



Faculty of Women for Arts, Science and Education
Biochemistry and Nutrition Department

Effect of Mulberry Extracts Supplementation on Improving Nephropathy Complications in Diabetic Rats

Thesis

Submitted for Faculty of Women for Arts, Science and
Education, Ain Shams University
In Partial Fulfillment for the Requirements of Master Degree of
Biochemistry and Nutrition

By

Huda Esmael Ahmed Mogahed

B.Sc. in Biochemistry and Nutrition
Biochemistry and Nutrition Department
Faculty of Women for Arts, Science and Education
Ain Shams University

2011

Under supervision of

Prof. Dr. Nora Mohamed Afifi El-Sheikh

Professor of Nutrition
Biochemistry and Nutrition Department,
Faculty of Women for Arts, Science and Education
Ain Shams University

Dr. Heba Adel Abd El- Hamid Barakat

Assistant professor of Biochemistry and Nutrition
Biochemistry and Nutrition Department,
Faculty of Women for Arts, Science and Education
Ain Shams University

2015

Dedication

I take this opportunity to express my profound gratitude towards my family and a special feeling of gratitude to my loving parents whose words of encouragement and push for tenacity ring in my ears.

Lovingly, I dedicate this work to my respective family who has been my constant of inspiration.

My dear husband, thank you for your support, love and for being close to me.

Last I would like to thank my friends for their unconditional support and guidance towards the completion.

Acknowledgement

First and foremost, thanks **Allah** for most Merciful and most Gracious who gave me the ability to carry out this work.

I express my gratefulness towards my guide **Pro. Dr. Nora Mohamed Afifi El-Sheikh** Prof. of Nutrition, Biochemistry and Nutrition Department, Faculty of Women for Arts, Science and Education, Ain Shams University, who's excellent guidance and dedicated efