b’ indicates values are significantly different from Normal Control rats with $p < 0.05$. ‘***’ indicates values are significantly different from Diabetic Control rats with $p < 0.001$. ‘*’ indicates values are significantly different from Diabetic Control rats with $p < 0.05$.

Vit C level decreased in diabetic rats compared to normal control. Administration of DK caused significant ($p < 0.001$ or $p < 0.05$) increase in Vit C level in all tissues of diabetic rats in a dose dependent manner, but the rate of increase varied in different tissues. The highest increase was observed in liver (185%) compared to diabetic rats which is highly significant ($p < 0.001$). In diabetic rats, the administration of DK helped to bring the vitamin C level to the normal level or higher in liver and kidney, but in heart, though there was an increase in vitamin C the increase was below normal for all the doses. Administration of DK to normal rats also caused an increase in vit C concentration, with the highest percentage increase observed in heart (54%) of dose A fed rat followed by kidney (48%) of dose B fed rats. Dose A caused a drastic increase in vit C level in liver of diabetic rats ($p < 0.001$). Administration of DK to normal rats also caused an increase in vit C concentration, but the highest increase was observed in liver and kidney of dose A fed rats. Increase in vit C in diabetic as well as normal rats due to the administration of DK was significant in all tissues compared to diabetic control rats ($p < 0.001$ or $p < 0.05$).

5.2.2.3: Vitamin C synthesis at mRNA level

Figure 5.2.2.2: Showing expression of gulo gene in different experimental groups.



Values are expressed as mean \pm SD of six rats. ‘a’ indicates values are significantly different from Normal Control rats with $p < 0.001$. ‘b’ indicates values are significantly different from Normal Control rats with $p < 0.05$. ‘***’ indicates values are significantly different from Diabetic Control rats with $p < 0.001$. ‘*’ indicates values are significantly different from Diabetic Control rats with $p < 0.05$.

As expected, the expression of gulo gene was markedly decreased (66%) in the liver of diabetic rats. There was no significant change in

the expression in the normal control rats fed with DK. At the same time DK administration enhanced gulo expression significantly in the DK fed diabetic rats in a dose dependent manner with the highest increase in expression in the dose C fed rats. The percentage increase in expressions from that of diabetic rats were 18% with dose A, 62% in dose B and 84% in dose C fed rats.

5.2.2.4: Vitamin E

Table 5.2.2.2: Effect of DK on non enzymatic antioxidant vitamin E in normal and experimental rats.

Sl:No	Groups	Plasma
1.	NC	6.64 ± 0.61 [*]
2.	DC	5.36± 0.49 ^b
3.	DDKA	7.48 ± 0.68 ^{**}
4.	DDKB	8.38 ± 0.77 ^{a**}
5.	DDKC	6.64 ± 0.61 [*]
6.	CDKA	6.84 ± 0.53 [*]
7.	CDKB	6.26 ± 0.57
8.	CDKC	7.97± 0.73 ^{b**}

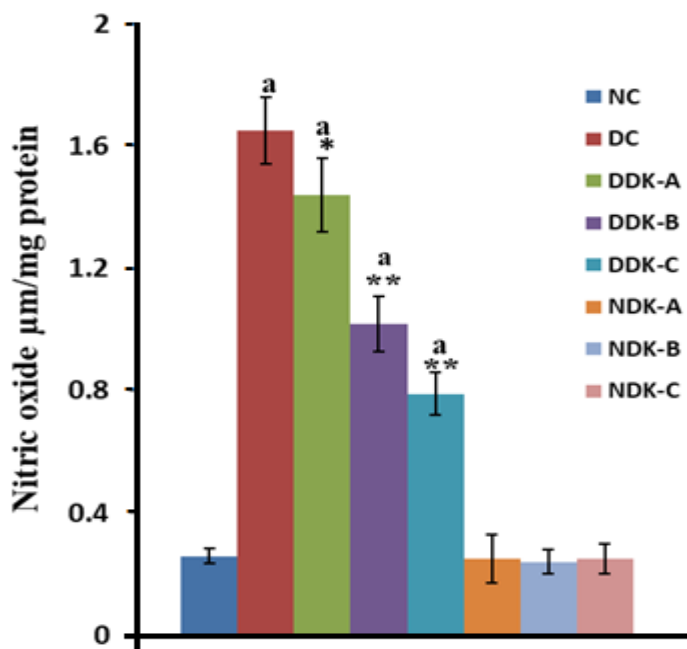
Values are expressed as mean ± SD of six rats. ‘a’ indicates values are significantly different from Normal Control rats with p<0.001. ‘b’ indicates values are significantly different from Normal Control rats with p<0.05. ‘**’ indicates values are significantly different from Diabetic Control rats with p<0.001. ‘*’ indicates values are significantly different from Diabetic Control rats with p<0.05. Vitamin E expressed as mg/dl.

Vit E concentration was significantly decreased in diabetic rats (P<0.05) as compared to NC rats. Administration of DK increased Vit E concentration in all the diabetic groups with the highest increase in

dose B fed rats. Vitamin E levels in all the DK administered diabetic rats were significantly higher ($p < 0.001$ and $p < 0.05$) than in diabetic control rats, with dose B producing the highest increase (56%) followed by dose A (40%) and dose C (23%). The increase in vitamin E level in diabetic rats fed with dose A and B was significantly higher than that in normal control as well as diabetic control rats. Dose C administration brought the vitamin E level in diabetic rats in par with that of normal control and this increase was significantly higher ($p < 0.05$) than in diabetic control rats. Normal rats administered DK also had a higher vitamin E level with dose C fed animals have the highest increase followed by dose B and dose A in decreasing order. These increases in vitamin E due to DK administration in normal rats were significantly different from that in diabetic rats with increasing doses producing increased effects.

5.2.3: Nitric oxide levels in liver tissue of normal and experimental rats

Figure 5.2.3.1: Effect of DK on nitric oxide levels in liver tissue of normal and experimental rats



Graph showing the levels of in the liver tissue of the rats of different groups. NO level was assessed by measuring the nitrite concentration in the liver tissue homogenates using Griess method. The results were expressed as μmol nitrite/mg protein content of the samples. Values are expressed as mean \pm SD of six rats. 'a' indicates values are significantly different from Normal Control rats with $p < 0.001$. 'b' indicates values are significantly different from Normal Control rats with $p < 0.05$. '**' indicates values are significantly different from Diabetic Control rats with $p < 0.001$. '*' indicates values are significantly different from Diabetic Control rats with $p < 0.05$.

The levels of NO in the liver tissues of diabetic rats were elevated significantly ($P < 0.001$) in comparison to control rats. The levels decreased significantly ($P < 0.001$ and $P < 0.05$) in diabetic rats when treated with DK. Administration of DK produced a dose dependent effect on the NO level with the highest dose resulting in maximum reduction in the NO level, however, it was still higher than that in the control rats ($P < 0.05$). DK administration failed to cause any effect in the NO levels in normal rats.

Fig 5.2.3.2: Melt peak of iNOS

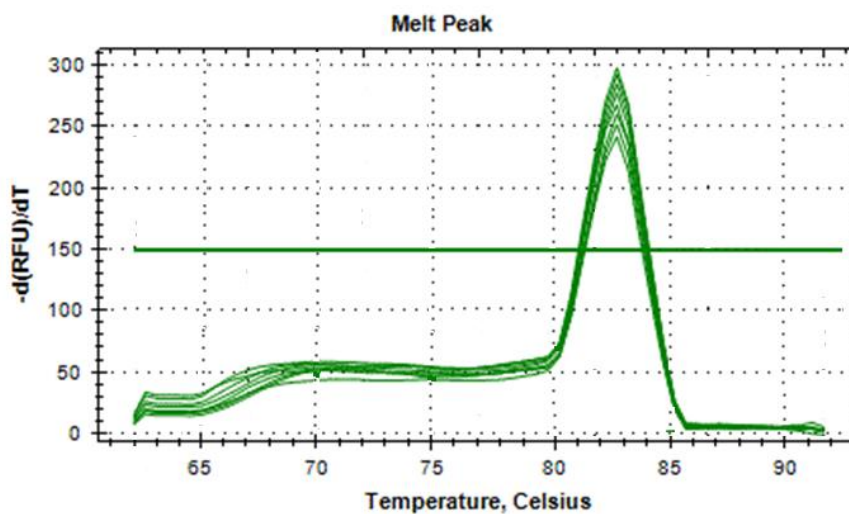


Table 5.2.3.1: Expression of iNOS at mRNA level in liver.

Sl:No	Groups	Fold increase in copy number
1	NC	1 ^{**}
2	DC	4.17 ^a
3	DDKA	3.52 ^{a**}
4	DDKB	2.09 ^{a**}
5	DDKC	1.5 ^{b**}
6	CDKA	1.08 ^{**}
7	CDKB	1 ^{**}
8	CDKC	0.9 ^{**}

Expression levels of the iNOS gene in the liver of rats of the different groups expressed as fold difference in copy number of mRNA of iNOS gene relative to the nontreated control. 'a' indicates values are significantly different from Normal Control rats with $p < 0.001$. 'b' indicates values are significantly different from Normal Control rats with $p < 0.05$. '**' indicates values are significantly different from Diabetic Control rats with $p < 0.001$. '*' indicates values are significantly different from Diabetic Control rats with $p < 0.05$.

The expression level of iNOS gene was highest in diabetic control rats with an increase of 317% from the normal level and in DDKC rats, the expression was lowest among the DK fed diabetic rats having an expression level which is only 50% higher from normal rats. In DK treated diabetic rats the expression level decreased in a dose dependent manner with the least effect observed in DDKB rats. Dose C resulted a reversal of the effect caused by the diabetic condition to a great extent and there was a change of 178%. Altered pattern of results was obtained in the case of DK fed normal rats. In DK treated normal control rats, the expression remained near to the normal value without any significant effect.

5.2.4: Serum Total Protein**Table 5.2.4: Effect of DK on Serum total protein in normal and experimental rats.**

SL.NO	GROUPS	SERUM
1	NC	7.37±0.26* *
2	DC	3.77±0.14 ^a
3	DDKA	4.19±0.15 ^{a*}
4	DDKB	5.44±0.19 ^{a**}
5	DDKC	4.41±0.16 ^{a**}
6	CDKA	6.70±0.24 ^{a**}
7	CDKB	7.14±0.26 ^{**}
8	CDKC	6.78±0.24 ^{a**}

Values are expressed as mean ± SD of six rats. ‘a’ indicates values are significantly different from Normal Control (NC) rats with p<0.001. ‘b’ indicates values are significantly different from Normal Control rats with p<0.05. ‘**’ indicates values are significantly different from Diabetic Control (DC) rats with p<0.001. ‘*’ indicates values are significantly different from Diabetic Control rats with p<0.05. Serum total protein expressed as gm/dl.

Serum protein levels decreased by 48.6% in diabetic rats when compared to that in normal control rats and the decrease was statistically significant. DK administration was able to restore serum protein levels in diabetic rats to a great extent. Interesting was to notice that the moderate dose (Dose B) of the DK was most potent in restoring the serum protein levels which was also up to 73.51% only. Through DK administration serum protein levels were significantly elevated (P<0.001 and P<0.05) in diabetic rats. Surprisingly, the drug did not induce any increase in control rats.

5.3: Discussion

Increased oxidative stress is a major causative factor in the development and progression of diabetes (Ceriello., 2000). In the present study we have observed a decrease in enzymatic and non-enzymatic antioxidant parameters in the diabetic and normal rats. Free radicals play a very important role in the complications of DM and increased ROS mediated damage to DNA, RNA, lipids and proteins (Marco et al., 2015). Oxidative stress is the common phenomenon of the pathogenesis of the β -cell dysfunction (Ceriello., 2008).

Antioxidants help biological system to repair damages caused by the free radicals (Iwara., 2013). Deleterious effects of free radicals depend both on their level as well as on the ability of the cells to detoxify these radicals by employing the enzymes involved in free radical metabolism (Michiels et al., 1994). The main function of the free radical scavenging enzymes such as CAT, SOD, GPx and GRd is to protect the biological system from free radical damage (Comporti., 1985, Rio., 2005). SOD converts the most dangerous superoxide anion to less toxic hydrogen peroxide and oxygen while the haem –containing ubiquitous enzyme CAT detoxifies H_2O_2 into water and oxygen (Selvan., 2008). The antioxidant enzyme GPx also scavenges H_2O_2 . In addition to that GPx is involved in decreasing the levels of lipid-peroxides and organic hydroperoxides (Sreekutty and Mini., 2016). Another important antioxidant enzyme GRd helps for the recycling of oxidised glutathione back into glutathione (Salvayre., 2009). Enzymatic and non- enzymatic antioxidants decreased in diabetic rats. STZ was reported to increase free radical production (Punitha et al.,

2005). These two physiological conditions increased the oxidative stress in rats with STZ induced diabetes.

DK administration increased the levels of the enzymatic as well as non- enzymatic antioxidants. Increase in antioxidant parameters in DK fed diabetic and normal rats may be due to two reasons. One may be that DK act as a powerful antioxidant in biological system. Because of this the con: of the free radicals might have decreased in DK fed rats. This might have decreased the turnover rate of antioxidants. Other may be due to direct stimulating effect of DK on the activity of CAT, SOD, GPx and GRd, they in turn remove the free radicals.

Non-enzymatic parameters like vit C, vit E and GSH play an important role as antioxidants in protecting the cells from oxidative damage (Li et al., 2016). Previous research reports established the role of blood GSH in maintaining the level of vit C and vit E, so the decreased level of GSH in diabetic rats would have affected the vit C and vit E levels (Nwanjo et al., 2007). In this study we observed a decrease in the non-enzymatic antioxidants vit C, vit E and GSH in diabetic rats and on administration of DK the level of this non enzymatic antioxidant increased in diabetic as well as in normal control rats. Increased con: of these non-enzymatic parameters may be due to direct stimulating effect of DK on normal control as well as diabetic rats and the decreased level in DC rats due to the increased use for the detoxification of the over produced ROS.

Vit C is a natural water-soluble antioxidant and has the capacity to act as a reducing agent in free radical mediated oxidation process (Lobo et al., 2010). Vit E is a potent fat soluble antioxidant generally found in

several foods, fats and oils. It also has the ability to detoxify superoxide and H₂O₂ free radicals, and offers membrane stability (Gultekin et al., 2001). The decreased level of vit C and vit E observed in diabetic rats is in agreement with the reports of Jayachandran et al (Jayachandran et al., 2018). This study for the first time is reporting an increase of vit C and vit E level in diabetic rats supplemented with DK. Rats synthesise vit C (Rice., 2000) and the increase in vitamin C in the DK fed rats might be due to the decreased requirement of it for detoxification of the excessively produced ROS as the components of the DK might be doing the job.

GSH is one of the main non enzymatic antioxidants present in tissues that help to counter balance free radical mediated damage (Sreekutty and Mini., 2016). As observed in this study previous studies had also reported decrease in the GSH level in diabetic rats and that GSH play a major role in the development and progression of diabetic complications (Obrosova et al., 2003, Lee et al., 2000). Jain et al (2002) had demonstrated the oxidative stress due to hyperglycemia and ketosis and the resultant glutathione depletion in type 1 diabetic patients (Jain et al., 1998, 1999). It has a direct scavenging activity, act as a co-substrate for Glutathione Peroxidase, an antioxidant enzyme and also have the ability to act as a cofactor. It helps in protecting the cell structure. This protection is extended by maintaining redox homeostasis or through its role in detoxification reactions (Sreekutty and Mini., 2016). Studies have already shown a decreased GSH level in STZ induced diabetic rats as compared to normal control rats (Ewis and Rahman., 1995). This study also reported same pattern of results in diabetic and normal control rats. Reduction in GSH in diabetic rats

may be due to increased degradation of GSH by oxidative stress or the decrease in its synthesis (Loven et al., 1986). Increased GSH in DK fed diabetic rats may be due to the reversal of the changes that led to its decreased levels.

The level of vit C, vit E, and GSH were reduced in diabetic rats and increased in diabetic and normal rats fed with DK. Increment of these parameters in diabetic and normal rats may be due to two reasons. One may be DK act as a powerful antioxidant in biological system. Because of this the con: of the free radicals were decreased in diabetic and normal rats fed with DK. So the turnover rate was decreased. Other may be due to direct stimulating effect of DK on the production of vit C, vit E and GSH. Increased protein synthesis observed in DK fed rats is another possibility as it might also had increased the concentration of enzymatic protein involved in the synthesis of vit C and GSH.

Pal et al., in (2007) reported that elevated levels of NO may react with superoxide anion to form peroxy-nitrite radical that binds to protein and interrupt its function (Pal et al., 2007). Maejima et al in (2001) suggested that increased NO level was observed in diabetic condition. Our data is in agreement with this report and NO level increased in diabetic rats as compared to the normal control rats. This may be due to the enhanced production of NO caused by increased glucose level and achieved through increased expression of eNOS and iNOS gene (Cosentino et al., 1997).

The decrease in serum total protein during diabetes was reversed to some extent by the administration of DK. Serum total protein level was also decreased in diabetic rats as compared to normal control rats.

This result was consistent with the previous report (Tuvemo et al.,1997). Administering DK to diabetic rats resulted reversal of the situation. The reduction in the serum total protein in diabetic rats may be due to increased protein catabolism as reported elsewhere (Mansour et al., 2002). Administration of DK increased serum protein concentration in diabetic as well as normal rats. This may explain the growth enhancing properties of DK (Warrier et al., 2013).

5.4: Conclusion

The present study has shown a decrease in the activity of antioxidant enzymes and nonenzymatic antioxidants in diabetic rats. Administering DK to diabetic rats resulted in a reversal of the situation. From these results, it can be concluded that DK, a poly herbal formulation (PHF) used in Ayurveda exerts significant antioxidant activity. This herbal product could be developed as a promising natural and safe remedy or as a preventive agent in diabetes mellitus.

Effect of Dhanwantaram Kashayam on Lipid profiles

6.1: Introduction

Lipids are one of the important biomolecules, which are insoluble in water and soluble in several organic solvents. Abnormal lipid metabolism leading to dyslipidemia (Wu and Parhofer., 2014) is a prominent characteristic of diabetes. Alteration in lipid profile like Total Cholesterol (TC), Free Fatty Acids (FFA), Phospholipids (PL) and Triglycerides (TG) is one of the important characteristics of diabetes mellitus (Kandasamy and Ashokkumar., 2014). Elevated total cholesterol and triglycerides are major risk factors of cardiovascular diseases. Previous results have already suggested that insulin deficiency or insulin resistance affects key enzymes and pathways in lipid metabolism (Taskinen., 2002). To prevent complications in patients with diabetes these risk factor needs to be strictly controlled (Turner et al., 1998).

Diabetes is a metabolic disease, which leads to several micro and macro vascular complications such as atherosclerosis, peripheral vascular disease, nephropathy, retinopathy and neuropathy (Lamb and Goldstein., 2008). It increases the chance of atherosclerosis and the mortality due to heart related complications is 2-4 times higher in diabetes than in nondiabetes (McEwen et al., 2006). Increased blood sugar level can also lead to atherosclerosis through the increased

glycosylation of all proteins, particularly collagen cross linking and matrix proteins of arterial wall. This eventually causes endothelial cell dysfunction, finally leads to atherosclerosis (Bhambhani et al., 2015).

Hypercholesterolemia is one of the important risk factors of atherosclerosis and subsequent coronary heart diseases (Wadhera et al., 2016). Cholesterol is a waxy substance produced by animal liver and also supplied in diet through animal products. Cholesterol is mainly divided in to two types; high density lipoprotein (HDL) and low density lipoprotein (LDL) (Ma and Shieh., 2006). In a layman's concept, the former is good and latter is bad for cardiovascular system (Tabas., 2002). Cholesterol is one of the important lipids in some membranes and major functions include insulating nerves, part of cell membranes and producing certain hormones. Cholesterol plays a major role in human heart health and at the same time, higher level of cholesterol is a leading risk factor for human cardiovascular disease such as coronary heart disease and stroke (Ma and Shieh., 2006).

Free fatty acids (FFA) are one of the major class of lipids seen in our body. Based on the presence or absence of double bonds free fatty acids are mainly classified in to three, saturated (without double bonds), monounsaturated (with one double bond) and polyunsaturated (with two or up to six double bonds) fatty acids. Poly unsaturated fatty acids are essential part of our body and mainly obtained from consumption of fish and fish oils (Orsavova et al., 2015). Unsaturated fatty acids again exist in cis and trans configuration and cis configuration is found in most of the naturally occurring unsaturated

fatty acids. These unsaturated fatty acids are potent inducers of adiposomes or lipid droplets. Lipid droplets are major sites for eicosanoid generation during the process of inflammation and cancer in cells. It also has important roles in secretion of inflammatory mediators, cell signaling and regulation of lipid metabolism (Bozza and Viola., 2014).

Under physiological conditions FFA are known to modulate insulin release through fatty acid metabolism. Long-chain FFA increase insulin secretion, where as short-chain FFA inhibit its secretion. Long-chain FFA bind and interact with the G-protein-coupled free fatty acid receptor (FFAR)-1 in the pancreatic β -cells and leads to the activation of phospholipase C (PLC). PLC hydrolyses Phosphatidylinositol-4,5-bisphosphate (PIP₂) to diacylglycerol and inositol-1,4,5-triphosphate (IP₃). IP₃ will induce the release of Ca²⁺ into the cytosol by docking on the calcium channel. This increases the intracellular Ca²⁺ concentration and triggers the secretion of insulin (Shapiro et al., 2005, Fujiwara et al., 2005, Salehi et al., 2005, Itoh et al., 2003). On the contrary glucose-stimulated insulin secretion is inhibited by short-chain FFA (Ximenes et al., 2007). Stress mediated increase in the cytosolic Ca²⁺ concentration also increase insulin secretion (Porte., 1967).

Phospholipids are another major lipids containing phosphate group and they are the major component of the lipid bilayer of cell membranes (Singh et al., 2017, Renne and de Kroon., 2018). The structure of phospholipids mainly consists of two hydrophobic fatty acid “tails” and a hydrophilic “head”. Based upon the nature of their backbone

phospholipids are divided into two groups. They are glycerophospholipids and sphingophospholipids (Chang et al., 2018) and former is the major class of naturally occurring phospholipids.

Blood glucose level was thought to affect the phospholipid symmetry in cell membrane which is crucial for the maintenance and in controlling the traffic through the plasma membrane. The choline-containing phospholipids phosphatidylcholine (PC) and sphingomyelin (SM) are found primarily on the outer layer of the membrane and inner monolayer contain the amine-containing phospholipids, phosphatidylserine (PS) and phosphatidylethanolamine (PE) (Bretscher., 1973, Verkleij et al., 1973). Alterations in PS asymmetry are reported to cause several pathologic conditions including unwanted thromboses. Increased membrane viscosity (Watala., 1992) and different abnormalities were reported in erythrocytes from diabetic rats. Increased procoagulant activity and resultant vascular occlusion was reported due to the loss of PS symmetry. Glucose level affect Ca^{2+} uptake by cells and intracellular Ca^{2+} levels affect transmembrane lipid scrambling (Willson et al., 1993) and phospholipid asymmetry in cell membrane. They also suggested that the hyperglycemic damage to blood cell membranes may be a significant contributor to vascular complications in diabetes

Triglycerides are the main constituents of the body fat in humans and other vertebrates but its excess level leads to the condition called hypertriglyceridemia, a prevalent risk factor for cardiovascular disease (CVD). High triglyceride level is also an important marker for several types of atherogenic lipoproteins (Talayero and Sacks., 2011). Such abnormalities due to the excess levels of these biomolecules makes it

imperative to control their levels in blood. Synthetic drugs currently used for this purpose have several side effects such as hypoglycemia, gastrointestinal disturbances, renal toxicity and hepatotoxicity (Caprio and Fonseca., 2014). Therefore, plant formulations with less toxicity have great importance and value for treating disease like diabetes mellitus. Many Type 2 diabetic patients are reported to have hypertriglyceridemia. Often diabetic patients in the postprandial state have an increased concentration of triglyceride-rich lipoproteins. Elevated concentration of triglyceride-rich lipoproteins is key to all manifestations of diabetic dyslipidemia. Hypertriglyceridemia cause abnormal HDL metabolism (Sugden and Holness., 2011).

Due to the importance of lipid metabolism and the altered levels of different lipid components during, diabetes, we have studied the levels of major lipids in the tissues and serum of experimental animals.

6.2: Results

6.2.1: Total Cholesterol

Level of cholesterol in tissues was in range of 3-6 mg/g of wet weight and 40 -53.6 mg/dl in serum. In diabetic rats the level increased by 19.2, 79.7, 37.7 and 23.2% in liver, kidney, heart and serum respectively. The increase in TC observed in all the tissues and serum of diabetic rats is significant ($P < 0.001$) compared to normal. Administration of DK reduced this increase in a dose dependent manner in liver and kidney. TC level was also decreased in heart and serum of DK fed diabetic rats, but the decrease was not in a dose dependent manner. Compared to diabetic, in serum the decrease was not significant in DK fed diabetic rats but in all the tissues (liver,

kidney and heart) decrease was significant. TC level was decreased in almost dose dependent manner in all tissues and serum. DK administration reduced the TC level in all the tissues of normal rats also. Except in kidneys, the high dose of DK administration reduced TC levels of normal rats to below normal level. In kidneys dose A fed normal rats showed decreased TC level than the high dose of DK fed normal rats.

Table 6.2.1: Effect of DK on Total cholesterol

Sl.No	Groups	Liver	Kidney	Heart	Serum
1	NC	5.21±0.18**	5.94±0.21**	6.10±0.22**	43.61±1.55**
2	DC	6.23±0.22 ^a	9.71±0.34 ^a	8.36±0.30 ^a	53.77±1.91 ^a
3	DDKA	5.17±0.18**	6.74±0.24 ^{a**}	7.02±0.25 ^{a**}	52.16±1.85 ^a
4	DDKB	4.60±0.16 ^{a**}	6.03±0.21**	5.95±0.21**	50.74±1.80 ^a
5	DDKC	4.46±0.16 ^{a**}	5.79±0.21**	6.23±0.22**	51.56±1.83 ^a
6	CDKA	4.85±0.17 ^{b**}	4.37±0.16 ^{a**}	6.18±0.22**	50.38±1.79 ^{a*}
7	CDKB	3.88±0.14 ^{a**}	5.61±0.19**	5.63±0.20 ^{b**}	41.83±1.48**
8	CDKC	3.92±0.14 ^{a**}	5.33±0.19 ^{a**}	4.98±0.18**	40.04±0.42 ^{b**}

Values are expressed as mean ± SD of six rats. ‘a’ indicates values are significantly different from Normal Control rats with p<0.001. ‘b’ indicates values are significantly different from Normal Control rats with p<0.05. ‘**’ indicates values are significantly different from Diabetic Control rats with p<0.001. ‘*’ indicates values are significantly different from Diabetic Control rats with p<0.05. Total Cholesterol expressed as mg/gm tissue/dL serum.

6.2.2: Free Fatty Acids

Levels of FFAs displayed tissue related variations. Its levels were 5.74±0.23, 6.07±0.24, 2.26±0.09 mg/g tissue in liver, kidney and heart respectively of normal rats. In serum of normal rats, the FFA was 67.6±2.68 mg/dl. Changes in FFA levels were in a similar pattern as seen in case of TC. Increases in the FFA by 53.4, 29.48 107.7 and 43.04% in liver, kidney, heart and serum respectively were seen in

diabetic rats and this increase in FFA level was statistically significant ($P<0.001$). Administration of DK to diabetic rats reduced the FFA level in all tissues studied. The decrease was in a dose dependent manner in heart, kidney and serum. In liver FFA level was brought to normalcy by dose A, but higher doses did not make any further effect. Hence this result demonstrates a tissue- specific effectiveness of the preparation. The highest dose brought down the FFA level to normal or even below in certain tissues of non-diabetic rats. Administration of DK in normal rats did not affect the FFA level in liver and heart whereas in kidney and serum the FFA level decreased significantly ($P<0.001$ and $P<0.05$).

Table 6.2.2: Effect of DK on Free Fatty Acids.

Sl.No	Groups	Liver	Kidney	Heart	Serum
1	NC	5.74±0.23 **	6.07±0.24 **	2.26±0.09 **	67.61±2.68 **
2	DC	8.83±0.35 ^a	7.86±0.31 ^a	4.70±0.19 ^a	96.66±3.83 ^a
3	DDKA	5.10±0.20 ^{a**}	5.64±0.22 ^{b**}	2.56±0.10 ^{a**}	85.38±3.38 ^{a**}
4	DDKB	6.11±0.2 **	4.03±0.16 ^{a**}	2.53±0.10 ^{b**}	78.13±3.10 ^{a**}
5	DDKC	5.33±0.21 **	3.52±0.14 ^{a**}	2.28±0.09 **	67.98±2.70 **
6	CDKA	5.24±0.21 ^{b**}	2.82±0.11 ^{a**}	2.54±0.10 ^{b**}	60.3±2.39 ^{b**}
7	CDKB	5.28±0.21 ^{b**}	2.90±0.11 ^{a**}	2.43±0.10 **	58.76±2.33 ^{a**}
8	CDKC	5.13±0.20 ^{a**}	2.56±0.10 ^{a**}	2.19±0.09 **	59.42±2.36 ^{a**}

Values are expressed as mean ± SD of six rats. ‘a’ indicates values are significantly different from Normal Control rats with $p<0.001$. ‘b’ indicates values are significantly different from Normal Control rats with $p<0.05$. ‘**’ indicates values are significantly different from Diabetic Control rats with $p<0.001$. ‘*’ indicates values are significantly different from Diabetic Control rats with $p<0.05$. Free fatty acids expressed as mg/gm tissue/dL serum.

6.2.3: Phospholipids

Table 5.2.3: Effect of DK on Phospholipids.

Sl. No	Groups	Liver	Kidney	Heart	Serum
1	NC	9.01±0.31**	10.11±0.34*	13.99±0.47**	95.27±3.22 **
2	DC	11.44±0.39 ^a	10.75±0.36 ^b	15.66±0.53 ^a	109.16±3.69 ^a
3	DDKA	10.35±0.35 ^{a**}	8.66±0.29 ^{a**}	15.29±0.52 ^a	107.51±3.63 ^a
4	DZKB	8.33±0.28 ^{b**}	8.46±0.29 ^{a**}	13.19±0.45 **	104.15±3.52 ^a
5	DDKC	7.48±0.25 ^{a**}	8.45±0.29 ^{a**}	12.65±0.45 ^{a**}	97.22±3.29 **
6	CDKA	6.75±0.23 ^{a**}	7.84±0.27 ^{a**}	12.18±0.41 ^{a**}	77.72±2.63 ^{a**}
7	CDKB	6.53±0.30 ^{a**}	7.58±0.26 ^{a**}	11.06±0.38 ^{a**}	78.17±2.64 ^{a**}
8	CDKC	6.63±0.22 ^{a**}	6.52±0.22 ^{a**}	10.53±0.37 ^{a**}	74.51±2.52 ^{a**}

Values are expressed as mean ± SD of six rats. ‘a’ indicates values are significantly different from Normal Control rats with p<0.001. ‘b’ indicates values are significantly different from Normal Control rats with p<0.05. ‘**’ indicates values are significantly different from Diabetic Control rats with p<0.001. ‘*’ indicates values are significantly different from Diabetic Control rats with p<0.05. Phospholipids expressed as mg/gm tissue/dL serum.

PL level increased significantly (p<0.05) in the liver, heart and serum of diabetic rats, but no significant change was observed in kidney. DK administration significantly (P<0.001 and P<0.05) decreased PL levels in all tissues in both diabetic and non diabetic rats in a dose dependent manner. In liver and heart, dose B was able to bring the PL to a level below normal, but in serum a higher dose (dose C) was need to bring the PL level to normalcy. The decreases in diabetic rats due to the highest dose of DK were 27%, 34%, 25% and 22% in liver, kidney, heart and serum respectively. All doses of DK decreased PL far below normal levels in the different tissues except heart and serum. In heart and serum the dose A and B resulted a decrease in PL level, but remained above or same as normal levels.

6.2.4: Triglycerides

Table 6.2.4: Effect of DK on Triglycerides.

Sl. No	Groups	Liver	Kidney	Heart	Serum
1	NC	2.78±0.10 ^{**}	3.32±0.12 ^{**}	2.23±0.08 ^{**}	50.36±1.79 ^{**}
2	DC	5.86±0.21 ^a	6.22±0.22 ^a	3.68±0.13 ^a	66.47±2.36 ^a
3	DDKA	1.78±0.06 ^{a**}	5.62±0.20 ^{a**}	2.77±0.10 ^{a**}	48.07±1.71 ^{**}
4	DDKB	1.50±0.05 ^{a**}	4.02±0.14 ^{a**}	2.62±0.09 ^{a**}	45.40±1.61 ^{a**}
5	DDKC	2.99±0.11 ^{b**}	3.51±0.13 ^{**}	2.28±0.08 ^{**}	40.27±1.43 ^{a**}
6	CDKA	2.76±0.10 ^{**}	2.82±0.10 ^{a**}	2.19±0.078 ^{**}	40.05±1.42 ^{a**}
7	CDKB	2.17±0.08 ^{a**}	2.89±0.10 ^{a*}	1.29±0.05 ^{a**}	30.21±1.07 ^{a**}
8	CDKC	2.77±0.10 ^{**}	2.55±0.09 ^{a**}	1.25±0.04 ^{a**}	25.11±0.89 ^{a**}

Values are expressed as mean ± SD of six rats. ‘a’ indicates values are significantly different from Normal Control rats with p<0.001. ‘b’ indicates values are significantly different from Normal Control rats with p<0.05. ‘**’ indicates values are significantly different from Diabetic Control rats with p<0.001. ‘*’ indicates values are significantly different from Diabetic Control rats with p<0.05. Triglycerides expressed as mg/gm tissue/dL serum.

Levels of TG varied in different target tissues. Its levels were 2.78±0.1, 3.32±0.12 and 2.23±0.79 mg/g of wet weight in liver, kidney and heart and 50.36±1.78 mg/dl in serum of normal rats. A significant increase in TG levels was observed in all the tissues of the diabetic rats. The increases were 112.8%, 87.4%, 65.02% and 32% in liver, kidney, heart and serum respectively. TG levels in the liver remained normal in DK administered normal rats. A significant (P<0.001 and P<0.05) decrease in the TG level in the liver, kidney, heart and serum was observed in DK fed diabetic rats compared to diabetic control rats. In kidney and heart of diabetic rats the dose C was sufficient to bring the TG level to normal. TG levels in the serum of all rats except DDKA showed

significant difference ($P < 0.001$ and $P < 0.05$) from NC. In the heart and serum of normal rats, the dose C brought the TG level to about 50% of normal value.

6.3: Discussion

Hyperglycemia is one of the major contributors for the development of cardiovascular diseases in both type I and II diabetes (Grundy et al., 1999). Disturbance in lipoprotein metabolism is another reason for the development of vascular complications in diabetic mellitus which accelerates atherosclerosis (Maser et al., 1991). Several previous studies have already established the close relationship between hyperglycemia and dyslipidemia (Brownlee., 2001, Taskinen., 2003). Present study clearly showed that lipid profiles such as TC, FFA, TG and PL concentrations were increased in diabetic rats as compared to normal control rats. Administration of DK decreased lipid profile values in normal control and diabetic rats.

Altered lipid profile metabolism and diabetes mellitus are the potent predictors for the development of dyslipidemia, hypertension and cardiovascular diseases (Goldberg., 2001). There is an interrelationship between carbohydrate and lipid metabolism. So any disease affecting carbohydrate metabolism leads to disorders in lipid metabolism and vice versa (Ozder., 2014).

This study revealed elevation of total cholesterol in diabetic rats and administration of DK reduced these levels to normal or below normal level in diabetic and normal control rats. The increased cholesterol

level in diabetic condition may be due to hyperphagia. This induced an increase in the activity of HMG-CoA reductase of the intestine and results increased synthesis of cholesterol, leading to raised levels in plasma. In addition dietary cholesterol also adds up to total cholesterol by increased absorption (Christopher et al., 1981).

Elevated FFA level was observed in diabetic rats and the administration of DK reversed the effect of diabetes on FFA. Growing evidences suggest detrimental effects of prolonged exposure to increased FFA concentrations on β -cells (Mc Garry and Dobbins., 1999, Zhou and Grill., 1994), which is a classical example of lipotoxicity. Experiments in rodent islets in presence of elevated FFA levels had shown a reduction in insulin secretion in response to hyperglycemia. Biosynthesis of proinsulin and decrease in insulin stores were also reported in such a condition (Mc Garry and Dobbins., 1999, Zhou and Grill., 1994, Bollheimer et al., 1998). Similar results were observed in the experiments with obese Zucker diabetic fatty rats where enhanced levels of circulating FFA was found to cause impairment in β -cell function and apoptosis of insulin-secreting cells leading to the development of diabetes (Milburn et al., 1995, Lee et ai., 1997, Shimabukuro et al., 1998). These findings supports the concept that in genetically predisposed human subjects, prolonged exposure of β -cells to higher levels of circulating FFAs may contribute to abnormalities in the function of pancreatic islet and lead to type 2 diabetes (Unger., 1995). Zhou and Grill (1995) found that 0.125 mmol/l palmitate or oleate had a marked cytostatic effect on glucose-stimulated insulin release after 48 h incubation. More recently, it was shown that elevated fatty acids increased the proinsulin/insulin ratio

(Bjorklund and Grill., 1999). Hypertriglyceridemia is an essential component of atherogenic lipid profile (Mazzone et al., 2008).

Influence of insulin on the production of liver apolipoprotein and in regulating the enzymatic activity of cholesterol ester transport protein and lipoprotein lipase leading to the development of diabetic dyslipidemia is already established (Ozder., 2014). Insulin deficiency also reduced the activity of hepatic lipase and production of biologically active lipoprotein lipase (Elinasri and Ahmed., 2008, Mooradian., 2009, Smith and Lall., 2008). Increased blood glucose level combined with diabetic dyslipidemia increase atherosclerosis-related inflammation and makes it more extensive (Taskinen and Borén., 2015).

Hypertriglyceridaemia and hypercholesteridaemia are the two dyslipidemic conditions seen in diabetes mellitus, which usually accompanies decreased HDL cholesterol (Howard., 1987) (Taskinen et al., 1992). The possible mechanism behind the hypertriglyceridaemia is delayed clearance of triglyceride rich lipoproteins and increased hepatic secretion of very low density lipoprotein (VLDL), which automatically increased the substrate for TG, FFA and glucose (Goldberg., 1996). Dyslipidemia is not an obligatory component of diabetes and normal lipid profile is often observed in well controlled diabetic conditions.

Insulin resistance and elevated glucose concentration can worsen dyslipidemia, especially hypertriglyceridemia. Reports had already shown the possibility to improve dyslipidemia considerably by lowering elevated glucose level. In this study we have seen a decrease

in the glucose level as well as TG level in diabetic rats administered with DK. Decreased hyperglycemia might have resulted the decrease in the triglyceride level. The exact mechanism of decreasing TG level along with decreasing glucose level is not yet known. As suggested by previous reports (Sugden and Holness., 2011) increased glucose flow to the liver might have induced this positive change. Increase in insulin level is another effect that we have observed due to DK administration. Expression and activity of lipoprotein lipase is regulated by insulin and hence during severe insulin deficiency the possibility of decreasing the catabolism of triglyceride-rich lipoproteins is high. Hyperglycemia and hypoinsulinemia will reduce the catabolism of TG which in turn results in increased TG levels during diabetes.

The relationship of TG levels with CVD is well reported in several reviews which suggests higher risk of CVD events with an increase in the TG level (Sarwar et al., 2007, Nordestgaard and Varbo., 2014).

Klempfner et al., (2016) had already reported the independent association of fasting serum TG levels with the risk of CVD, even after adjustment for other lipid parameters in general population. Existing reports suggests the importance of the proper control of TG levels to reduce the risk of CVD especially during diabetic condition. Here in this study we have observed the TG lowering effect of DK in diabetic rats with hypertriglyceridemia.

A clinical study conducted in lean control and overweight but non-diabetic patients showed that baseline fasting plasma insulin and HOMA-IR index were positively correlated with erythrocyte membrane phosphatidylethanolamine and phosphatidylcholine content

in the whole population (Younsi et al., 2002). Poitout and Robertson (2008) had reported the influence of fatty acids on insulin resistance and pancreatic lipotoxicity.

Lipid metabolic pathways represent potential therapeutic targets to prevent or delay the onset and progression of metabolic disease. Further animal studies and clinical trials are required to define the stage of disease at which modulation of lipid metabolism will have maximal efficacy (Meikle and Summers., 2017).

DK is rich in phytochemicals such as polyphenols, tannins, flavanoids etc (Sindhu and Sruthi., 2012). Polyphenols have anti-lipidemic (Fenercioglu., 2010, ungerStephen., 2012) activity. Tannins decrease dietary absorption of cholesterol (Park., 2002). There are several mechanisms responsible for the hyper triglyceridaemia in diabetes. One mechanism increases the activity of hormone sensitive lipase that catalyses the mobilization of fatty acids from the stored triacylglycerols in the adipose tissue (Cullen et al., 1999, Reynisdottir et al., 1997). As a result, large amount of FFA returns to the liver. Recent studies showed that altered phospholipids metabolism also associated with pathogenesis and progression of metabolic disorders (Chang et al., 2018). An increase in phospholipid level was observed in diabetic rats which was reversed by the administration of DK. This study revealed the positive effect of DK administration on phospholipid levels in diabetic rats and normal control rats.

Kandasamy and Ashokkumar in (2014) reported an increase in lipid profiles like TC, FFA, TG in diabetic nephropathy condition and its reversal by the administration of myricetin, a natural flavonoid. The

present study also reports a similar pattern of results. Administration of flavonoids rich DK nullified the effect of diabetic condition on lipids by bringing the levels to normal or below normal level in all tissues studied. Similar observation is recorded in normal control rats also.

Lipid profiles like TC, FFA, TG and PL were increased in diabetic rats. The increased lipid levels have a suppressing effect on insulin production (Hao et al., 2007). DK administration decreased lipid profiles in diabetic rats and also had reduced blood glucose level. This decrease in lipid levels might also have removed the inhibition on insulin production which in turn resulted in reduced glucose levels. Dyslipidaemia which is a common phenomenon observed in both insulin deficiency and insulin resistance (Aluwong., 2016) play a major role in developing cardiovascular diseases (CVD) in diabetic patients (Yadav., 2007). CVD was found to be the most prevalent causative agent in diabetes related mortality (Matheus AS et al., 2013). The observed decrease in lipid levels due to DK administration may also reduce CVD in diabetic patients and hence the related mortality.

6.4: Conclusion

DK is rich in various phytochemicals. DK administration set right the abnormal lipid metabolism in diabetic rats. This positive effect might have caused by their synergistic action.

Effect of Dhanwantaram Kashaya on Lipid peroxidation

7.1: Introduction

Lipid peroxidation is a process in which oxidants such as reactive oxygen species (ROS) or free radicals attack lipids containing carbon-carbon double bonds especially seen in polyunsaturated fatty acids (PUFAs) (Yin et al., 2011). Lipids are mainly classified in to two types, polar lipids and apolar lipids. Triglycerides are one of the important forms of apolar lipids stored by the adipose tissue in the form of fat. It is one of the major types of energy storage form in mammals (Fruhbeck et al., 2001, Frayn., 1998). Polar lipids are the structural components of cell membranes. It participates in the formation of the permeability barrier of cells and subcellular organelles in the form of a lipid bilayer. Lipid bilayer are mainly made up of glycerol based phospholipids (Vance and Vance., 2002). Diabetes cause severe derangements in different metabolic process in body which include lipid metabolism and lipid peroxidation leading to free radical generation (ROS). These ROS triggers the different diabetic complications affecting the cardiovascular system, nervous system, urinogenital system etc. (kumar et al., 2014).

Different diabetic complications such as persistent hyperglycemia, glucose autoxidation, obesity, protein glycation and derangement in the activities of the enzymes such as nitric oxide synthase and xanthine

oxidase cumulatively contribute to free radical pool. (Valko et al., 2007, Rashid et al., 2015). These increased free radicals attack the unsaturated fatty acids, low-density lipoprotein (LDL), and total cholesterol (TC), found in higher level in DM and lead to the production of undesirable oxidized products contributing to initiation and progression of the disease along with complications (Rashid et al., 2015). ROS stimulate oxidation of LDL producing oxidized LDL (ox-LDL), which along with the involvement of macrophages lead to foam cell formation and atherosclerotic plaques (Valko et al., 2007).

One of the main consequences of uncontrolled oxidative stress is injury to cells, tissues and organs. Large amount of ROS or free radicals directly damage membrane lipids. Mitochondria, plasma membrane, endoplasmic reticulum and peroxisomes are the major sites of endogenous ROS production (Moldovan and Moldovan., 2004). Hydroxyl radicals ($\text{HO}\cdot$) and hydroperoxyl ($\text{HO}\cdot 2$) radicals are two most prevalent ROS that can affect the lipids profoundly. The hydroxyl radicals are small, short lived, highly mobile, water-soluble, and chemically most reactive species of activated oxygen. They are formed from O_2 during the time of cellular metabolism (Lane., 2002), unspecifically attack biomolecules and cause oxidative damage to cells (Halliwell and Gutteridge., 1984). Hydroperoxyl radical ($\text{HO}\cdot 2$) is a much stronger oxidant than superoxide anion-radical and plays a crucial role in lipid peroxidation. It could initiate the chain oxidation of polyunsaturated phospholipids and lead to impairment of membrane function (Bielski et al., 1983, Schneider et al., 2008, Browne and Armstrong., 2000).

Lipid peroxidation produces mainly two types of oxidation products, lipid hydroperoxides (LOOH) are the primary one and MDA is secondary. MDA is the end product formed from decomposition of larger PUFAs and arachidonic acid (Esterbauer et al., 1991). It is a convenient biomarker for lipid peroxidation of omega-3 and omega-6 fatty acids and also a most mutagenic product of lipid peroxidation (Esterbauer and Cheeseman., 1990, Esterbauer et al., 1991, Pryor., 1989). MDA is one of the most popular and reliable markers for determining oxidative stress in clinical situations (Giera et al., 2012). Because of this reason this oxidative stress marker is most relevant to biomedical research community.

In non enzymatic method during the lipid peroxidation process a mixture of lipid hydroperoxides are formed and the peroxy radical of the hydroperoxides with a cis-double bond undergo a series of reaction to form a new free radical. The intermediate free radicals again undergo further reactions to form bicycle endoperoxides which finally undergo cleavage to produce MDA (Ayala et al., 2014). Hydroperoxides also react with transition metals like iron or copper to form stable aldehydes like MDA and damage cell membranes (Halliwell and Chirico., 1993). Stress conditions induce the production of MDA and it is highly reactive with different biomolecules such as proteins and DNA leading to the formation of adducts (Zarkovic et al., 2013, Blair., 2008, Luczaj and Skrzydlewska., 2003). Excessive MDA production is associated with various pathological conditions (Garcia et al., 2013).

Dyslipidemia observed in diabetic patients is reported to be associated with increased lipid peroxidation which further is strongly correlated with higher levels of systemic inflammation (Bastos et al., 2016). Lipid peroxidation associated with hyperglycemia lead to cellular damage and cytoprotective enzyme inactivation (Ceriello and Motz., 2004). Glucose stimulated insulin secretion (GSIS) in islets is regulated by MDA through Wnt pathway (Ayala et al., 2014). Elevated levels of serum MDA have been reported in type 2 diabetes and it was reported to cause many adverse physiological consequences which includes alteration in the structural integrity of cell membrane and resultant leakiness. This also inactivate many of membrane bound enzymes and surface receptor molecules leading to the errors in cellular regulation.

Peroxisome proliferator-activated receptor (PPAR) is found in three isoforms, α , γ , and δ , (Dreyer., 1992) and are reported to play pivotal roles in lipid metabolism, glucose and energy homeostasis, and involved in inflammation and oxidative stress (Lee et al 2013). Peroxisome proliferator-activated receptor alpha (PPAR α) is reported to regulate myocardial lipid metabolism and control inflammation and oxidative stress (Palomer et al. 2013). Decreased expression of PPAR α was reported in diabetic mouse models (Hu et al., 2013). All these PPAR isoforms are involved in the regulation of various biological processes by functioning as ligand-activated transcription factors (Feige., 2006). Large number of genes involved in regulating the intermediary metabolism of glucose and lipids, insulin sensitivity etc are controlled and regulated by these isoforms (Fajas et al., 2001, Willson et al., 2001). Many PPAR agonists have been in use for the

treatment of metabolic diseases like diabetes mellitus (Inzucchi., 2015). Genes responsible for insulin-dependent glucose uptake (GLUT4, IRS-1, IRS-2, and c-Cbl associated protein) and adipokines (adiponectin, resistin, leptin, and tumor necrosis factor- α) of adipocytes are PPAR γ responsive (Ahmadian et al., 2013). Activation of these genes in adipocytes enhance systemic insulin sensitivity and helps to control diabetes. Genes such as GLUT4, IRS-1, IRS-2, and c-Cbl associated protein in adipocytes also respond to PPAR and hence play an important role in insulin signaling (Ahmadian et al., 2013).

Expression level of PPAR α in pancreatic islets and beta cells depends on glucose level (Gremlich et al., 2005). In another study hyperglycemia was found to downregulate expression of PPAR α in isolated rat islets and INS-1E cells (Roduit et al., 2000, Ravnskjaer et al., 2006). Taylor et al., (2013) had further demonstrated the role of PPAR α in regulating insulin mRNA level. Pdx-1, a transcription factor involved in the development of pancreas and beta cell development was also upregulated by PPAR α (babu et al., 2007). PPAR α induce genes encoding enzymes of fatty acid oxidation (FAO) in mitochondria and peroxisomes and activates fatty acid catabolism. Proteins involved in cellular fatty acid import are also activated by PPAR α . It can also stimulate gluconeogenesis (Roberts et al., 2002).

Role of PPAR- δ in regulating β -cell function is extensively studied. Prolonged treatment of diabetic *db/db* mice with a PPAR- δ agonist reduced blood glucose levels and improved insulin sensitivity and pancreatic islet function (Winzell et al., 2010). Ravnskjaer et al. (2010)

had suggested a fatty acid–sensor role to PPAR- δ , which helps in improving insulin secretion in β -cells. In another study, Cohen et al (2011) had reported an increase in 4-hydroxy-2E-nonenal (4-HNE) levels in β -cells exposed to high glucose, along with a marked release of arachidonic (AA) and linoleic acid (LA) from membrane phospholipids. 4-hydroxynonenal (4-HNE), a lipid peroxidation product of AA and LA functions as an endogenous ligand for PPAR- δ , and boost insulin secretion by β -cells.

Considering the importance of PPARs in diabetic condition and the possibility of ameliorating diabetic complications through the manipulation of its expression levels, we have studied the effect of DK on the expression of PPAR α and δ at mRNA level.

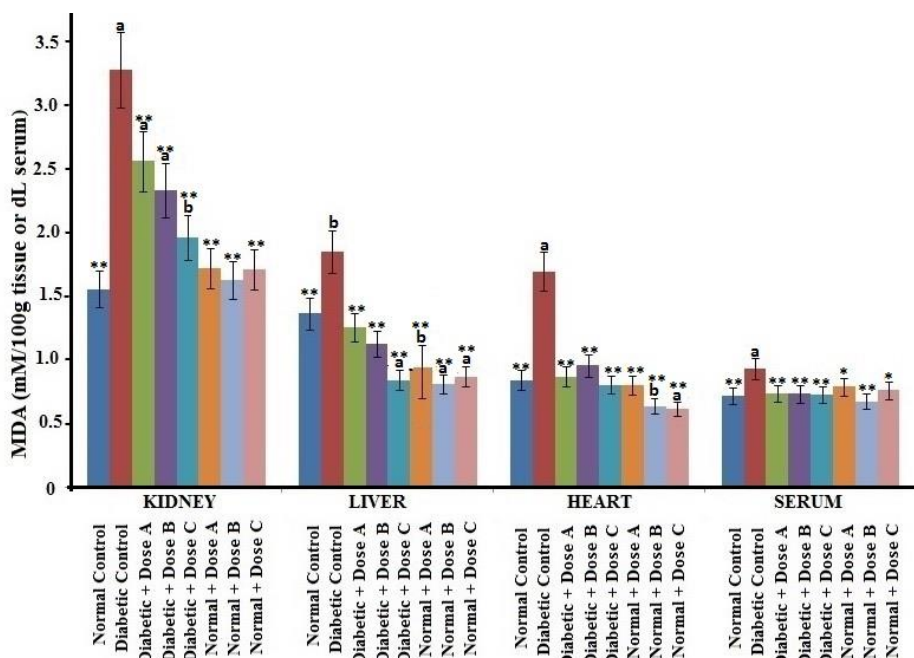
7.2: Results

7.2.1: MDA levels

MDA level was significantly increased in diabetic rats ($P < 0.001$) as compared to normal control rats (Fig 7.2.1). Highest increase was observed in kidneys (110%), followed by heart (101%), liver (35%) and serum (30%). Administration of DK decreased MDA level in all tissues of diabetic rats in a dose dependent manner. DK in dose C fully mitigated the increase in MDA levels in liver, heart and serum of diabetic rats. A decrease in MDA level was also observed in the kidneys of DK administered diabetic rats, but even with dose C the MDA level remained 26% higher than in normal control rats. Administration of DK to normal rats decreased the levels of MDA in

the liver, heart and serum, but in kidney the MDA level showed an increase, but was not significant.

Figure 7.2.1: Effect of DK on Concentration of MDA in different experimental groups of rats.



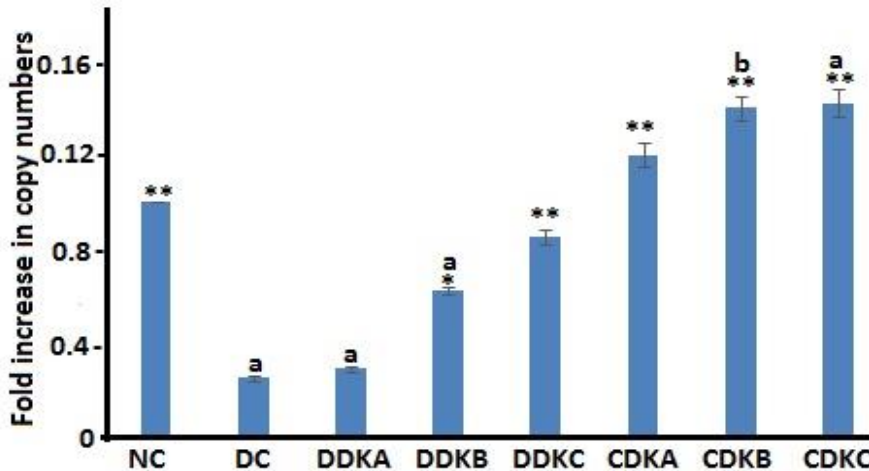
Values are expressed as mean \pm SD of six rats. ‘a’ indicates values are significantly different from Normal Control rats with $p < 0.001$. ‘b’ indicates values are significantly different from Normal Control rats with $p < 0.05$. ‘**’ indicates values are significantly different from Diabetic Control rats with $p < 0.001$. ‘*’ indicates values are significantly different from Diabetic Control rats with $p < 0.05$.

7.2.2: Expression of PPAR- α at mRNA level

Expression of PPAR- α was significantly decreased in DC rats ($P < 0.001$) as compared to NC rats. Administration of DK increased its expression level in a dose dependent manner in pancreatic tissues of diabetic rats and normal control rats. Compared to DC rats PPAR- α

expression level was significantly increased ($P < 0.001$ and $P < 0.05$) in the pancreatic tissues of all DK administered diabetic and normal rats except DDKA rats. In diabetic rats the expression level decreased by 75% and administration of DK resulted a dose dependent increase with the highest increase in dose C fed rats. In diabetic rats dose C increased the expression by 240%. At the same time the level of PPAR- α expression in dose C fed diabetic rats was 15% less than in normal rats. In normal rats DK administration in dose C increased PPAR- α expression by 40% and dose B by 20%. Increase resulted by dose A was insignificant.

Figure 7.2.2: Expression of PPAR α at mRNA levels in the pancreas of rats

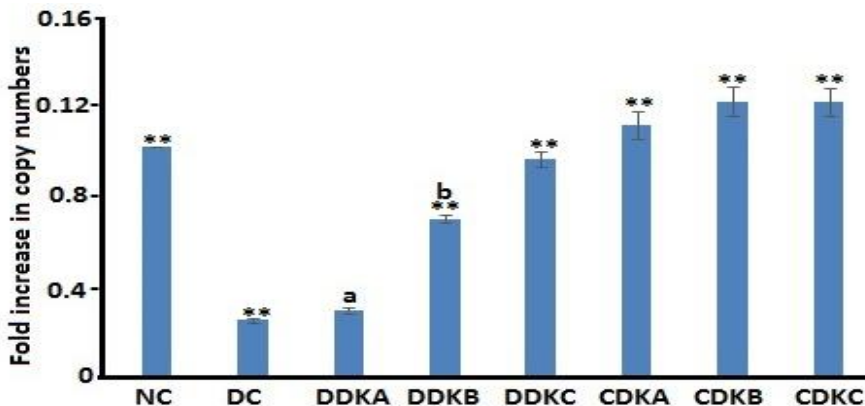


Values are expressed as mean \pm SD of six rats. 'a' indicates values are significantly different from Normal Control rats with $p < 0.001$. 'b' indicates values are significantly different from Normal Control rats with $p < 0.05$. '**' indicates values are significantly different from Diabetic Control rats with $p < 0.001$. '*' indicates values are significantly different from Diabetic Control rats with $p < 0.05$.

7.2.3: Expression of PPAR- δ at mRNA level

Expression of PPAR- δ was also significantly decreased in DC rats ($P < 0.001$) as compared to NC rats. Administration of DK increased its expression level in a dose dependent manner in pancreatic tissues of diabetic rats and normal control rats. Compared to DC rats PPAR- α expression level was significantly increased ($P < 0.001$ and $P < 0.05$) in all pancreatic tissues of DK administered diabetic and normal rats. In diabetic rats the expression level decreased by 75% and the administration of DK increased PPAR- δ mRNA expression level in all diabetic rats. The highest expression level was observed in dose C fed diabetic rats and the percentage of increase was 280% in comparison with diabetic rats. Dose C administration almost fully mitigated the change in expression level of PPAR- δ in diabetic rats. In normal rats increase due to DK administration was not significantly higher.

Figure 7.2.3: Expression of PPAR δ in pancreas of rats



Values are expressed as mean \pm SD of six rats. 'a' indicates values are significantly different from Normal Control rats with $p < 0.001$. 'b' indicates values are significantly different from Normal Control rats with $p < 0.05$. '**' indicates values are significantly different from Diabetic Control rats with $p < 0.001$. '*' indicates values are significantly different from Diabetic Control rats with $p < 0.05$.

7.3: Discussion

MDA is a primary biomarker for free radical mediated lipid damage and oxidative stress (Shodehinde and Oboh., 2013). Enormous previous studies had reported significant changes in lipid metabolism and membrane structure in patients with diabetes especially vascular complications (Fowler., 2008). Jain et al (2002) had demonstrated the oxidative stress due to hyperglycemia and ketosis and the resultant increase in lipid peroxidation in type 1 diabetic patients. Increased concentration of MDA that we observed in diabetic condition suggest decline in defense mechanisms by enzymatic and nonenzymatic antioxidants (Saddala et al., 2013). Several previous studies have already reported elevated MDA level in plasma, serum and other tissues of diabetic patients (Moussa., 2008, Bandeira et al., 2012). Present study revealed similar pattern of results in diabetic rats. In this study elevated level of MDA was observed in diabetic rats in comparison to normal control rats. Lipid peroxidation produce aldehydes such as malondialdehyde (MDA) (Begrache et al., 2006) which attack biomolecules like proteins, DNA, and phospholipids (Catala., 2009). The change in protein and phospholipid levels that we have observed in this study is a possible outcome of elevated lipid peroxidation.

Administration of DK decreased MDA level in a dose dependent manner in all tissues (liver, kidney and heart) and serum of diabetic and normal rats. Increased level of lipid peroxidation is attributed to the enhanced production of ROS. ROS play a crucial role in the

pathogenesis of diabetic complications and the hyperglycemic condition can stimulate free radical production. Membrane lipids are easily damaged by the actions of reactive oxygen species (Reiler., 1995). The elevated level of lipid peroxidation is a convenient method to monitor oxidative damage (Viani et al., 1991). From the results, it is evident that lipid peroxidation has occurred which is the consequences of oxidative stress (Arabmoazzen et al., 2015). It is also evident that DK may exert antioxidant property and protect the tissues from lipid peroxidation as its administration had fully reversed the abnormality.

Previous studies had suggested antioxidant role to PPAR α . This along with our data suggests the contribution of PPAR α deficiency in ROS production. Inhibition of PPAR α level in diabetic rats observed in this study might have increased peroxide radicals and this along with hyperglycaemia may have resulted in β - cell damage. Further studies are needed to establish the various mechanisms of increased oxidative stress and β - cell damage observed in diabetic rats.

Bauer et al., (2004) had reported an important role for PPAR α in protecting hepatocytes from potential oxidative damage. PPAR α expression was decreased by 75% in diabetic rats as compared to normal rats. Previous reports had already suggested the induction in the expression of PPAR α by insulin (Wang et al., 2012) and hence the decreased level of it could be explained by the decreased insulin level observed in diabetic rats. High glucose level observed in diabetic rats might also have repressed the expression of PPAR α (Ravnskjaer et al.,

2006). DK administration increased insulin level as well as PPAR α , which might have contributed for the beneficial impact of DK.

PPAR δ is the most abundant form of PPAR isoform in pancreatic islet cells (Ravnskjaer et al., 2010). There are conflicting reports about this isoform. PPAR δ is reported to be a negative regulator of insulin secretion as knock out mice had an increased number of pancreatic islets and a 2-fold increase in beta cell mass (Iglesias et al., 2012). At the same time there are other reports citing PPAR δ as a positive regulator, which suggested the role of a promoter of stem cells in to β -cells through the upregulation of Pdx-1 (Li et al., 2015). PPAR δ is deemed to sense oxidative stress through a feed-back control mechanism (Benedetti et al., 2014).

PPAR δ is thought to protect the cells from oxidative damage through different mechanisms like inhibiting the production of ROS and lipid intermediates and by increasing their degradation. Data from gene expression studies points to the possibility of the involvement of PPAR δ -dependent metabolism and detoxification of lipid intermediates in this process (Coleman et al., 2007). Enzymes such as aldehyde and alcohol dehydrogenases and glutathione S-transferases are involved in the biotransformation of 4-hydroxynonenal (4-HNE), a lipid peroxidation product of AA and LA to less toxic intermediates (Awasthi et al., 2003). Activation of PPAR δ increased mRNA for aldehyde dehydrogenase and glutathione -S- transferase (Coleman et al., 2007). Aldehyde dehydrogenase and glutathione -S- transferase are reported to have a major role in offering protection against lipid

peroxidation and toxicity (Awasthi et al., 2003).The involvement of these enzymes in DK administration needs investigation.

7.4: Conclusion

From these results, it can be concluded that DK, a poly herbal formulation used in Ayurveda exerts significant effects in suppressing lipid peroxidation. DK exerts its effect by suppressing MDA production, upregulating PPAR α and PPAR δ and by consequent metabolic changes. This herbal product could be developed as a promising natural and safe remedy for the prevention or delay of diseases caused by imbalance in ROS metabolism.

Effect of Dhanwantaram Kashaya on expression of various genes associated with diabetic complications

8.1: Introduction

Diabetes is associated with various health complications many of which are due to the alterations in the expression of different genes. The change in the expression of the different categories of genes like the genes involved in immune response, glucose homeostasis, apoptosis etc is reported to cause diabetic condition or the diabetic condition was found to alter their expression. We already had observed different beneficial impacts of DK in diabetic rats and most of the diabetic complications are mitigated by DK administration. We were interested to see whether the beneficial effect exerted by DK is through its influence on the expression levels of different genes. In this study the expression levels of some candidate genes are assessed to see the impact of DK in controlling the expression of those genes. This should help us to reveal the mode of action of DK in ameliorating the complications that we have observed in diabetic rats.

8.1.1: Nuclear factor- κ B (NF- κ B)

Nuclear factor- κ B (NF- κ B) is a group of structurally related and evolutionarily conserved proteins belonging to the Rel family and found in all cell types. They are regulated in response to cell stimulation by shuttling from the cytoplasm to the nucleus (Birbach et al., 2002).

Autoimmune attack on pancreatic β -cells mediated in part by cytokines such as interleukin-1 (IL-1) and interferon lead to type I diabetes. NF- κ B activity is normally not detected in resting β -cells, but its activation occurs on exposure to IL-1. This activation causes its translocation to the nucleus (Cardozo et al., 2001) and lead to the modification of the expression of hundreds of genes. This transcription factor is involved in various responses of cells to stimuli such as stress, cytokines, free radicals, ultraviolet irradiation, and microbial antigens. NF- κ B pathway has been found to be increased in skeletal muscle of patients with type 2 diabetes. Activation of NF- κ B in skeletal muscle was reported to cause insulin resistance (Sriwijitkamol et al., 2006).

A recent study demonstrated the activation of NF- κ B by hyperglycemia-dependent overproduction of mitochondrial superoxide (Nishikawa et al., 2000). Activation of NF- κ B induce oxidative stress, chronic inflammation, fibrosis, and cell death which are instrumental and exacerbate diabetic cardiomyopathy (Falcão-Pires and Leite-Moreira., 2012). Inhibition of the NF- κ B pathway was reported to protect β -cells of pancreas from apoptosis caused by the injection of low-dose streptozotocin, which was suggested as a potential strategy for protecting β -cells (Eldor et al., 2006). Various other studies revealed the possibility of preventing the cytokine-induced cell death in human islet cells (Giannoukakis et al., 2000) as well as in mouse insulin producing MIN6 cells (Baker et al., 2001) by inhibiting the cytokine induced activation of NF- κ B.

Mice deficient in NF- κ B1 (p50) were resistant to streptozotocin-induced diabetes, which further disclose the effect of NF- κ B on pancreatic β -cells (Giannoukakis et al., 2000). If reactive oxygen species (ROS) are not detoxified, cellular components are damaged and stress-sensitive intracellular signalling pathways mediated by nuclear factor-kappa B (NF κ B), p38 and Jun N-terminal kinase (JNK); also known as stress-activated kinases (SAPK) are altered (Newsholme et al., 2007). This could easily happen during diabetic condition as there is high oxidative stress induced by hyperglycaemia.

On the contrary certain other studies had reported the possible beneficial impact of NF- κ B on β cells. iNOS and MnSOD genes which may participate in β -cell defence are activated by NF- κ B (Cardozo et al., 2001). NF- κ B, however, also acts as a protective factor against programmed cell death by inducing antiapoptotic genes (Badrichani et al., 1999, Bach et al., 1997). NF- κ B “decoy” was reported to inhibit alloxan-induced β -cell death and diabetes mellitus in mice. Conflicting reports on the roles of NF- κ B in diabetic conditions induced the investigation on the expression of this gene in normal and diabetic rats as well as in DK fed normal and diabetic rats.

8.1.2: Tumour necrosis factor - α (TNF- α)

We have observed an increase in the expression of NF κ B in diabetic rats. A growing number of studies have shown that NF κ B activation induces the expression of several inflammatory cytokines, including TNF- α (Suzuki et al., 1997, Ho and Bray., 1999). Therefore, it is likely

that TNF- α production could be increased during diabetic condition. To test this hypothesis, we studied the expression levels of this gene at mRNA level. TNF- α was the first proinflammatory cytokine reported to have involvement in pathogenesis of insulin resistance and T2DM (Akash et al., 2018). TNF- α inhibits insulin mediated uptake of glucose and hence affect glucose metabolism (Aguirre et al., 2000). TNF- α metabolism is disturbed in metabolic disorders, such as obesity and insulin resistance (Groop et al., 1991). Previous reports also suggest the impact of the perturbations of TNF- α metabolism in the onset and progression of type 2 diabetes. Effective utilisation of TNF- α activity neutralisation can reverse the inflammatory responses and the progression of diabetes related complications. TNF- α was found to reduce the expression of insulin-regulated glucose transporter type 4 (GLUT4) (Akash et al., 2018) and affect glucose uptake. Elevated levels of serum TNF- α was observed in patients with type 1 and type 2 DM (Liu et al., 2016, Qiao et al., 2017) which was positively correlated with insulin resistance (Liu et al., 2016).

8.1.3: COX-2

COX-2 is an enzyme involved in the production of prostaglandins (PGs) during disease and inflammation and is rapidly inducible (Fujita et al., 2007). Sorli et al had demonstrated that COX-2 is predominantly expressed in islets (Sorli et al. 1998). Its expression is upregulated by hyperglycaemia (Persaud et al., 2004). Prostaglandins E2 (PGE2) is derived from COX-1 and COX-2 and hence decreased concentration of

these enzymes will decrease the production of PGE₂. PGE₂ was found to inhibit glucose-induced insulin secretion in pancreatic β -cell line (Robertson et al., 1987).

These facts point to the possibility of treating or preventing diabetes by altering the expression of COX-2. In line with this a recent in vitro study had reported increased glucose-stimulated insulin release in the pancreatic β -cell line, INS-1E cells through selective COX-2 inhibition by celecoxib (Luo et al., 2002). In hyperglycaemic conditions, IL-1 β is released from β -cells and it upregulate COX-2 expression (Maedler et al., 2002, Corbett et al., 1993). Influence of COX-2 in diabetic condition is getting revealed and based on the observation in our study it was felt important to study the expression of this gene in different groups of rats which might help to unravel the mode of action of DK.

8.1.4: Caspase 8

β -cell loss through apoptosis is an important pathological mechanism in type 1 and type 2 diabetes (*Mathis et al., 2001, Butler et al., 2003*). Caspases are the major components of the cell suicide machinery (Chang and Yang., 2000). Caspases also have important roles in other cellular processes like proliferation, differentiation, and survival and the role of caspases in these cellular processes is highly cell type and stimulus dependent. (Kang et al., 2004, Chun et al., 2002).

Expression of Cellular FADD-like IL-1 β -converting enzyme (FLICE)-inhibitory protein (c-Flip), a molecule known to inhibit caspase-8-

mediated apoptosis in β -cells, was shown to inhibit Fas-induced apoptosis and induce β -cell proliferation (Maedler et al., 2002). The activation of extracellular death receptors leads to the recruitment of FADD (Fas-associated death domain) and procaspase 8. This leads to the cleavage of procaspase 8 by self-cleavage or by another caspase 8 molecule (Kaufmann and Earnshaw., 2000, Muzio et al., 1998).

Activated caspase 8 which is an initiator caspase, activates downstream executioner caspases that cleave cell death substrates or directly induces apoptosis (Krammer., 2000, Lavrik and Krammer., 2012). Expression of caspase 8 was studied to see the effect of DK on caspase mediated apoptosis initiation during diabetes.

8.1.5: Caspase 3

Caspase-3 is the major effector caspase involved in apoptotic pathways and is thought to have important role in the apoptosis of β -cell in the pathogenesis of type 1 diabetes. Reddy et al., in (2003) had reported caspase-3 immuno reactivity predominantly in intraislet macrophages of NOD mice during diabetes which was not observed in islet cells. This suggested rapid clearance of apoptotic β cells after the onset of diabetes (Reddy et al., 2003). Liadis et al., (2005) had reported Caspase-3-dependent β -cell apoptosis. They also observed the importance of caspase-3 in the development of autoimmune diabetes. (Liadis et al., 2005). Investigation on the expression of caspase 3 in diabetic rats and the effect of DK on it might elaborate the possibility of developing DK as a therapeutic or preventive aid against diabetes.

8.2: Results

8.2.1: RNA extraction

Expression of the different genes under study were assessed at mRNA level. For this total RNA was isolated by using Trizol and the concentration and the quality was assessed by spectrophotometry. The purity and concentration of RNA were analysed by using procedure as mentioned in materials and methods. Results obtained for one of the estimations are given in Table 8.2.1.1. We got good RNA preparation from all the samples. An OD 260/280 ratio above 1.8 ensured good quality RNA preparation.

Table: 8.2.1.1: Showing the quality (260/280 ratio) and yield of RNA isolated from liver tissue.

Groups	OD at 260 nm	OD at 280 nm	Ratio 260/280	Conc. RNA (µg RNA /ml)
NC	0.8753	0.4858	1.801	1750.6
DC	0.5453	0.3016	1.808	1090.6
DDKA	0.9402	0.5163	1.821	1880.4
DDKB	0.8606	0.4733	1.818	1721.2
DDKC	1.0137	0.5593	1.812	2027.4
CDKA	0.5407	0.2962	1.825	1081.4
CDKB	1.3215	0.7225	1.829	2643
CDKC	0.9814	0.5288	1.856	1962.8

RNA concentration expressed as µg RNA per ml, calculated by using the equation: Concentration = A₂₆₀ x Dil. factor x 40.

Table: 8.2.1.2: Showing the quality (260/280 ratio) and yield of RNA isolated from bone marrow cells.

Groups	OD at 260 nm	OD at 280 nm	Ratio 260/280	Conc. RNA (µg RNA /ml)
NC	0.1597	0.0840	1.901	319.4
2µl	0.0982	0.0523	1.877	196.4
3 µl	0.1351	0.0736	1.835	270.2
5µl	0.1733	0.099	1.750	346.6
10µl	0.1839	0.102	1.802	367.8
20µl	0.1679	0.0912	1.841	335.8
50µl	0.1437	0.0801	1.794	287.4
100µl	0.1574	0.0864	1.821	314.8

RNA concentration expressed as µg RNA per ml, calculated by using the equation: Concentration = A₂₆₀ x Dil. factor x 40.

The RNA was successfully isolated from heart tissue of rats. The purity and concentration of RNA were analysed by using procedure as mentioned in materials and methods.

Table: 8.2.1.3: Showing the quality and yield of RNA isolated from rat heart tissue.

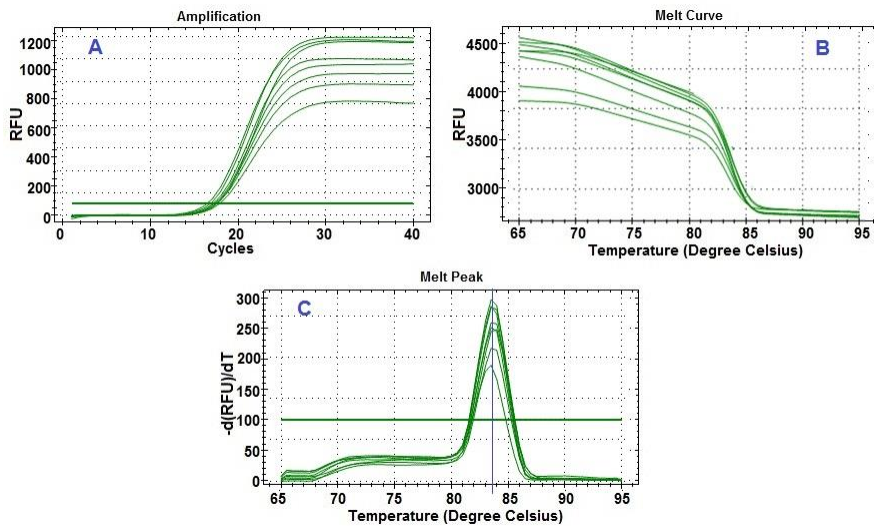
Groups	OD at 260 nm	OD at 280 nm	Ratio 260/280	Conc. RNA (µg RNA /ml)
NC	0.4661	0.2583	1.804	932.2
DC	0.5300	0.289	1.833	1060
DDKA	0.5510	0.3039	1.813	1102
DDKB	0.4585	0.2536	1.807	917
DDKC	0.1645	0.0794	2.071	329
CDKA	0.3912	0.214	1.828	782.4
CDKB	0.4482	0.2486	1.802	896.4
CDKC	0.4574	0.2523	1.812	914.8

RNA concentration expressed as µg RNA per ml, calculated by using the equation: Concentration = A₂₆₀ x Dil. factor x 40.

8.2.2: Real Time PCR Analysis of gene expression at mRNA level

8.2.2.1 Expression of β actin gene used as reference gene in this study

Figure 8.2.2.1: Expression of β actin gene in the liver tissue of rats



Expression of β actin gene in the liver tissue of rats belonging to the different groups studied by real-time PCR. (A): Shows the graphical representation of amplification curve, (B) shows the melt curve and (C) represents the melt Peak.

Melting curve analysed revealed only one peak and the melting point of the product was shown as 83.7. The observed single peak confirmed the amplification of a single product and the melting point shown is same as the calculated melting point of the PCR product. This ensured the authenticity of the amplified product.

Real-time quantitative polymerase chain reaction (RT-qPCR) is a powerful technique to assess the relative changes in gene expression (VanGuilder et al., 2008) at mRNA level. At the same time changes in sensitivity of the estimations need to be normalised using an endogenous control (reference gene) to ensure the reliability of the data (Panina 2018). At present, housekeeping genes such as β actin, Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) or ribosomal genes etc are thought to be universally required and to be constitutively and stably expressed in varying experimental conditions. In this study β actin gene was used as internal candidate to normalise the gene expression levels observed in real time PCR.

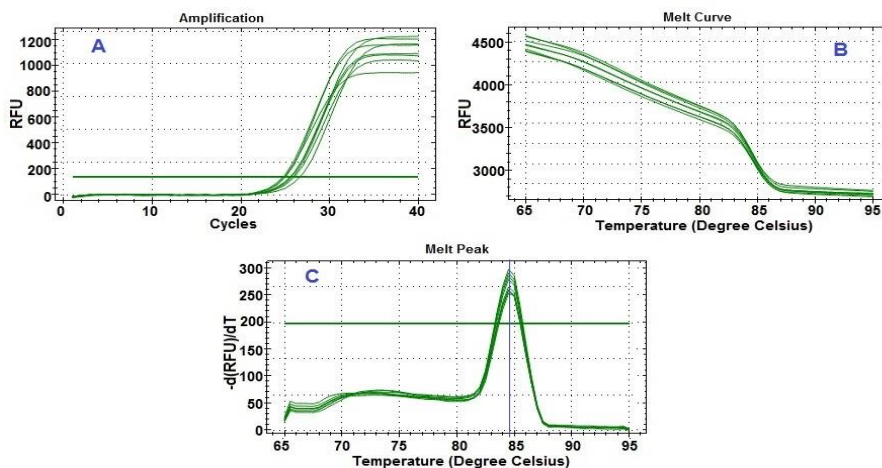
8.2.2.2: Effect of DK on the expression of NF- κ B

Expression of NF- κ B in liver at mRNA level was studied by real-time PCR and the results are shown in table 8.2.2.2.1. The authenticity of the amplified product was assured by melting curve analysis. Melting curve analysis revealed only one peak and the melting point of the product was 84.6. The observed single peak confirmed the amplification of a single product and the melting point shown is same as the calculated melting point of the PCR product. This ensured the authenticity of the amplified product.

We assumed that the β actin is unaffected by our treatment and will give same amplification efficiency for all groups without any variation in expression. All the values are regularised with the expression level of β actin. The expression level of NF- κ B was significantly higher

($p < 0.001$) in diabetic control rats and least in DK treated normal rats fed with dose C. In the case of DDKA rats the expression level decreased in comparison to DC rats, but remained higher than normal rats ($p < 0.05$). Dose B and C also showed decrease in proportion to increasing dose. Dose C brought the expression of NF- κ B very close to normal level and the difference between NC and DDKC rats were not significant. Expression of NF- κ B in bone marrow, heart and liver followed the same pattern with highest increase due to diabetes seen in bone marrow followed by heart and liver. DK administration decreased the expression in a dose dependent manner in all the three tissues with the highest percentage of decrease in liver (48%) followed by bone marrow (37%) and heart (18%).

Figure 8.2.2.1: Expression of NF- κ B gene in the liver tissue of rats



Expression of NF- κ B gene in the liver tissue of rats belonging to the different groups studied by real-time PCR. (A): Shows the graphical representation of amplification curve, (B) shows the melt curve and (C) represents the melt Peak.

Table 8.2.2.2.1: Expression levels of the NF-κB gene in the liver of rats of the different groups.

Sl No	Groups	CT	Beta actin	ΔCT	ΔΔCT	2 ^{-ΔΔCT}
1	NC	25.99	17.59	8.4	0	1**
2	DC	24.98	17.95	7.03	-1.37	2.58 ^a
3	DDKA	24.70	17.19	7.51	-0.89	1.85 ^b
4	DDKB	24.87	17.31	7.56	-0.84	1.79 ^{b*}
5	DDKC	25.56	17.57	7.99	-0.41	1.32 [*]
6	CDKA	26.59	18.25	8.34	-0.06	1.04 [*]
7	CDKB	25.77	17.22	8.55	0.15	0.9 [*]
8	CDKC	25.55	16.95	8.6	0.20	0.87 [*]

Showing the calculation of the expression levels of the NF-κB gene in the liver of rats of the different groups. Where ΔCT = CT of NF-κB gene- CT of β-actin, ΔΔCT = ΔCT treated -ΔCT non treated (corresponding control) and 2^{-ΔΔCT} = Fold difference in NF-κB gene relative to the nontreated control. ‘a’ indicates values are significantly different from Normal Control rats with p<0.001. ‘b’ indicates values are significantly different from Normal Control rats with p<0.05. ‘***’ indicates values are significantly different from Diabetic Control rats with p<0.001. ‘*’ indicates values are significantly different from Diabetic Control rats with p<0.05.

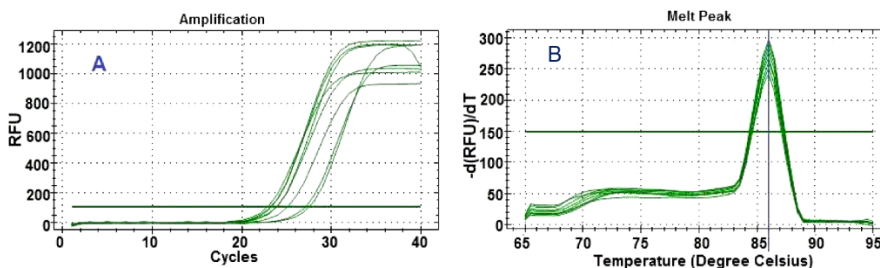
Table 8.2.2.2.2: Expression levels of the NF-κB gene in the bone marrow, heart and liver of rats of the different groups

Sl:No	Groups	Bone marrow	Heart	Liver
1	NC	1**	1*	1**
2	DC	3.5 ^a	3.26 ^a	2.58 ^a
3	DDKA	2.93 ^a	3.12 ^a	1.85 ^b
4	DDKB	2.42 ^a	2.84 ^a	1.79 ^{b*}
5	DDKC	2.19 ^a	2.67 ^a	1.32 [*]
6	CDKA	1.02 [*]	0.99 [*]	1.04 [*]
7	CDKB	0.98 [*]	0.96 [*]	0.9 [*]
8	CDKC	0.96 [*]	0.92 [*]	0.87 [*]

Showing the expression levels of the NF-κB gene in the bone marrow, heart and liver of rats of the different groups expressed as fold difference in copy number of mRNA of NF-κB gene relative to the nontreated control. ‘a’ indicates values are significantly different from Normal Control rats with p<0.001. ‘b’ indicates values are significantly different from Normal Control rats with p<0.05. ‘***’ indicates values are significantly different from Diabetic Control rats with p<0.001. ‘*’ indicates values are significantly different from Diabetic Control rats with p<0.05.

8.2.2.3: Expression of TNF- α gene in diabetic and DK treated rats

Figure 8.2.2.3.1: Expression of TNF- α gene in the liver tissue of rats belonging to the different groups



Expression of TNF- α gene in the liver tissue of rats belonging to the different groups studied by real-time PCR. (A): Shows the graphical representation of amplification curve and (B) represents the melt Peak.

Melting curve analysed revealed only one peak and the melting point of the product was shown as 86. The observed single peak confirmed the amplification of a single product and the melting point shown is same as the calculated melting point of the PCR product. This ensured the authenticity of the amplified product.

Table 8.2.2.3.1: Expression levels of the TNF- α gene in the rats of the liver of the different groups

Sl:No	Groups	Fold increase
1	NC	1**
2	DC	3.52 ^a
3	DDKA	2.23 ^{a*}
4	DDKB	1.92 ^{b*}
5	DDKC	1.4 ^{b**}
6	CDKA	0.97 ^{**}
7	CDKB	0.88 ^{**}
8	CDKC	0.85 ^{**}

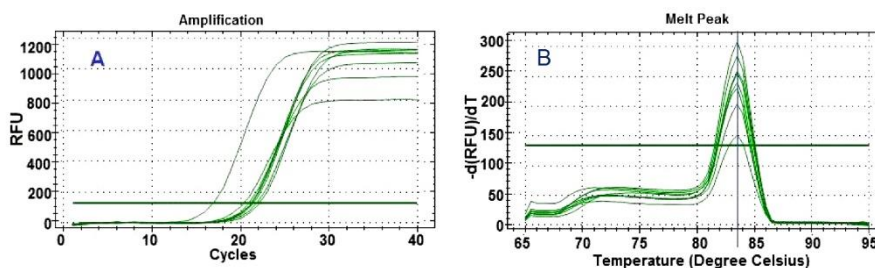
Showing the expression levels of the TNF- α gene in the rats of the liver of the different groups expressed as fold difference in copy number of mRNA of TNF- α gene relative to the nontreated control. 'a' indicates values are significantly different from Normal Control rats with $p < 0.001$. 'b' indicates values are significantly different from Normal Control rats with $p < 0.05$. '**' indicates values are significantly different from Diabetic Control rats with $p < 0.001$. '*' indicates values are significantly different from Diabetic Control rats with $p < 0.05$.

8.2.2.3.1: Expression of TNF- α gene in different groups of rats

As observed with other genes the expression of TNF- α was also hyped in diabetic rats with a percentage increase of 252% from normal control rats. DK exerted a dose dependent effect on the expression with increasing doses suppressing the expression more and more. In dose C fed diabetic rats the expression was only 40% higher than normal rats. In diabetic rats the dose C decreased the expression by 212%, dose B by 160% and dose A by 129%. A dose dependent decrease in expression of TNF- α was observed in normal rats also, but this decrease was not significant.

8.2.2.4: Gene expression of COX-2 on treatment with DK.

Figure 8.2.2.4.1: Expression of Cox-2 gene in the liver tissue of rats belonging to the different groups



Expression of Cox-2 gene in the liver tissue of rats belonging to the different groups studied by real-time PCR. (A): shows the melt curve and (B) represents the melt Peak.

Melting curve analysed revealed only one peak and the melting point of the product was shown as 83.5. The observed single peak confirmed the amplification of a single product and the melting point shown is

same as the calculated melting point of the PCR product. This ensured the authenticity of the amplified product.

8.2.2.4.1: Data analysis of COX-2

Table 8.2.2.4.1: Expression levels of the COX-2 gene in the rats of the liver of the different groups

Sl:No	Groups	Fold increase
1	NC	1**
2	DC	3.29 ^a
3	DDKA	2.8a
4	DDKB	2.30 ^a
5	DDKC	1.74 ^{b*}
6	CDKA	1.1*
7	CDKB	0.98*
8	CDKC	0.91*

Showing the expression levels of the COX-2 gene in the rats of the liver of the different groups expressed as fold difference in copy number of mRNA of COX-2 gene relative to the nontreated control. 'a' indicates values are significantly different from Normal Control rats with $p < 0.001$. 'b' indicates values are significantly different from Normal Control rats with $p < 0.05$. '**' indicates values are significantly different from Diabetic Control rats with $p < 0.001$. '*' indicates values are significantly different from Diabetic Control rats with $p < 0.05$.

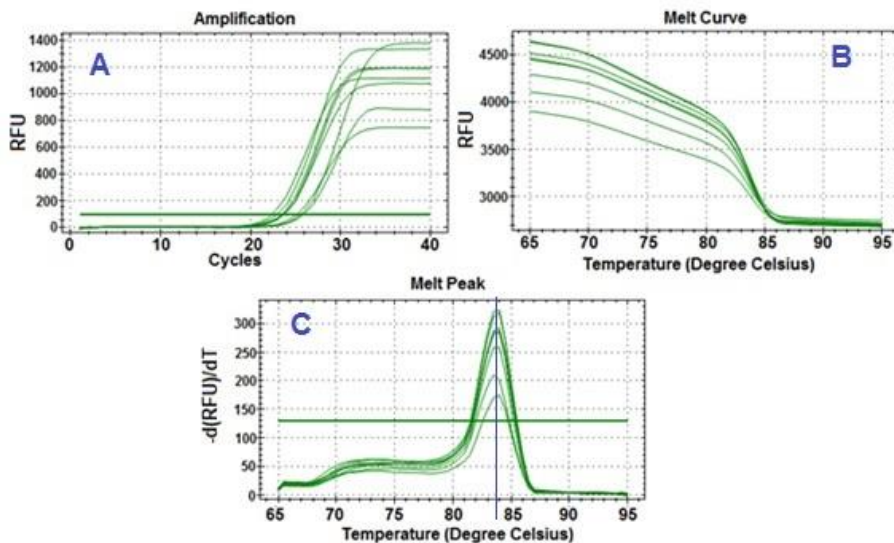
8.2.2.4.2: Expression of COX-2 gene on treatment with DK.

The expression of COX-2 was also studied by Real-time PCR. The expression levels of COX-2 increased significantly ($p < 0.001$) in diabetic rats. The expression of COX-2 was 229% higher in diabetic rats compared to normal control rats. Administration of DK decreased

the diabetes induced expression of COX-2 in a dose dependent manner. Compared to normal control rats the expression of COX-2 was only 79 percent higher in dose C fed diabetic rats. Dose C reduced the hyped expression of COX-2 due to diabetes from 229% to 79%. DK administration to normal rats also decreased the expression of COX-2, but this change was not significant. These data indicated that DK positively regulates COX-2 expression.

8.2.2.5: Expression of Caspase 8 in diabetic and DK treated rats

Figure 8.2.2.5.1: Expression of caspase 8 gene in the liver tissue of rats belonging to the different groups



Expression of caspase 8 gene in the liver tissue of rats belonging to the different groups studied by real-time PCR. (A): Shows the graphical representation of amplification curve, (B) shows the melt curve and (C) represents the melt Peak.

Melting curve analysis revealed only one peak and the melting point of the product was shown as 83.8. The observed single peak confirmed the amplification of a single product and the melting point shown is same as the calculated melting point of the PCR product. This ensured the authenticity of the amplified product.

Table 8.2.2.5.1: Expression levels of the Caspase 8 gene in the rats of the liver of the different groups

Sl: No	Groups	Fold increase
1	NC	1 [*]
2	DC	5.97 ^a
3	DDKA	4.81 ^a
4	DDKB	3.15 ^{a*}
5	DDKC	1.85 ^{b*}
6	CDKA	0.97 [*]
7	CDKB	0.95 [*]
8	CDKC	0.82 [*]

Showing the expression levels of the Caspase 8 gene in the rats of the liver of the different groups expressed as fold difference in copy number of mRNA of Caspase 8 gene relative to the nontreated control. ‘a’ indicates values are significantly different from Normal Control rats with $p < 0.001$. ‘b’ indicates values are significantly different from Normal Control rats with $p < 0.05$. ‘***’ indicates values are significantly different from Diabetic Control rats with $p < 0.001$. ‘*’ indicates values are significantly different from Diabetic Control rats with $p < 0.05$.

8.2.2.6.2: Expression of Caspase 8 in diabetic and DK treated rats

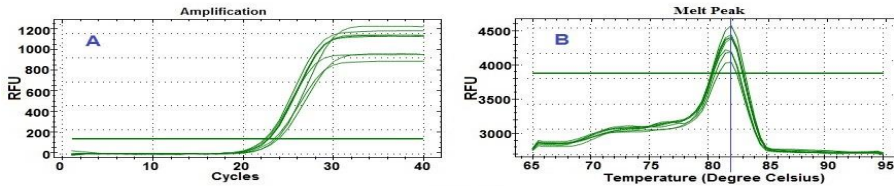
The expression level of Caspase 8 at mRNA level showed a very high increase (497%) in diabetic control rats ($p < 0.001$). Expression of Caspase 8 gene decreased in DK treated diabetic rats in a dose

dependent manner. Administration of dose C to diabetic rats decreased the expression of caspase 8 and reached close to normal and the difference was only 85% with NC rats, i.e. a change of 412% (497-85). In DK treated normal control rats, the expression was significantly decreased with increase in dose administered.

8.2.2.6: Expression of Caspase- 3 gene in diabetic and DK treated rats

Caspase 3 is the most important member of caspase family and plays an important role in apoptosis. Destruction of β cells in the pancreatic islets of diabetic rats by streptozotocin lead to diabetes or hyperglycaemia. To evaluate the effect of caspase in this destruction of β cells, we studied the expression levels of caspase 3 at mRNA level (Table 9.2.2.3.1). Expression of caspase was found to increase in liver of diabetic rats ($p < 0.005$). There was a hundred percent increase in the level of caspase 3 mRNA. Administration of DK decreased this increase in a dose dependent manner. Dose C was able to reverse the effect of diabetes and the expression level of caspase gene reached near normal value.

Figure 8.2.2.6.1: Expression of caspase 3 gene in the liver tissue of rats belonging to the different groups.



Expression of caspase 3 gene in the liver tissue of rats belonging to the different groups studied by real-time PCR. (A): Shows the graphical representation of amplification curve and (B) shows the melt Peak.

Melting curve analysed revealed only one peak and the melting point of the product was shown as 83.8. The observed single peak confirmed the amplification of a single product and the melting point shown is same as the calculated melting point of the PCR product. This ensured the authenticity of the amplified product.

Table 8.2.2.6.1: Expression levels of the Caspase 3 gene in the rats of the liver of the different groups

Sl:No	Groups	Fold increase
1	NC	1*
2	DC	2.07a
3	DDKA	1.56b
4	DDKB	1.32
5	DDKC	1.24
6	CDKA	1.02*
7	CDKB	0.93*
8	CDKC	0.81*

Showing the expression levels of the Caspase 3 gene in the rats of the liver of the different groups expressed as fold difference in copy number of mRNA of Caspase 3 gene relative to the nontreated control. 'a' indicates values are significantly different from Normal Control rats with $p < 0.001$. 'b' indicates values are significantly different from Normal Control rats with $p < 0.05$. '**' indicates values are significantly different from Diabetic Control rats with $p < 0.001$. '*' indicates values are significantly different from Diabetic Control rats with $p < 0.05$.

8.3: Discussion

Expression of NF- κ B, TNF- α , COX-2, Caspase3 and Caspase 8 were studied in diabetic rats and diabetic rats administered with DK. In diabetic rats there was an increase in the expression of all the studied genes. DK administration ameliorated these diabetic complications to a great extent in a dose dependent manner. There were minor variations in the extent of the effect of DK between these genes.

Nuclear factor- κ B (NF- κ B) is a nuclear transcription factor found in all types of cells. As in case of TNF- α , it is also involved in cellular responses to stimuli such as stress, cytokines, free radicals, ultraviolet irradiation, and microbial antigens. (Patel and Santani., 2009). Several cytokines are involved in the destruction of β cells. Exposure to some of these cytokines cause the activation and translocation of NF- κ B into the nucleus (Cardozo et al., 2001). Many genes are activated by these cytokines and lead to β cell inactivation or apoptosis. Many of these genes are activated through NF- κ B. iNOS is one of such genes activated by NF- κ B. The activation of NF- κ B can trigger pro-apoptotic cascades in β cells (Barkett and Gilmore., 1999). Inhibition of NF- κ B by stable transfection with a dominant negative inhibitor of NF- κ B was found to prevent cytokine-induced cell death in human islet cells and cultured rat β cells. (Giannoukakis et al., 2000). Role of NF- κ B in β cell apoptosis was further revealed by the experiments of Quan et al (2001) using NF- κ B decoys (Quan et al., 2001) as well as by using mice deficient in NF- κ B (Mabley et al., 2002). NF- κ B

activation by hyperglycemia is observed in peripheral blood mononuclear cells (PBMCs) of patients with type 1 diabetes. (Hofmann et al., 1998). Certain other reports are contra indicating where the researchers found the protective role of NF- κ B in pancreatic β cells. Studies by Norlin et al (2005) with transgenic mice had reported the blocking of NF- κ B expression by hyperglycaemia and the decrease in the total number of endocrine cells in the adult pancreas. This prolonged suppression of NF- κ B was also found to reduce the expression of key genes in the insulin-secretion pathway. (Norlin et al., 2005). Similar studies had reported a decreased time course for the development of diabetes in animals where NF- κ B activation was prevented. All these points to the role of NF- κ B in β cell development and glucose homeostasis and hence the critical control of gene expression by NF- κ B is physiologically important. In another study the attenuation of NF- κ B activation by the co-expression of NF κ B Inhibitor Alpha ($I\kappa$ B α) in the liver was found to reduce the expression of NF- κ B dependent genes and at the same time it reversed many of the type 2 diabetic complications (Cai et al., 2005). Hyperglycaemia was found to activate NF- κ B and this activation of NF- κ B antagonizes the function of PPAR γ (Suzawa et al., 2003) that regulates lipid and glucose homeostasis. (Cai et al., 2005).

Hyperglycemia was found to increase the release of ROS which activate expression of NF- κ β and initiate pro-inflammatory response leading to endothelial dysfunction (Paneni et al., 2013). Increased release of NF- κ B leads to the release of proinflammatory cytokines,

chemokines and vascular cell adhesion molecules (VCAMs) (Patel., 2009). This up-regulation of cytokines and chemokines such as TNF- α , IL1 β , IL6, CD36, MCP-1 induce inflammatory process and the apoptosis of endothelial cells (Reddy and Natarajan., 2011). Over-activation of NF- κ B will also cause abnormal transcription as well as altered expression of various genes involved in vascular complications (Zheng et al., 2010, Kitada et al., 2010). All these answers the vascular complications associated with diabetes.

In this study we observed an increase in the expression of NF- κ B in diabetic rats. Along with that increased oxidative stress was also evident in diabetic rats. Hyperglycaemia and the increased oxidative stress might have resulted in the up regulation of NF- κ B. NF- κ B up regulation might have induced apoptosis of β cells in diabetic rats. This might further increase blood glucose level and lead to the abnormal glucose metabolism in diabetic rats and the resultant complications like cardiovascular diseases and renal abnormalities. Though there are conflicting reports on the effect of NF- κ B during diabetic conditions our results clearly shows the up regulation of NF- κ B expression during diabetes. Administration of DK down regulated the NF- κ B expression in diabetic rats in a dose dependent manner. In dose C fed diabetic rats most of the diabetic complications like hyperglycemia, increased oxidative stress, diuresis, etc disappeared on day 21. Bringing down the expression of NF- κ B might have reduced the damage on the β cells. This might increase the number of functioning β cells which increase insulin production. Availability of enough insulin will reduce

blood glucose level to normalcy and glucose homeostasis will be achieved.

Macrophages and other cell types in our body produce Tumor necrosis factor- α (TNF- α) in response to different stimuli (Ming et al., 1987, Kern et al., 2001). TNF- α in turn stimulate the release of the interleukins, prostaglandins and chemokines. TNF- α acts as a cofactor in different physiological processes like apoptosis, neutrophil activation, activity of lipoprotein lipase etc. (Ming et al 1987, Kern et al 2001, Halse et al., 2001). Recent research had revealed the casual link between the increased TNF- α production and decreased insulin sensitivity (Hotamisligil et al., 1994, Saghizadeh et al., 1996, Solomon., 1975).

Elevated circulating levels of TNF- α can induce the development of vascular diseases and atherosclerosis through the induction of proinflammatory cytokines and adhesion molecules which increase monocyte-endothelial cell adhesion (Solomon., 1975, Cavender et al., 1987, Jiala and Devaraj., 1996). Vascular inflammation in diabetic patients is also linked to elevated levels of TNF- α (Gamble et al., 1985). Induction of TNF- α production due to hyperglycemia is reported in diabetic rats and mice (Tanaka et al., 1992) as well as in streptozotocin-induced diabetic rats (Sagara et al., 1994). Further this effect on TNF- α secretion due to hyperglycemia is reported by Guha et al (2000) in cell culture studies using monocytes (Guha et al., 2000). Elevated TNF- α predicts future risk of myocardial infarction and is

also one of the risk factors of vascular inflammation seen in diabetic patients (Ridker et al., 2000).

We too observed an increased expression of TNF- α in diabetic rats. This might have caused the different abnormalities observed in the diabetic rats. Administration of DK down regulated the TNF- α expression in a dose dependent manner. There was a decrease in the diabetic complications in the DK fed rats in a dose dependent manner. The change in expression of TNF- α might have contributed for this beneficial impact of DK. Further investigations on the expression levels of proinflammatory cytokines and adhesion molecules during diabetes and on DK administration are needed to reveal the complete picture of the role of TNF- α during diabetes and the impact of DK in controlling its expression and the resultant metabolic changes in body.

Pancreatic islet constitutively and dominantly expresses COX-2 while in most other tissues during normal condition COX-2 expression is undetectable. COX-2 is significantly induced by stimuli such as lipopolysaccharide, cytokines such as interleukin-1 (IL-1) and TNF- α (Vane et al., 1998, Wadleigh and Herschman., 1999, Robertson., 1998). COX-2 is involved in the conversion of arachidonic acid into prostaglandin E2 (PGE2). PGE2 is suspected to play a role in inflammation, islet destruction, and inhibition of insulin secretion (Tran et al., 1999).

Excess production of ROS during hyperglycaemia was reported to increase COX-2 expression in mesangial cells (Kiritoshi et al., 2003).

Cosentino et al (2003) had reported a similar effect in endothelial cells, where high glucose was found to increase COX-2 expression (Cosentino et al., 2003). Increased NF- κ B expression due to hyperglycaemia could also increase the expression of COX-2. As explained earlier in this chapter, we have observed an increase in the NF- κ B expression during diabetic condition, which should be one of the causes of hyped COX-2 expression. Increased COX-2 level will increase PGE2 production which turns to be an underlying factor behind the vascular complications associated with diabetes (Shanmugam et al., 2004). Endothelial dysfunction might lead to an early manifestation of diabetes-related macro- and microvascular complications (De Vriese et al., 2000). Cyclooxygenase-2 (COX-2) - dependent synthesis of prostaglandins affect vasodilator mechanisms (Bagi et al., 2005, Guo et al., 2005).

Oxidative stress and lowgrade inflammation which are implicated in the pathogenesis of diabetes mellitus might be the underlying mechanism responsible for the upregulation of COX-2 in diabetes (Helmersson et al., 2004, Kiritoshi et al., 2003, Cosentino et al., 2003).

COX-2 has been implicated in many inflammatory diseases such as atherosclerosis and islet dysfunction. This reduced function of islets leads to hypoinsulinemia (Robertson et al., 1998, Burleigh et al., 2002). Our study also demonstrated the increased expression of COX-2 at mRNA level, hypoinsulinemia and hyperglycaemia and the administration of DK reversed this. This change in COX-2 expression

due to DK administration might be one of the underlying factors in the beneficial impact of DK.

Human islets cultured in diabetes-like high glucose medium were found to undergo apoptosis (Federici et al., 2001). Uncontrolled cell death through apoptosis or necrosis may lead to organ failure and chronic diseases. Studies in both humans as well as animals had revealed the hyperglycemia induced β -cell dysfunction and the resultant decrease in insulin synthesis and secretion (Harmon et al., 1999, Donath et al., 1999). Another study conducted in diabetic nude mice by Jansson et al (1995) had reported reduced graft insulin content in the animals transplanted with non-curative amounts of human islets cells. Progressive fall in β -cell mass and persisting hyperglycemia even after islet transplantation was also reported in animals treated with small numbers of human islets cells (Jansson et al., 1995). Our observation of degenerated islets in diabetic rats, decreased blood insulin level and the concurrent hyperglycemia points to the possibility of β cell death due to apoptosis.

Electron microscopy analysis of islets cultured under hyperglycaemic conditions reported totally degranulated β cells after exposure to high glucose which strengthen the concept that hyperglycaemia might direct dysfunctional cells to undergo apoptosis (Federici et al 2001). To investigate the role of DK in apoptosis induced by hyperglycaemia we have assessed the expression levels of two pro apoptotic genes; caspase 3 and 8. Caspases are cysteine proteases which are primary effectors in

apoptosis and proteolytically dismantle cellular structures including cytoskeleton, mitochondria, golgi apparatus, endoplasmic reticulum, nucleus etc. Their key role in apoptosis is well reported. More than 14 caspases are known and some of which are involved in apoptosis and others in the activation of cytokines (Liadis et al., 2005).

The essentiality of caspase-dependent apoptotic pathways in the apoptosis of β -cell is already established (Federici et al., 2001). In another study Fas-induced apoptosis of β -cells were suppressed by the expression of c-Flip, a molecule known to inhibit caspase-8-mediated apoptosis (Maedler et al., 2002), which sheds light into the importance of caspase-8 in apoptosis induction in β cells. There are extrinsic and intrinsic apoptotic pathways. In this study we have investigated the effect of DK on two of the caspases, caspase 8 and caspase 3. Caspase-8 is an upstream caspase involved in the extrinsic pathway, whereas caspase-3 is an effector caspase downstream of both pathways. Caspase 8 initiates apoptosis and caspase 3 is an executioner. Both caspases were up regulated indicating the apoptotic death of β cells in STZ diabetic rats. Administration of DK reversed this up regulation of the caspase 3 and 8 pointing to the possibility of one or other components of it showing anti apoptotic effect. The action might be directly on the caspase expression or indirectly through the removal of the oxidative stress. Further studies are needed to establish the real nature of the action of DK.

8.4: Conclusion

We have observed the hyped expression of all the genes studied in diabetic rats and this hype in expression is mitigated by the administration of DK. These genes are activated by different cytokines and some of which are activated through NF- κ B. The increased expression of NF- κ B observed might have up regulated the expression of other genes. iNOS is another gene activated by NF- κ B. This activation is the main underlying cause of diabetic complications. DK increased insulin production which reduced blood glucose level as well as antioxidant stress which together might have helped to inhibit the expression of the major genes involved in the development of diabetic complications. Hypoinsulinemia, hyperglycaemia and oxidative stress and the expression of the genes involved in the development of diabetic complications are interrelated. They affect each other and produce the diabetic phenotype. Proper management of these factors are important in the treatment or prevention of diabetes and this could be achieved by the administration of Ayurvedic drugs like DK.

Chapter 9

Effect of Stem Enhance 2 (SE2) on diabetic rats

9.1: Introduction

Health maintenance and disease prevention are cost effective ways to ensure better quality of life. This could be achieved through the consumption of high-quality nutrients, probiotics, dietary supplements, phytochemicals that are known to have stem cell enhancing properties and also by incorporating nutrient-dense fruits and berries (Drapeau et al., 2019). Along with various other mechanisms, these bioactive natural substances work wonders in the body through the mobilisation of stem cells from the various niches in the body. Scientists have already identified several pharmaceuticals and natural substances with the ability to mobilise adult stem cells (ASCs) from human bone-marrow niches. The possibility of homing and utilising the mobilised ASCs for the repair of damaged tissue and producing the desired tissue repair or regeneration are extensively and successfully evaluated (Ismail et al., 2013).

Scientists have been working on the potential use of mobilized in situ adult stem cells (ASCs) as a noninvasive form of stem cell treatment. Many researches have led to the idea that endogenous or in situ ASCs can be mobilized from their niches (inhabitant areas) in the body. The result is migration of ASCs to various organs where their homing and resultant tissue repair or regeneration occur (Redondo et al., 2017). This noninvasive method has an obvious advantage over the processes

of harvesting and reintroduction of ASCs. It also represents a new horizon in stem cell based treatments that could make stem cell therapies more portable and cost limited. Several natural substances and pharmaceuticals have the ability to mobilize adult stem cells from the human bone-marrow. Stem cell enhancer (SE2) is one of this kinds of natural product developed from *Aphanizomenon flos aquae* (AFA). This product was reported to enhance the mobilization of the stem cells from the bone marrow to the damaged tissue and help for regeneration or tissue repair (Ismail et al., 2013).

Stem Enhance 2 (SE2) is one of the important natural product extracted from *Aphanizomenon flos aquae* (AFA). AFA is a unicellular prokaryotic microorganism included under the category of blue- green algae and belong to the phylum cyanobacteria (Nuzzo et al., 2018). Blue- green algae are one of the most nutrient rich food and consists of different types of enzymes, vitamins, minerals and also contain all essential and nonessential amino acids. In addition to it, this also have antibacterial, antioxidant properties and glucose and cholesterol regulatory effects (Sanaei et al., 2015). Numerous previous studies had already proved that it has beneficial effects in promoting the health and also controlling various diseases in humans (Parikh et al., 2001). Stem Enhance tablet is enriched with the cytoplasmic and cell wall fractions of AFA plant and also contains L-selectin ligand (LSL) (Ismail et al., 2013). This ligand helps the release of stem cells (CD34+ cells) from the bone marrow and its effect was detected on bone marrow stem cell mobilization (Jensen et al., 2007). One of the most important

characteristics of SE2 is that it enhances the stem cells homing in animals (Ismail et al., 2013).

Supplementation with AFA extract was reported to increase the total number of CD34⁺ peripheral cells (Merino et al., 2020). Ability of Hematopoietic Stem cells (HSCs) to self-renew and differentiate into various lineages of hematopoietic progenitor cells is well reported. CD34, a transmembrane protein expressed almost exclusively on certain types of stem cells is a marker of hematopoietic stem cells. CD34 is expressed on certain types of immature hematopoietic colony-forming cells in bone marrow (Drapeau et al., 2019).

Significant reduction of total circulating CD34⁺ cells were reported in Type 2 diabetes. (Fadini et al., 2007). These CD-34⁺ cells are more generic and immature population of progenitor cells originated from bone marrow and could be of endothelial or nonendothelial progenitors. (Yeh et al., 2003). Decrease in the cell count of CD34⁺ was reported to increase cardiovascular risks in patients with metabolic disorders (Fadini et al., 2009) and its role in type 2 diabetic patients are critically assessed. Many drugs that may influence progenitor cells are in use for the treatment or prevention of diabetes (Fadini et al., 2010). Peripheral-blood CD34⁺ are extensively used as the main source of repopulation of hematopoietic stem cells (HSCs), which is helping us to replace bone marrow (BM) cells for autologous/allogenic transplantation (Viswanathan et al., 2017).

Phytochemicals served as base or prototype for many modern drugs. There is a surge in the search for newer and more effective

drugs among the various phytochemicals which resulted in the assessment of many herbs used in traditional medicine as well some unidentified ones using modern techniques. Many plant or seaweed derived drugs are in use now a days for the treatment of diseases which have no permanent cure (Renganathan et al., 2019, 2020, Scarpa ES and Ninfali P, 2015). Search for effective drugs with less side effects among phytochemicals has gained momentum recently and many of them are being systematically tested on cell lines as well as on animal models and also are in different stages of clinical trials. In spite of that, screening for effects of phytochemicals on stem cells are scantily reported.

The potential of stem cells for the treatment of degenerative diseases are well understood by the scientific world, but the expense involved in their use are too high to be afforded by common people, which limit their use in therapeutic tissue engineering. More over growth factors and other biochemicals used for the mobilisation and differentiation of stem cells may induce malignant transformation of cells in due course of time (Volarevic et al., 2018). On the other hand, the use of phytochemicals for stem cell recruitment and functional transformation could avoid the chance of this type of malignant transformation (Raghavan et al, 2015). The scientific use of phytochemicals for stem cell mobilisation and differentiation and their use for the treatment of degenerative diseases is still in its infancy. There is still a long way to go for defining the ways of manipulation of stem cells for therapeutic use and to get a clear idea about structure-activity relationship of phytochemicals and stem cell differentiation.

SE2 is another plant derived compound which has action on stem cells. Likewise DK also may have similar effects as both of them share some common phytochemical as well as physiological properties such as rich in phytochemicals, antioxidant activity, rejuvenating effect, hypoglycemic and hypolipidemic effect etc. This research analysed the effect of SE2 on diabetic rats as a comparative tool to evaluate the effect of DK on diabetic rats and the extent of the involvement of stem cells for diabetic cure.

Various parameters associated with diabetes, body weight, blood glucose level, serum insulin level, expression of certain diabetes related gene etc were studied. Effect of SE2 on lipid peroxidation was assessed by analysing the level of MDA in different tissues of the rats administered various doses of SE2. Possible migration of bone marrow stem cells to the pancreas of diabetic rats after SE2 administration was assessed by RT PCR of CD34⁺ in peripheral blood. Gene specific primers designed using the mRNA sequence from the GenBank accession number XM_008769857.2 (David et al., 2019) were used for the PCR. The observations in this study points to the possibility of DK mobilising stem cells and aid in the regeneratin of pancreatic islets.

9.2: Results

9.2.1: Effect of SE2 on serum insulin levels

Streptozotocin induced diabetes resulted in a significant decrease in the serum insulin levels in the diabetic rats in comparison to normal control rats. In diabetic rats, it dropped by 53.77% (from 18.28 μ U/ml

to 8.45 μ U/ml). Administration of SE2 slightly increased its levels in both diabetic and normal rats, in a dose dependent pattern. In diabetic rats, the increases induced by low, moderate, and high doses were only 6.1, 9.7 and 25.20% respectively. The increases were significant only in the dose C fed rats, ($p < 0.05$). On the other hand, in non-diabetic rats the increase in serum insulin level was not significant in any of the groups.

Table 9.2.1: Serum insulin levels on 21st day in different groups of rats

Sl.No	Groups	Serum
1	NC	18.28 \pm 0.654**
2	DC	8.45 \pm 0.48 ^a
3	DSE2A	8.97 \pm 0.66 ^a
4	DSE2B	9.27 \pm 1.12 ^a
5	DSE2C	10.58 \pm 1.74 ^{a*}
6	CSE2A	18.59 \pm 1.12**
7	CSE2B	18.85 \pm 1.36 ^{b**}
8	CSE2C	19.85 \pm 1.43**

Showing the Serum insulin level in the rats of control and different experimental groups. Serum insulin was expressed in μ u/ml. Values are expressed as mean \pm SD of six rats. 'a' indicates values are significantly different from Normal Control rats with $p < 0.001$. 'b' indicates values are significantly different from Normal Control rats with $p < 0.05$. '**' indicates values are significantly different from Diabetic Control rats with $p < 0.001$. '*' indicates values are significantly different from Diabetic Control rats with $p < 0.05$.

Table 9.2.2: Body weight of different groups of rats during the experimental period

Sl.No	Groups	0 th Day	7 th Day	14 th Day	21 st Day
1	NC	209.65±7.44	212.49±9.09*	211.79±7.20**	216.64±7.69**
2	DC	189.67±7.09	157.45±6.74 ^a	149.75±5.09 ^a	138.77±4.93 ^a
3	DSE2A	186.84±6.45	162.70±7.03 ^b	154.15±7.63 ^a	142.82±8.46 ^a
4	DSE2B	179.62±6.56	160.74±7.28 ^b	153.87±8.66 ^a	144.46±8.57 ^a
5	DSE2C	186.94±7.36	167.78±7.85 ^b	159.90±6.15 ^a	151.54±8.46 ^a
6	CSE2A	189.65±6.38	192.28±6.95**	195.38±7.29**	198.35±8.36**
7	CSE2B	192.86±6.86	196.75±7.79**	200.81±7.66**	203.64±7.54**
8	CSE2C	201.56±7.36	204.43±7.88**	207.25±7.57**	210.77±7.56**

Showing the change in body weights of rats belonging to different groups during the study period expressed in grams. Values are expressed as mean ± SD of six rats. ‘a’ indicates values are significantly different from Normal Control rats with $p < 0.001$. ‘b’ indicates values are significantly different from Normal Control rats with $p < 0.05$. ‘**’ indicates values are significantly different from Diabetic Control rats with $p < 0.001$. ‘*’ indicates values are significantly different from Diabetic Control rats with $p < 0.05$.

Though not significant, the NC rats gained weight during the experimental period, but in diabetic rats, body weight gradually decreased throughout the entire experimental period with a total accumulated decrease of 26% on the 21st day. Feeding with SE2 did not make a significant change in the body weight loss due to diabetes though it exerted minor effect. In the case of SE2 fed diabetic rats, the decrease in body weight was lesser and effect of SE2 was concentration dependent. The lowest dose (dose A) fed rats lost 23% of body weight in 21 days, whereas in dose B fed rats the decrease in weight was only 19% and in dose C fed rats diabetic rats the loss was only 18%. Though it was not significant, the SE2 administration was beneficial to the diabetic rats as there was an 8% decrease in weight loss.

Table 9.2.3: Fasting Blood Sugar Level

Sl.No	Groups	0 th Day	7 th Day	14 th Day	21 st Day
1	NC	120.20±3.51**	115.81±4.11**	109.83±3.54**	115.83±10.57**
2	DC	463.77±13.55 ^a	515.14±18.29 ^a	533.11±18.93 ^a	542.23±49.48 ^a
3	DSE2A	485±17.46 ^a	505±19.85 ^a	512.15±19.81 ^a	496.52±49.32 ^a
4	DSE2B	463.21±22.98 ^a	472.54±19.90 ^a	448.53±18.65 ^a	435.9±23.57 ^a
5	DSE2C	445.63±22.98 ^a	443.76±19.89 ^a	448.24±18.65 ^a	435.9±23.57 ^a
6	CSE2A	138.85±4.73**	139.83±4.58**	132.51±4.42**	126.78±6.54**
7	CSE2B	135.61±4.08**	138.41±4.396**	127.61±4.32**	125.61±8.97**
8	CSE2C	132.64±3.49**	127.86±4.537**	120.2±4.85**	118.8±9.32**

Fasting blood sugar in the rats of different groups at different time points during the experimental period. Fasting Blood Sugar was expressed in mg/dl. Values are expressed as mean ± SD of six rats. ‘a’ indicates values are significantly different from Normal Control rats with p<0.001. ‘b’ indicates values are significantly different from Normal Control rats with p<0.05. ‘**’ indicates values are significantly different from Diabetic Control rats with p<0.001. ‘*’ indicates values are significantly different from Diabetic Control rats with p<0.05.

Blood sugar level significantly increased in DC rats (P<0.001) as compared to NC rats. SE2 administration was found to decrease the blood sugar level in both diabetic and normal rats. Blood sugar in DC rats remained significantly high during the experimental period. In the case of SE2 fed diabetic rats there was a decrease in the blood sugar level, but the decrease was not significant. Fasting blood sugar (FBS) of normal control rats over the course of the 3 weeks was not much different and they are not significantly different from one another. Diabetic control rats showed steep increase in FBS level and at the end of the experimental period FBS level was increased to 542.23 mg/dl (17% of the level on day 0 in diabetic rats). In dose A fed diabetic rats SE2 administration did not make any decrease in FBS level in the first

two weeks and a small decrease happened in the third week. In the case of dose B fed diabetic rats FBS level was increased in first week and after that it was decreased. In dose C fed diabetic rats SE2 decreased FBS level and at the end of the experimental period FBS level was 435.9 mg/dl. But the decrease was not significant ($p < 0.05$). SE2 fed normal rats showed decrease in FBS level on day 21 and this decrease was also not significant.

Table 9.2.4: MDA level in the different tissues of the rats of different groups on 21st day.

Sl.No	Groups	Kidney	Liver	Heart	Serum
1	NC	1.55±0.07	1.36±0.06	0.84±0.04	0.71±0.04
2	DC	3.27±0.16 ^a	1.84±0.09 ^b	1.7±0.08 ^b	0.93±0.05 ^b
3	DSE2A	3.01±0.15 ^a	1.8±0.08 ^b	1.69±0.07 ^b	0.91±0.04 ^b
4	DSE2B	2.97±0.14 ^b	1.78±0.07 ^b	1.62±0.07 ^b	0.88±0.04
5	DSE2C	2.53±0.15 ^{b*}	1.63±0.05 [*]	1.58±0.06 ^b	0.85±0.05
6	CSE2A	1.48±0.06 [*]	1.32±0.03 [*]	0.82±0.04 [*]	0.68±0.03 [*]
7	CSE2B	1.5±0.07 [*]	1.29±0.03 [*]	0.79±0.04 [*]	0.64±0.03 [*]
8	CSE2C	1.32±0.05 [*]	1.27±0.02 [*]	0.76±0.03 [*]	0.59±0.03 [*]

Showing the level of MDA in different tissues of control rats and rats exposed to different treatments MDA is expressed in mM/100gm tissue or dL serum. Values are expressed as mean ± SD of six rats. ‘a’ indicates values are significantly different from Normal Control rats with $p < 0.001$. ‘b’ indicates values are significantly different from Normal Control rats with $p < 0.05$. ‘***’ indicates values are significantly different from Diabetic Control rats with $p < 0.001$. ‘*’ indicates values are significantly different from Diabetic Control rats with $p < 0.05$.

MDA level was increased significantly ($p < 0.05$) in diabetic rats as compared to normal control rats. Highest increase was observed in kidneys (110%), followed by heart (101%), liver (35%) and serum (30%). Administration of SE2 decreased MDA level in all tissues of diabetic and normal rats in a dose dependent manner. Significant

decrease in MDA level was only observed in the kidneys of SE2 administered (dose C) diabetic rats. liver and serum MDA levels also decreased due to SE2 administration in a dose dependent manner, but the decrease was not significant. Administration of SE2 to normal rats also decreased the levels of MDA in the liver, heart and serum, but the decrease was not significant in any of the tissue.

9.2.5: NO levels in the liver of rats of different groups

Figure 9.2.1: NO levels in the liver of rats of different groups

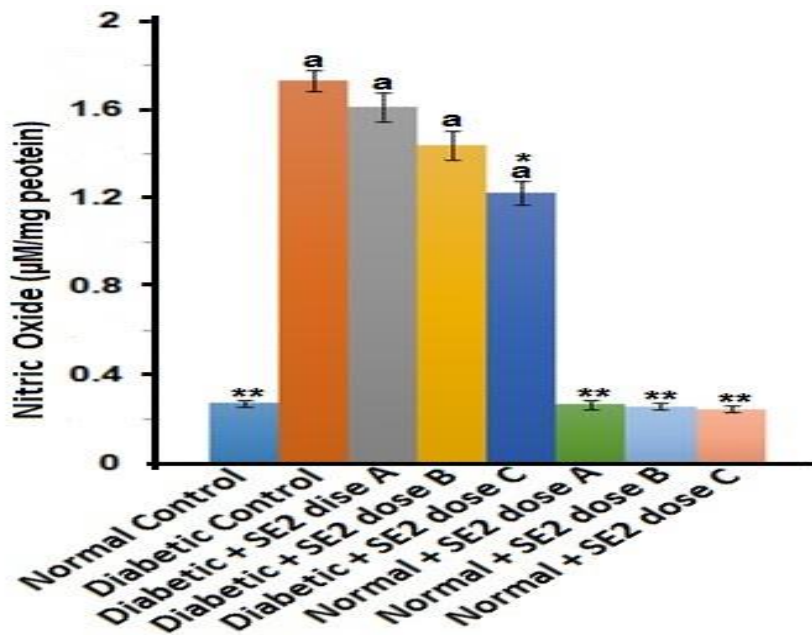


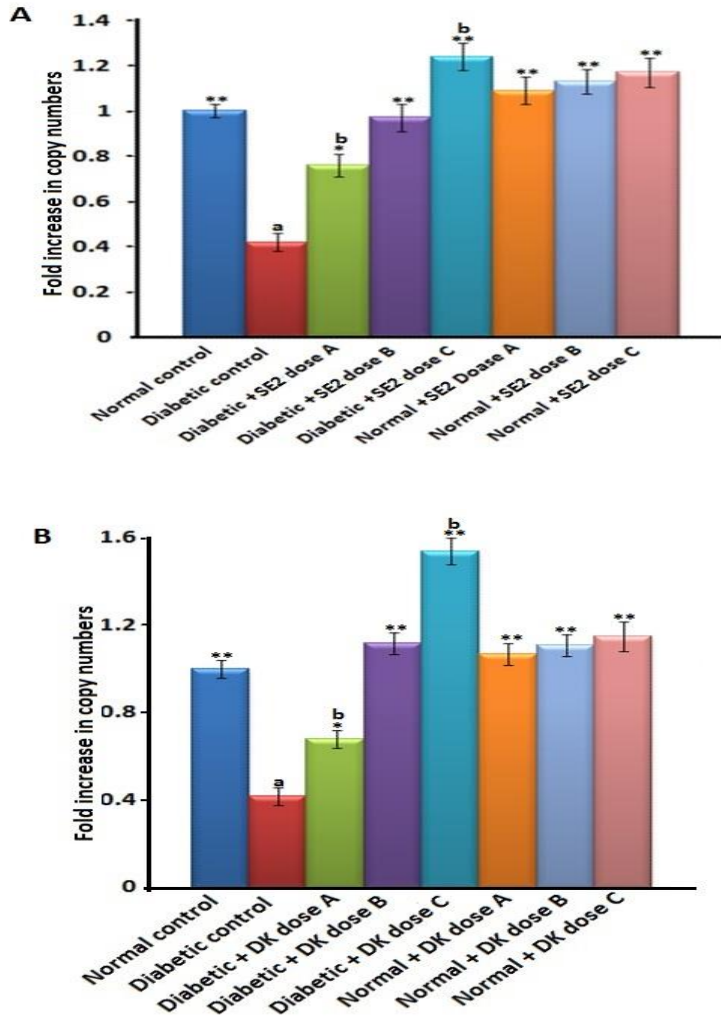
Figure 9.2.1: Graph showing the levels of NO in the liver tissue of the rats of different groups. The results were expressed as μmol nitrite/mg protein content of the samples. Values are expressed as mean \pm SD of six rats. 'a' indicates values are significantly different from Normal Control rats with $p < 0.001$. 'b' indicates values are significantly different from Normal Control rats with $p < 0.05$. '**' indicates values are significantly different from Diabetic Control rats with $p < 0.001$. '*' indicates values are significantly different from Diabetic Control rats with $p < 0.05$.

The levels of NO in the liver tissues of diabetic rats were elevated significantly ($P < 0.001$) in comparison to control rats. SE2 administration decreased the NO levels in the diabetic rats in a dose dependent manner. The decrease was significant only in the dose C fed rats, however, it was still higher than that in the control rats ($P < 0.001$). SE2 administration failed to produce any effect in the NO levels in normal rats.

9.2.6: Mobilisation of bone marrow stem cells

The percent of circulating CD34+ve cells were assessed by studying the expression of CD34 gene by real-time PCR and was considered as an indicator of the extent of mobilization of stem cells. The expression of this gene was upregulated by the administration of SE2 (Figure 9.2.2 A) as well as DK (Figure 9.2.2 B) in a dose dependent manner both in the diabetic as well as normal rats. The effect of the highest dose of DK was slightly higher than that of SE2, but the difference between SE2 and DK was not significant. This increase in gene expression might be due to the increased number of circulating CD34+ve cells as compared to the number of circulating CD34+ve cells in control rats.

Figure 9.2.2 A&B: Showing the copy numbers of CD34⁺ gene at mRNA level in rats



Expression of CD34 gene at mRNA level in peripheral blood of different groups of rats. A: SE2 administered and B: Dhanwantaram Kashaya administered rats. Change in gene expression is shown as fold increase in copy numbers in comparison to normal animals. Values are expressed as mean \pm SD of six rats. 'a' indicates values are significantly different from Normal Control rats with $p < 0.001$. 'b' indicates values are significantly different from Normal Control rats with $p < 0.05$. '**' indicates values are significantly different from Diabetic Control rats with $p < 0.001$. '*' indicates values are significantly different from Diabetic Control rats with $p < 0.05$.

9.3: Discussion

The present investigation studied the effect of SE2 in STZ induced diabetic and normal rats. Oxidative stress plays an important role in pathogenesis and progression of type-I and type- II diabetes. Elevated levels of free radicals declined endogenous enzymes and there by stimulated lipid peroxidation in our body (Mahajan et al., 2013). Various chemicals or other preparations of natural origin and are reported to have beneficial impact on human body especially due to their antioxidant, hypoglycaemic, hyperinsulinemic, hypolipidemic and the ability to mobilise stem cells are being extensively used for the treatment of diseases like diabetes. Lipid peroxidation is one of the main reason for production of free radicals and oxidative stress in diabetes mellitus (Halliwell et al., 1994). MDA is the major marker of lipid peroxidation and elevated MDA level is associated with impairment of enzymatic and non- enzymatic antioxidant defense mechanisms (Sameni et al., 2016). In agreement with previous reports we have also observed an increase in MDA level in diabetic rats. This shows the increased lipid peroxidation in diabetic rats. Administration of SE2 decrease MDA level in a dose dependent manner which is in agreement with previous reports. The ability of SE2 to decrease MDA level in diabetic rats is comparatively lesser than the ability of DK in this respect. The DK decreased the MDA level near to normal value in dose C fed diabetic rats.

Concentration of MDA and FBS levels were increased in diabetic rats and administration of SE2 decreased both these parameters in DC and

NC rats. But the decrease was not significant. Several previous studies had already suggested the ability of many of the pharmaceuticals and natural substances to mobilize adult stem cells (ASCs) from human bone-marrow. These mobilized ASC can home into damaged tissue and helps for tissue repair and regeneration. Damaged tissues initiate complex signals that attract migrating regenerative stem cells (Jensen and Drapeau., 2002). Stem Enhance is a natural product extracted from *Aphanizomenon flos aquae* (AFA) plant. This formulation have the ability to mobilize adult stemcells from human bonemarrow because of the presence of L-selectin ligand and it helps to release stem cells (CD34+ cells) from the bonemarrow (Jensen et al., 2007).

Other studies in human also reported significant increase in the circulating percentage of CD34+ve cells after the consumption of one gram of StemEnhance. Mobilisation of BM CD34+ve cells was related to L-selectin ligand contained in StemEnhance through the down regulation of CXCR4 chemokine receptor. Interruption in the binding of SDF-1 with CXCR4 chemokine receptor through the L-selectin in SE2 might have lead to the mobilisation of CD34+ve stem cells. Binding of SDF-1 to CXCR4 leads to the externalization of adhesion molecules that anchor the stem cell in the bone marrow. Here SE2 interferes with CXCR4 binding and reduce the adhesion of stem cells to the bone marrow which could help in the mobilisation of stem cells (Ismail et al 2013).

The total number of peripheral CD34+ cells after supplementation of DK and SE2 to diabetic rats increased. Both the formulation contains

several antioxidants (Renganathan et al., 2020 (a) and (b), Merino et al., 2020, Ismail et al., 2013), which could synergistically have contributed to increase in the stem cell (SC) mobilisation. This observations concur with the various reports on the protective effects shown by different antioxidants (Saheera S et al., 2019).

In our study supplementation with SE2 as well as DK increased the total number of CD34+ve peripheral cells, which concur with enhanced HSC mobilization observed with the supplementation of cyanophyta *Aphanizomenon flos-aquae*. This induced mobilisation was reported beneficial in severe human diseases like cardiomyopathy, diabetes, rheumatoid arthritis, kidney failure and Parkinson disease (Drapeau et al., 2019, Aksoy et al., 2003, Merino et al., 2020).

A growing amount of studies have demonstrated decreased levels of circulating stem cells during type I and type II diabetes (Fujimaki et al., 2015, Fadini et al., 2006). Our observation of increased oxidative stress in diabetic rats is in agreement with previous reports. This increased oxidative stress leads to the decrease in the mobilisation of stem cells from bone marrow which in turn reduce numbers of circulating endothelial progenitor cells (EPCs). (Tousoulis et al., 2008). Increase in oxidative stress during diabetes has been well reported (Aksoy et al., 2003, Yao et al., 2006). Increasing evidences suggested the influence of the oxidative stress during diabetes in mediating the mobilisation of stem cells from the bone marrow of experimental animal models and humans (Qiu et al., 2012).

Involvement of endothelial nitric oxide (NO) synthase through the increased production of NO help in the mobilisation of stem cells or progenitor cells from the bone marrow. NO is produced in the bone marrow stroma by the cells in the vascular walls. This NO acts in a paracrine manner and induce mobilisation of stem cells. This leads to the increase in the number of circulating stem cells (Aicher et al., 2003, 2004). We have already reported increased levels of NO in different tissues of diabetic rats (Renganathan et al., 2020). The administration of SE2 as well as DK decreased the NO levels with DK exerting a much higher effect. This increased NO concentration might have initiated cascade of reactions leading to diabetic complications.

NO was found to mediate the action of cytokines like TNF α (Maciejewski et al., 1995, Punjabi et al., 1992), which is noteworthy in the light of enhanced level of this cytokine in diabetic rats which was cancelled by the administration of DK. Inhibition of NOS activity *in vivo* was reported to increase the number of stem and early progenitor cells in the BM (Michurina et al., 2004). Administration of SE2 and DK suppressed the increase in the NO level and also observed an increase in the circulating CD34⁺ cells. These observations clubbing with the reports of NO as an important regulator of hematopoietic stem cells indicate the possibility to expand the number of cells in the stem cell pool by suppressing the activity of NOS.

Decrease in the NO production by the administration of these phyto-products might have contributed the amelioration of diabetic

complications and one of the ways might be by the mobilization of stem cells from bone marrow or from different tissue niches (Michurina et al., 2004).

Increased oxidative stress indicated by decreased SOD and increased MDA observed in diabetic rats would also have resulted in impaired mobilisation of SCs from the bone marrow and decrease in their number in peripheral circulation (Que X et al., 2012). We have observed the mitigation of oxidative stress and other diabetic complications in diabetic rats fed with DK and SE2. Possible explanations include; SE2 and one or other components of DK acts as antioxidant or exert antioxidant effect through the activation of major antioxidant enzymes, including SOD. We already have reported (Renganathan et al., 2020) the role of DK as antioxidant and its ability to mitigate the damage induced by various free radical-generating processes.

We have not studied the antioxidant effect on bone marrow, but as the anti-oxidative effect exerted by DK or SE2 is systemic and would have affected bone marrow too. So the demonstrated increase in the circulating stem cells might be due to the antioxidant property of these compounds in the bone marrow.

Administration of DK and SE2 reduced oxidative stress level in diabetic rats. Attenuated oxidative stress is one of the possible mechanisms involved in increased circulating SCs in DK/SE2-treated diabetic rats, as oxidative stress has a significant role in mediating stem cell mobilisation from bone marrow. DK or SE2 treated diabetic rats

had enhanced level of insulin and reduced blood glucose levels. Increased oxidative stress and the resultant metabolic as well as histologic changes in pancreatic islets would have helped the homing of the circulating SCs in the islets and this might have caused the formation of new β cells. New β cells should have produced more insulin which resulted an increased level of blood insulin. This improved insulin level helped in glucose homeostasis. It is interesting to note that DK exerted a better effect by reducing the FBS and increasing serum insulin levels than SE2. This enhanced effect was visible on body weight as well as on other diabetic complications like hyperglycemia, hyperlipidemia, diuresis, food intake etc. By day 21 the body weight of DK fed rats reached near normal, but SE2 administration did not make such a noticeable effect. In the case of SE2 dose C only produced a noticeable effect on body weight.

Here in this study both DK and SE2 are found to improve the carbohydrate metabolism in diabetic rats. They are also found to have a beneficial impact on the histology of pancreas with improved islets structure and function. This had improved the synthesis of insulin and hence a better glucose homeostasis. Though both have positive effects, DK is found slightly better than SE2. DK improved the blood glucose level within three weeks, but SE2 was not that efficient. Even after two weeks SE2 did not produce much decrease in the blood glucose level.

The potential effect of natural products with antioxidant effects like DK or SE2 on improving the mobilisation of SCs from bone marrow

could provide an alternative opportunity for the treatment of diabetes and associated derangements or cardiovascular disease in humans.

Empirical observations of these studies suggest the possibility of improvement in disease conditions through the long-term consumption of DK and SE2 with DK offering a better effect. This might bring significant improvement in different health conditions including degenerative diseases like diabetes, kidney insufficiency, chronic obstructive pulmonary disease etc.. However rigorous studies are needed to examine the real effect and other related side effects.

9.4: Conclusion

SE2 and DK were found to exert different beneficial impact on diabetic rats and the DK was found more efficient in ameliorating diabetic complications. In conclusion, observations in this study reveal the possibility of altering the proportion of stem cells through simple methods like consumption of herbal extracts. The extent to which these extracts exert their influence on the number and proportion of the circulating stem cells and how far they are beneficially utilised by the body for the regeneration of damaged tissue and the extent to which different degenerative diseases are cured need further investigations and are on the run.

Effect of Bone Marrow Stem cells in diabetic rats

10.1: Introduction

Stem cells are unique cell types having the ability to differentiate into any type of specialized cells under appropriate microenvironment. Depending upon the environment and requirement of the organism, stem cells divide rapidly and may take two growth pathways. In the first pathway the newly produced stem cell remains as the same type of stem cells continuing the lineage and in the second pathway the cell undergo further differentiation to become a more specialized cell with specific function. Stem cells are rapidly divided in certain organs such as gut and bone marrow where there is a need for repair and replace the damaged cells or form new type of cells like blood cells, but in organs like heart and pancreas stem cells remain as a resident cell and divide only under certain specific conditions (Abdulazeez., 2015).

Stem cells are unspecialized cells and have self- renewal ability. Based upon their potential ability stem cells are classified into several types such as unipotent stem cell, totipotent stem cells, pluripotent stem cells, multipotent stem cells and oligopotent stem cells (Kalra and Tomar., 2014). Unipotent stem cells have the ability to differentiate into only one type of cell and repeated division is one of the important characteristics of this type of cells. Dermatocytes are the best example of unipotent stem cells (Zakrzewski et al., 2019). Totipotent stem cells

divide and differentiate to form the whole organism. They have the ability to form embryo and extra-embryonic structures and zygote is the example for this. Unipotent stem cells have the narrowest and totipotent stem cells have the highest differentiation potential ability. Pluripotent stem cells have the capacity to form all germ layers but not have the ability to form extra embryonic structures (Zakrzewski et al., 2019). Embryonic stem cells are example for this. Multipotent stem cells have the ability to form different cell types and it has a narrower spectrum of differentiation than pluripotent stem cells. They can specialize in discrete cells of specific cell lineages and hematopoietic stem cell is the example for it (Zakrzewski et al., 2019). After differentiation hematopoietic stem cells becomes oligopotent stem cells and differentiation abilities are restricted to cells of its lineage. Example myeloid stem cell and they can divide to form only white cell types not red blood cell types (Zakrzewski et al., 2019).

Mesenchymal stem cells (MSCs) are multipotent stromal cells, play a crucial role in the development of different stem cells *in vivo* (Krebsbach et al., 1999). It has the ability to enhance the development and function of various cell types and isolated from variety of sources including bone marrow and various human organs (Busser et al., 2015, Moroni and Fornasari., 2013). It also has self- renewal ability and immunomodulatory effects. Because of these reasons MSCs are widely used and ideal for stem cell therapy of various diseases (Rasmusson et

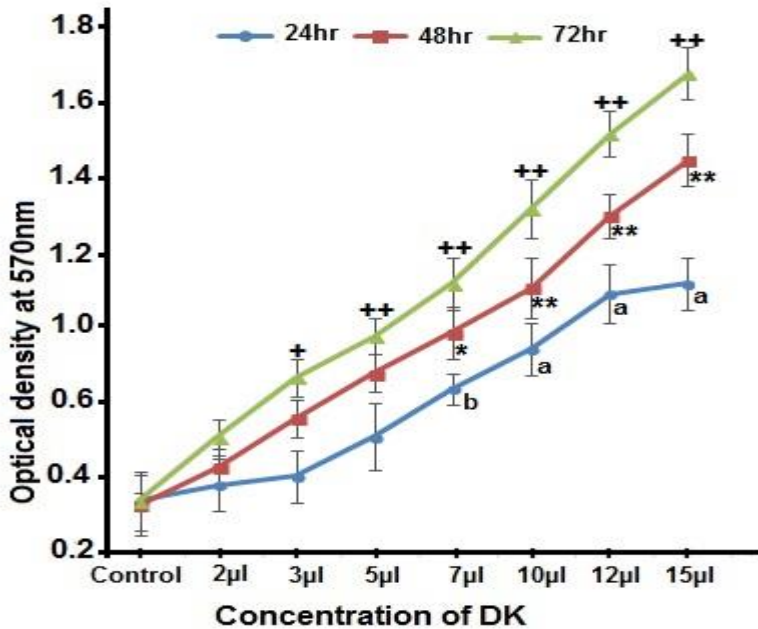
al., 2003, Gao et al., 2016, Papazova et al., 2015, Zhang and He et al., 2019).

Bone marrow mainly consists of two types of cells including hematopoietic stem cells and mesenchymal stem cells (Pittenger et al., 1999, Colter et al., 2000). Bone marrow derived stem cells can be differentiated into pancreatic β cells (Li and Ikehara., 2014). Yang in 2006 reported the differentiation potential of hepatic stem cells expressing duodenum homeoboxprotein-1 to form pancreatic β cells, and they improving hyperglycemia in diabetic mice (Yang., 2006). Bone marrow cells have the ability to differentiate into functionally competent β - cells *in vivo* (Janus et al., 2003). Insulin like growth factor 1 (IGFI) is the important morphological factor secreted by MSCs (Chen et al., 2008) and it mediate the proliferation and differentiation of pancreatic β - cells (Lingohr et al., 2002). Present study was planned to evaluate the effect of DK on bone marrow derived MSCs *in vitro* and also to determine the ability of these cells to home in pancreas of diabetic rats. Effect of IV injection of BMSCs on hyperglycemia and hypoinsulinemia observed in diabetic rats was also studied along with the effect of this injection of BMSCs on the blood cell composition.

10.2: Results

10.2.1: Viability of bone marrow cells

Figure 10.2.1: Viability of bone marrow cells by MTT method treated with increasing doses of DK.



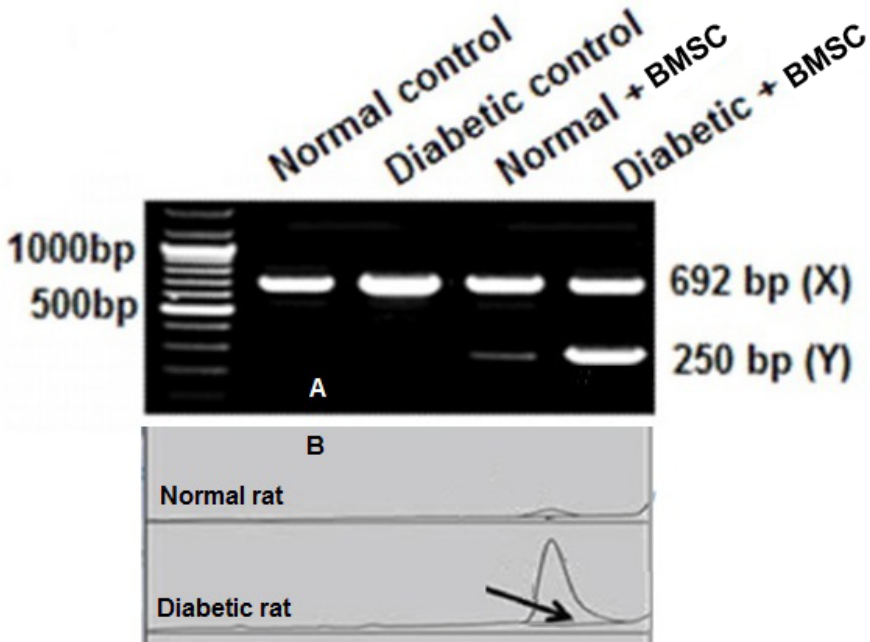
Graphical representation of the change in the optical density observed in cell viability test by MTT of BMSC cells treated with increasing doses of DK. Values are the mean of six estimations. Error bars indicate the Standard Deviation (+SD). “a” indicates values are significantly different from control with $p < 0.001$ for 24hr DK exposure and “b” indicate significance level at $p < 0.05$ for the same doses and duration. “*” indicates values are significantly different from control with $p < 0.001$ for 48hr DK exposure with “**” indicate significance level at $p < 0.05$ for the same dose and duration and “++” indicates values are significantly different from control with $p < 0.001$ for 72hr DK exposure and “b” indicate significance level at $p < 0.05$ for the same doses and duration.

Bone marrow cells isolated and cultured as per the protocol described in materials and methods section. After 8 passages the cells were plated (4000 cells/ well) in 96 well culture plates and were treated with different doses of DK for varying duration (24, 48 and 72hr). Density of viable cells were determined by MTT assay. There was increase in the OD of the wells having BMSC cells treated with different doses of DK. Significant increase in the number of viable cells was observed in all the wells treated with DK. The increase in the number of viable cells indicated an increased proliferation of the BMSC cells on treatment with DK. The increase in proliferation was dose dependent.

10.2.2: Determining the presence of bone marrow cells in pancreas by PCR

Homing of injected bone marrow cells in pancreas was confirmed by PCR for “Y” chromosome using the primers described in the materials and methods. Bone marrow cells from male rats were injected into female rats. Tissues invaded by male bone marrow cells will show positive signal for “Y” chromosome. PCR results reported the presence of injected bone marrow cells in the pancreas of diabetic as well as normal rats. In the normal pancreas the intensity of PCR band was too light and thin whereas in pancreas of diabetic rats the signal strength of the PCR product was very thick and bright.

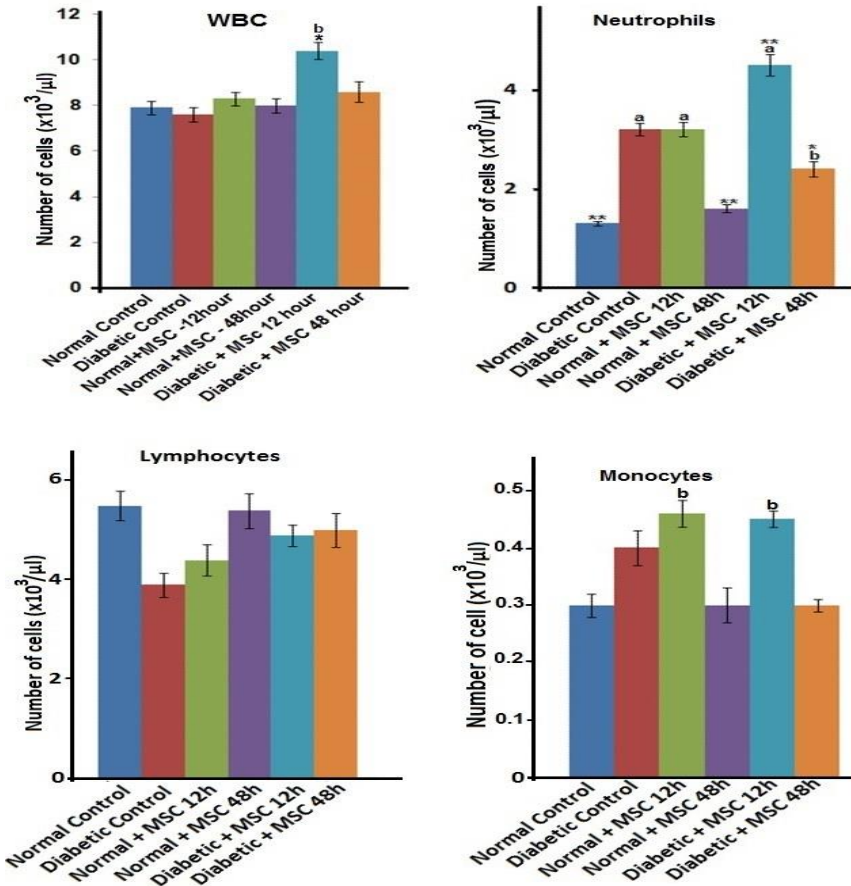
Figure 10.2.2: Determining the presence of bone marrow cells in pancreas by PCR



A. Gel electrophorogram showing the PCR bands of X chromosome, Y chromosome and 1Kb ladder. B. Showing the intensity peak of the corresponding PCR bands for Y chromosome protein in Figure A obtained by using “ImageJ” software (NIH). The peaks depict the densities of the PCR bands for Y and X chromosome in BMSC injected diabetic rats. The arrow indicates the line that specifies the area of measurement within the peak. The PCR band density is automatically determined by the ImageJ software using the specified area.

10.2.3: Blood cells

Figure 10.2.3: Change in the number of different blood cells in response to the injection of bone marrow stem cells (MSC).



Showing the change in the number of different blood cells in response to the injection of bone marrow stem cells (MSC). Values are expressed as average (\pm SD) of six estimations. The number of blood cells after 12 and 48hr of BMSC was determined microscopically. “***” indicates significance in comparison with diabetic rats at a significant level of $p < 0.001$ and “**” indicates significance in comparison with diabetic rats at a significant level of $p < 0.05$. “a” indicates significance in comparison with normal rats at a significant level of $p < 0.001$ and “b” indicates significance in comparison with diabetic rats at a significant level of $p < 0.05$.

To assess if there are any variations in the blood cell populations due to intravenous injection of BMSCs, the blood was collected from the tail vein before and 12 and 48 hr after BMSC injection. There were noticeable changes in the number of different types of WBCs 12hr after BMSC injection; however this change was found reversed after 48hr of injection. A decrease in the number of WBC and lymphocytes were observed in diabetic rats, whereas the number of neutrophils increased significantly in diabetic rats. The increase observed in the number of monocytes in diabetic rats was not significant. BMSC injection did not produce any significant difference in the number of WBC in normal rats. The number of neutrophils was found increased in diabetic rats as well as normal rats after 12h of BMSC injection. Neutrophil levels in both the groups decreased to normal level after 48h of BMSC injection. Lymphocyte numbers decreased in diabetic rats as well as after 12h of BMSC injection in normal rats. This decrease in the number of lymphocytes in normal rats due to BMSC injection was found normalized after 48h of injection. Injection of BMSC increased the number of lymphocytes in the diabetic rats to near normal value. After 48h of BMSC injection there was no significant difference in the number of monocytes in normal as well as diabetic rats from the normal.

10.2.4: Change in body weight of rats treated with bone marrow stem cells

Table 10.2.1: Change in body weight of the rats of different groups during the experimental period.

Sl. No	Groups	0 th Day	7 th Day	14 th Day	21 st Day
1	NC	208.65±8.44 ^{**}	214.49±709 ^{**}	216.79±8.20 ^{**}	218.64±8.69 ^{**}
2	DC	201.74±8.15 ^a	159.55±7.74 ^a	147.75±6.09 ^a	134.77±5.93 ^a
3	DC+BMSC	187.45±7.56 ^{a**}	177.70±7.03 ^{a**}	182.13±5.26 ^{a**}	186.84±5.62 ^{a**}
4	NC+BMSC	191.64±7.56 ^{b**}	195.71±7.51 ^{b**}	196.73±7.82 ^{a**}	199.94±6.67 ^{a**}

Body weight was expressed in a grams. Values are expressed as average (\pm SD) of six estimations. ‘a’ indicates values are significantly different from Normal Control rats with $p < 0.001$. ‘b’ indicates values are significantly different from Normal Control rats with $p < 0.05$. ‘**’ indicates values are significantly different from Diabetic Control rats with $p < 0.001$. ‘*’ indicates values are significantly different from Diabetic Control rats with $p < 0.05$.

There was no significant change in weight between normal control and BMSC injected normal rats in weight gain. Diabetic rats lost weight by 33%. Whereas diabetic rats received BMSC injection did not show weight loss at this level. In BMSC injected diabetic rats there was an initial weight loss on day 7, but after this they started gaining weight and by day 21 the weight of these rats reached near normal level. BMSC injection prevented weight loss in diabetic rats.

10.2.5: Blood glucose level and serum insulin level in bone marrow cells injected rats

Table 10.2.2: Fasting blood glucose and insulin levels in rats of different groups on 21st day.

SL NO:	GROUPS	FBS Level (21st day)	Serum insulin levels (21st day)
1	Normal Control	115.83±10.58 ^{**}	18.43 ± 1.2
2	Diabetic Control	542.23±49.48 ^a	10.66 ± 1.54 ^a
3	Normal + BMSC	110.75±9.84 ^{**}	19.88 ± 1.86 ^{**}
4	Diabetic + BMSC	239.48±45.43 ^{a*}	14.66 ± 2.6 ^{a*}

Showing the fasting blood glucose (mg/dl) and insulin (μ u/ml) levels in rats of different groups on 21st day. Values are expressed as mean \pm SD of six rats. 'a' indicates values are significantly different from Normal Control rats with $p < 0.001$. 'b' indicates values are significantly different from Normal Control rats with $p < 0.05$. '**' indicates values are significantly different from Diabetic Control rats with $p < 0.001$. '*' indicates values are significantly different from Diabetic Control rats with $p < 0.05$.

On 21st day, high fasting blood sugar (FBS) and decreased serum insulin levels were observed in diabetic rats as compared to normal control rats. The intensity of hyperglycemia and hypoinsulinemia increased in untreated diabetic rats during the three weeks of study. A significant increase in serum insulin level was observed in diabetic rats injected with bone marrow derived mesenchymal stem cells. A concurrent decrease in FBS was also observed in rats injected with bone marrow derived mesenchymal stem cells. There was no significant difference in FBS or insulin level in normal rats injected with bone marrow derived MSCs.

10.3: Discussion

Ikehara et al., (1985) had suggested the possibility of treating autoimmune diseases by the transplantation of allergenic bone marrow without serious immune reactions. Clinical symptoms of insulin dependent diabetes mellitus (IDDM) develop as pancreatic β -cell mass decreases to 10% of normal (Lafage and Peck., 1989). Normal glucose tolerance profile and absence of insulinitis were observed after 3 months of transplanting bone marrow of BALB/C nu/nu mice into lethally irradiated 5- to 6-month-old NOD mice (Ikehara et al., 1985, Ikehara et al., 1987). Another study with 8-month-old untreated NOD mice revealed the ability of allogeneic bone marrow transplantation in preventing glucose intolerance and hyperglycemia (Yasumizu et al., 1987).

We examined the possibility of ameliorating the diabetic complications by the systemic injection of BMSCs and observed a beneficial impact. The body weight as well as physical activities of the BMSC injected rats improved. There was a decrease in the amount of urine produced as revealed by the wetting intensity of the paper bed used in the cages. The water content in the paper bed in the cages of BMSC injected rats was in par with that of the cages of normal rats. Water consumption by the diabetic rats injected with BMSCs also decreased by 25% from that of untreated diabetic rats. These beneficial changes are explained by the improved blood glucose and serum insulin levels. Ameliorating diabetic symptoms like hyperglycemia and hypo insulinemia might have improved the body metabolism and hence better protein synthesis

and metabolic enzyme activities. This caused the general improvement in the body physiology and the improved physical status.

We have observed an increase in insulin level in diabetic rats received BMSC. Insulin treatment was found to decrease nitrogen loss through urine (Atchley et al., 1933) along with increased lean body mass (Walsch et al., 1976) and decreased circulating amino acids. Previous studies had already reported anticatabolic effect especially its role in reducing whole body protein breakdown (Nair et al., 1995). *In vitro* studies had also revealed the stimulatory effect of insulin on protein synthesis (Jefferson., 1980, Proud., 1994).

The effects of BMSCs in ameliorating diabetic symptoms were studied in female diabetic rats after IV injection of the cells. The homing of male cells in pancreas tissues was evaluated by PCR amplification of the male cell-specific sex-determining region on Y-chromosome (SRY) gene (Dhakal and Soares., 2018). The intensity of the PCR bands in BMSC injected female diabetic rats were compared with BMSC injected normal rats to see the difference if any of homing. The band intensity for SRY gene was multiple times higher in diabetic rats compared to normal rats. This shows the possibility of certain changes in diabetic pancreas enhancing the homing process. Increased homing of BMSCs into the pancreas of diabetic rats compared to normal rats when both were injected with the same number of BMSC cells points to the possibility of the stem cells reaching the diabetic pancreas and differentiating into β cells in the islets. This increased number of β cells might have increased the production of insulin which

in turn had helped to reduce the FBS level. FBS level as well as serum insulin level reached near normal in BMSC treated diabetic rats. Due to lack of biochemicals as well as time, it was not possible to investigate the histological effect of BMSC treatment.

It was interesting to note that IV injection of BMSCs into the circulation resulted an alteration in the composition of blood after 12 h of injection. A significant increase was observed in the number of all the blood cells studied. Out of this increase, the increase observed in the number of neutrophils, the white blood cells which are the first responders to inflammation is of particular interest (Jin et al., 2012). The change in blood cells due to BMSC injection was reversed after 48 h of injection. Rat body itself was able to manage and maintain homeostasis.

There are some limitations in the present study as we only had studied the early phase of cell distribution. It is necessary to study the effect or role of transplanted cells to see the complete effect of injecting BMSCs into the body. We also did not study the infiltration of injected BMSCs into other organs which remains as a major drawback of this study, though it is of at most importance (Dai et al., 2005). Assessment of the fate of cells after several weeks is important as many of the effect of these cells within the body come into occurrence after many weeks (Zhang et al., 2007). For example the myogenic differentiation of grafted cells was seen several weeks after cell injection (Dai et al., 2005). Allogenic male BMSCs were used as cell resource in this study as BMSCs are supposed to be immune privileged. Still there is a

chance of cell rejection due to immune response of the host against the “Y” chromosome antigen. We have not specifically studied the immune response of host. The study was only for 21 days and that might be the reason for not seeing any immune response. Hence we suggest studying the immune response as well as the use of cells of same sex for transplantation. We were forced to inject male cells into female animals for ease of determining the cell homing through RT-PCR and also due to the lack of required facilities or reagents for other means like MRI and using cells transfected with fluorescent protein genes or by means of flow cytometry for determining cell homing.

10.4: Conclusion

Injecting bone marrow cells to the diabetic rats produced certain beneficial effects which ameliorated many of the diabetic complications to a great extent. The effect produced by bone marrow cells were similar to that achieved by the administration of DK. It is interesting to note that the consumption of a drug which is a polyherbal product is able to produce beneficial impact which is comparable with the beneficial impact achieved by the complicated process like bone marrow injection. The importance of DK is of high significance as it is non-toxic or less toxic whereas the bone marrow injection may result very serious side effects such tumorigenesis.

Chapter 11

Summary and Conclusion

Diabetes Mellitus is a metabolic disease which leads to the uncontrolled condition known as hyperglycaemia in blood over a prolonged period of time. It is mainly classified into type I and II. The former occurs due to autoimmune destruction of pancreatic β - cells and the latter due to the body failing to respond to insulin correctly because of insulin resistance. The main objective of the present study is to find out the effect of Dhanwantaram Kashaya on STZ induced diabetic rats which is evident from the changes observed in various parameters in tissues (Liver, Heart, Kidney and Pancreas), blood, plasma and serum.

A comparative study of the effect of DK, SE2 and Bonemarrow derived stem cells was also made.

Investigations on glucose metabolism revealed that the parameters like fasting blood sugar level, Homa-IR index, OGTT, HbA1c were increased in DC rats as compared to NC rats. Administration of DK reversed these effects in diabetic rats with some beneficial effects in normal rats. Another important effect of DK was in serum insulin and Hb levels. The levels of these were reversed by DK administration. DK helped to maintain normal body weight in diabetic rats. The increase in organ weights due to diabetes was reversed by DK administration. Histopathological findings also suggested the beneficial effects of DK supplementation to diabetic rats through the regeneration of pancreatic β -cells in damaged and normal pancreas. Histopathology of pancreas

revealed the curative effect of DK not only on pancreatic β -cells but also on hepatocytes. Expression of insulin gene was upregulated in DK administrated DC and NC rats and hence present study reveals the efficiency of DK as an excellent hypoglycaemic agent.

The present study revealed that DK is rich with phytochemicals such as total tannins, total flavonoids and total phenolic contents. Various *in vitro* antioxidant power assays such as Ferric Reducing Antioxidant Power (FRAP), 2,2-Diphenyl-1-Picryl Hydrazine (DPPH) scavenging activity, 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging assay, Phosphomolybdenum assay, Hydroxyl radical scavenging activity, Hydrogen peroxide-scavenging activity and Nitric oxide scavenging activity of DK revealed its excellent antioxidant activity and free radical scavenging ability. The strong antioxidant and free radical scavenging activity of DK suggested by the presence of various phytochemicals may be the reason for its beneficial impact in biological system as reported in Ayurvedic system of medicine.

The activities of enzymatic antioxidants like catalase, SOD, GPx and GRd and the levels of non-enzymatic antioxidants such as GSH, Vit C and Vit E were found to be decreased in diabetic rats as compared to normal control rats which was reversed by administration of DK. DK was able to produce this beneficial impacts in normal control rats also. The expression of genes such as GPx, GRd and gulo were decreased and the iNOS was increased in DC rats. DK administration increased expression of GPx, GRd and gulo gene in DC and NC rats in a dose

dependent manner. At the same time the expression of iNOS gene was decreased in DK administrated DC and NC rats. A decrease in nitric oxide level in liver tissue as well as serum total protein was observed in DC rats which was also reversed by the administration of DK. From these observations, it can be concluded that DK, a poly herbal formulation (PHF) used in Ayurveda exerts significant antioxidant activity which could effectively used in managing diabetic complications.

Lipid profiles like TC, FFA, TG and PL were elevated in diabetic rats. DK administration reduced these lipid profile levels in DC and NC rats. In the case of some tissues DK administration reversed the changes fully and the levels of these lipid parameters reached normal level and in some cases it even went below normal level.

Lipid peroxidation product MDA was also shown the same pattern of results. Elevated level of MDA was seen in DC rats as compared to NC rats and the administration of DK decreased these effects in both normal and DC rats. The expression patterns of the pancreatic gene peroxisome proliferator-activated receptor, PPAR- α and PPAR- δ were also studied which revealed a decreased expression of these genes in DC rats. Here also DK exerted its beneficial impact by increasing the expression of these genes in both diabetic and normal control rats. From these results, it can be concluded that DK, a poly herbal formulation used in Ayurveda exerts significant anti lipid peroxidation activity.

Expression pattern of NF- κ B, TNF- α , COX-2, Caspase3 and Caspase 8 were also studied in normal rats, diabetic rats and the DK administered normal and diabetic rats. In diabetic rats there was an increase in the expression of all these genes. DK administration normalised expression pattern of these genes to a great extent in a dose dependent manner in DC with some beneficial impacts on NC rats.

The present investigation also studied the effect of SE2 in STZ induced diabetic and normal rats. Serum insulin level and body weight of different groups of rats during the experimental period were analysed. SE2 administration also decreased these two parameters in DC and NC rats. However, the administration of SE2 failed to make significant change in the body weight loss due to diabetes. In the case of SE2 fed diabetic rats, the decrease in body weight was slightly lesser and the effect of SE2 was concentration dependent. The increase in MDA, FBS and nitric oxide level observed in diabetic rats was reversed to a small extent by SE2 administration in DC rats. The decrease due to SE2 administration was significant in FBS and nitric oxide level but in the case of MDA the decrease was not significant except in kidney. The effect of SE2 in the above parameters was comparatively lesser than that produced by DK when both were used in a dose equivalent to human consumption.

The expression of CD34 gene at mRNA level was also analysed. The expression of this gene was upregulated by the administration of SE2 as well as DK in a dose dependent manner both in the diabetic as well

as normal control rats. The effect of the high dose of DK is slightly higher than that of SE2, but the difference was not significant.

In another study the DK was found to significantly increase the viability of bone marrow cells revealed by the increase in optical density in MTT assay with increase in concentration of DK. Presence of injected bone marrow cells in pancreas was confirmed by PCR for “Y” chromosome gene. PCR results reported the presence of injected bone marrow cells in the pancreas of diabetic as well as normal rats. In the normal pancreas the intensity of PCR band was too light and thin whereas in pancreas of diabetic rats the signal strength of the PCR product was very thick and bright. This reveals the homing of the injected cells in diabetic pancreas which could be the reason for the observed beneficial impact by repairing the degenerated pancreas by the formation of new β cells in the islets.

Blood cell analysis was also done to assess if there is any variations in blood populations due to intravenous injection of BMSCs. There were noticeable changes in the number of different types of WBCs 12hr after BMSC injection; however this change was found reversed after 48hr of injection. Significant changes were also observed in the case of neutrophil but in the case of lymphocyte no significant difference was observed in normal as well as diabetic rats with and without BMSC injection. After 48h of BMSC injection there was no significant difference in the number of monocytes in normal as well as diabetic rats from the normal.

Change in body weight of rats treated with bone marrow stem cells were also analysed. There was no significant change in weight between normal control and BMSC injected normal rats in weight gain. But diabetic rats lost their weight by 33% by the end of the study period. BMSC injection prevented this weight loss in diabetic rats.

On 21st day there was a significant increase in fasting blood sugar (FBS) and significant decrease in serum insulin level in diabetic rats as compared to normal control rats, which was also reversed by the IV injection of bone marrow derived mesenchymal stem cells. A concurrent decrease in FBS was also observed in rats injected with bone marrow derived mesenchymal stem cells. There was no significant difference in FBS or insulin level in normal rats injected with bone marrow derived MSCs.

DK exerted a comparable to better effect than that produced by SE2 as well as bone marrow derived MSCs. Like SE2, DK is also a product derived from plants and hence the chance of it causing serious toxic effect is less. Mere consumption of DK produced excellent hypoglycemic effect which was the result of regeneration of β cells of the islets of pancreas as well as its antioxidant effect. This study was only for a short duration and more studies need to be conducted to reveal the mode of action in full as well as its side effects if any. Our study expose the beneficial impacts of DK in diabetic condition and points to the possibility of developing DK as an effective less toxic drug for managing diabetic complications. It may also be taken as a dietary supplement to prevent developing diabetic condition.

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Presentations

- **Smitha Renganathan and Radhakrishna.G. Pillai.** Dhanwantharam Kashayam- a poly herbal Formulation Regenerates Pancreatic Islets and Ameliorates Diabetic complications in Diabetic Rats. Presented a paper in the oral session in the 28th Kerala Science Congress held at University of Calicut, Malappuram during 28-30th January 2016.
- **Smitha Renganathan and Radhakrishna.G. Pillai** delivered an oral presentation entitled Dhawantaram Kashayam Ameliorates Symptoms of Diabetes in Rats. At the National seminar on Recent Trends and Advancements in Regenerative medicine & the Role of Omics and Biomarkers in Health held at department of Zoology, University of Kerala, Thiruvananthapuram on 28th February- 2nd March, 2019.

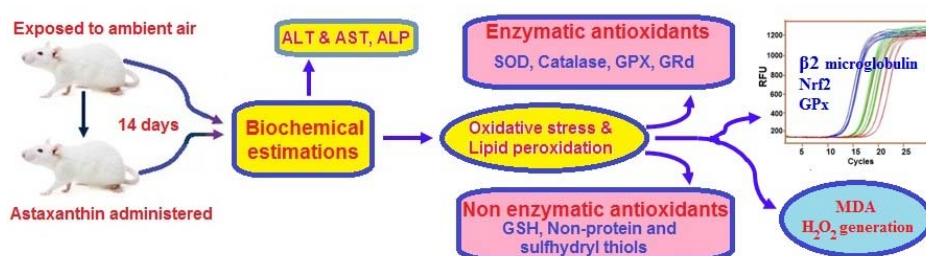
Beneficial impacts of Astaxanthin on biomarkers of antioxidant status and oxidative damage in Rats exposed to ambient air

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ABSTRACT



Effect of astaxanthin from *Haematococcus pluvialis* on lipid peroxidation and oxidative stress induced by ambient air exposure was studied. Wistar albino rats were exposed to ambient air was administered with astaxanthin in doses varying between 0.5 to 2% of food intake. Various biological parameters like ALT, AST, ALP, malondialdehyde, hydrogen peroxide, superoxide dismutase, catalase and glutathione reductase, were estimated biochemically and the expression of Nrf2 and glutathione peroxidase genes were estimated by reverse transcriptase PCR. Plasma ALT, AST, ALP, MDA and the activity of antioxidant enzymes; SOD, GRd, catalase were found increased significantly in ambient air exposed rats. Ambient air exposure decreased the levels of glutathione, non protein thiols and GPx expression whereas total thiols and expression of Nrf2 increased. However the concurrent administration of astaxanthin was found to reverse these changes in a dose dependent manner. The results of this study revealed the ability of astaxanthin to alleviate liver toxicity and oxidative stress induced by ambient air exposure and points to the possibility of developing astaxanthin as a dietary supplement that reduce the ill effect of toxic chemicals from ambient air.

Keywords: Astaxanthin, ALT, AST, lipid peroxidation, air pollution, oxidative stress, antioxidants

INTRODUCTION

Nearly 90% of the world population is living with below the air quality standards that WHO recommend.¹ Annually around 4.2 million people die due to the ill effects of air pollution. Increasing air pollution is a major concern as it is associated with a range of respiratory and cardiac diseases along with increased

mortality² and it also leads to various degenerative diseases like diabetes, Parkinsons³ etc. Air pollution is also a causative agent for cancer, asthma and chronic obstructive pulmonary disease (COPD).⁴ Exposure to ambient air was reported to cause oxidative stress which triggers a number of signalling pathways.⁵

Different components of polluted air like CO, nitrogen oxides, ozone, aromatic hydrocarbons, heavy metals, other fine and ultra fine particles are toxic to living organisms and many of them lead to the generation of free radicals and produce reactive oxygen species (ROS) and reactive nitrogen species (RNS). Imbalance between ROS and antioxidants cause oxidative stress which damage lipids and DNA.⁶ Enzymes of phase I and II play a big role in detoxifying these toxins and ROS. Glutathione peroxidase (GPx), a phase II enzyme breaks down peroxides including H₂O₂ into less toxic compounds.⁷ GPx also form glutathione disulphide

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Antioxidant activities of Dhanwantaram Kashayam –an Ayurvedic poly herbal formulation alleviates diabetic complications in rats

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Abstract

Purpose Phytochemicals of ethno medicines are being developed as effective drugs with minimum or no toxic side effects. Dhanwantaram Kashayam (DK) is a polyherbal formulation used as a potent general health tonic and is found to have antioxidant activities, but there are no proper scientific studies on its possible benefits. In this study we investigated the antioxidant and antidiabetic properties of DK and trying to explore the possibility of employing DK for the treatment of diseases like diabetes mellitus caused by the imbalance in Reactive Oxygen Species (ROS).

Methods We investigated the effect of DK on normal and rat model of diabetes. Rats were fed with DK for 21 days. Fasting blood glucose level, haemoglobin, glycosylated hemoglobin, non enzymatic antioxidants like vitamin C, vitamin E, reduced glutathione, and MDA were evaluated.

Results DK was found to ameliorate the disease symptoms of diabetes. A significant decrease in non enzymatic antioxidants – vitamin C, vitamin E, reduced glutathione and an increase in MDA and fasting blood glucose level was observed in diabetic rats. Administration of DK resulted significant increase in non enzymatic antioxidant levels both in diabetic and normal rats and a decrease in MDA, fasting blood glucose, glycosylated hemoglobin and HOMA-IR index was observed.

Conclusion This study reveals the protective role of DK in diabetic condition through its antioxidant and antihyperglycemic activity. Our results strongly indicate the possibility of DK being developed as a potent antidiabetic drug. DK could be an alternative in the treatment of diseases with ROS imbalance to expensive and toxic synthetic ‘medicines’.

Keywords Reactive oxygen species · Diabetes mellitus · Ayurveda · Kashayam · Antioxidants · MDA

Introduction

Oxidative stress is an important contributing factor in the pathogenesis of type 2 diabetes mellitus (DM) [1]. Several previous studies had already shown that oxidative stress is the major causative factors for diseases such as DM [2], cancer [3] etc. Hyperglycemia induced abnormal metabolism can result in the overproduction of reactive oxygen species (ROS) such as hydroxyl and superoxide radicals [4]. Balance between production and removal of ROS is necessary for proper functional integrity of cells. ROS formed from oxygen and nitrogen cause damages to the complex antioxidant system that

exists in Mammals [5] and are implicated as causative agents for many diseases [6].

Changes in glucose metabolism due to diabetes are reported to induce cell damage through different metabolic pathways which includes increase in glycation of proteins [7]. Most of the diabetic complications are caused by the mitochondrial over production of reactive oxygen species (ROS) leading to oxidative stress. High glucose in diabetic condition due to insufficient insulin activity is linked to different metabolic abnormalities. Excessive amounts of ROS oxidizes biomolecules, such as, DNA, protein, carbohydrates and lipids after surpassing various endogenous anti-oxidative defensive mechanisms which leads to oxidative stress [8]. Oxidative stress and the resultant tissues damages lead to diabetes associated complications [9]. Glycosylation of haemoglobin (HbA1c) elevated levels of glycosylated hemoglobin (GHb) and reduced total hemoglobin concentrations are salient features of diabetes [10]. The measurement of GHb is one of the well established means of monitoring glycemic control in

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Dhanwantaram kashayam, an Ayurvedic polyherbal formulation, reduces oxidative radicals and reverts lipids profile towards normal in diabetic rats



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ABSTRACT

Background: Hyperglycemia and hyper oxidative stress are indicators of diabetes mellitus which is also accompanied with decreased levels of antioxidant enzymes. While oxidative stress is important in increasing insulin secretion and controlling blood sugar level at the same time excess oxidative stress leads to the destruction of beta cells of pancreas resulting in to low insulin production and hyperglycemia. A balance between the levels of oxidative radicals and insulin production is needed, but is not defined yet. Hyperglycemia also leads to hyperlipidemia which can contribute to various health conditions like cardiovascular diseases.

Objectives: This study was designed to study the oxidative stress and lipid levels in diabetic rats. This also was designed to elucidate the effect of Dhanwantaram Kashayam, an Ayurvedic polyphenolic derived from plants on lipid metabolism and oxidative radical scavenging in diabetic rats.

Methods: Rats were made diabetic by injecting streptozotocin. Different enzymes involved in oxidative radical scavenging and lipid profiles including triglycerides, total cholesterol, free fatty acids and phospholipids were estimated using standard methods reported elsewhere.

Results: Level of antioxidant enzymes were lower in diabetic rats compared to normal controls. Administration of Dhanwantaram Kashayam restored the enzyme activity as well as reduced levels of different lipids in diabetic rats.

Conclusions: Administration of Dhanwantaram Kashayam increased the activity levels of antioxidant enzymes and reduced the levels of total cholesterol, phospholipids and triglycerides. The results of this study point to the possibility of developing Dhanwantaram Kashayam as a dietary supplement which can alleviate the complications associated with diabetes or prevent them altogether.

1. Introduction

At global level incidences of metabolic disorders and related diseases are increasing at a rampant rate because of easy availability of rich food stuff and decreasing physical activity. Obesity and diabetes are considered as two main outcomes of metabolic disorders [1]. Disturbances in the metabolism of carbohydrate, lipid and protein are well reported in these metabolic disorders [2]. The β -cells of pancreas are highly metabolically active and depend on oxidative metabolism for generating ATP for their energy requirements [3]. Elevated glucose level stimulates the production of insulin [3] and at the same time the enhanced production ROS is an unavoidable process in mitochondrial metabolism during high glucose tition [4] Enzymes of antioxidant metabolism are expressed at very low level in pancreatic β -cells [5]. These two conditions combined together render the β -cells highly susceptible

to damages caused by oxidative stress [3].

Oxidative stress plays an important role in tissue damages associated with diabetes [6]. An increase in reactive oxygen species (ROS) such as hydrogen peroxide, superoxide anion, and hydroxyl radicals have been reported in different cells exposed to high glucose [7,8]. Healthy cells have developed defensive mechanisms to manage the over production of ROS which include both enzymatic or non-enzymatic antioxidant systems. Enzymatic antioxidant defense mechanisms include enzymes such as superoxide dismutase (SOD), Catalase (CAT), Glutathione peroxidase (GPx), Glutathione Reductase (GRd) etc [9,10]. SOD converts the highly reactive radicals O_2^- to less reactive molecules H_2O_2 , which is then converted to harmless molecules like water and oxygen by CAT and GPx [11].

Dyslipidemia due to abnormal lipid metabolism is a prominent characteristic of diabetes [12]. This includes elevated levels of total

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Preliminary analysis of phytochemicals and in vitro free radical scavenging activity of *Dhanwantaram Kashayam*

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Dhanwantaram Kashayam, Total Phenolic content, FRAP, DPPH, Flavonoid, Ayurveda.

ABSTRACT

Dhanwantaram Kashayam (DK) is a polyherbal decoction used in Ayurveda for the postnatal care of mothers and for treating gynaecological diseases. It is also used as a growth stimulant in children as well as a regenerative medicine. Present study was to assess the various phytochemicals present in DK and to elucidate the role of DK as an effective antioxidant and free radical scavenger. Phytochemicals such as total phenolic content, total flavonoids and total tannins were assessed using standard biochemical methods. This study also investigated its *in vitro* antioxidant activity by ferric reducing antioxidant power (FRAP) assay and the free radical scavenging activity by assessing the scavenging activities on 2,2-Diphenyl-1-Picryl Hydrazine (DPPH), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), phosphomolybdenum, hydrogen peroxide, nitric oxide and the hydroxyl radical. Phytochemical assay reported fairly high levels of tannins 0.22 ± 0.015 mg/g, total flavonoids 1.23 ± 0.043 mg/g and total poly phenolic content 10.05 ± 0.94 mg/g. DK was found to have very good antioxidant activity and it scavenged the different free radicals in a dose dependent manner with low IC₅₀ values (DPPH: 2.08 ± 0.051 , ABTS: 38.46 ± 2.75 , phosphomolybdenum 50.4 ± 2.63 , H₂O₂: 57.9 ± 3.15 , NO: 57.25 ± 3.7 , all expressed in μ g/ml. Values were significant with $p < 0.05$). Results of this study clearly revealed that the DK is rich in phytochemicals and is a good source of natural antioxidants and is an efficient scavenger of peroxide radicals. This supports the use of DK in Ayurveda as a regenerative medicine, but further studies are needed to correlate the *in vitro* observations with its pharmacological effects *in vivo*.

INTRODUCTION

Plants synthesize different phytochemicals [1, 2] which are good antioxidants help in the fight against damages caused by reactive oxygen species (ROS) in animals [3, 4]. The vitamins A, C, E, and different phenolic compounds such as flavonoids, tannins, and lignins are the major phytochemicals with significant antioxidant capacity [3]. Other phytochemicals such as beta carotene, ascorbic acid and different phenolic compounds found in plants were found to have anti-inflammatory activity [5], and prevent or ameliorate symptoms of degenerative diseases like diabetes, Alzheimer's [6], Parkinson's [7], and certain cancers [8, 9].

Oxidative stress is reported to be the major cause of the pathogenesis of chronic diseases like diabetes. Reactive oxygen species (ROS) and free radicals play major roles in the progression and development of diseases like cancer, asthma and diabetes [10]. Phytochemicals are found to protect our body by lessening oxidative stress as well as by removing ROS [11]. *Dhanwantaram Kashayam* (DK) is a poly herbal formulation in the form of a decoction and the herbs used for its preparation are having regeneration property [12]. DK has excellent antioxidant properties as well [13].

Drugs of plant origin from Ayurveda have already proved to be excellent leads for drug development [14]. DK is used in Ayurveda for many ailments and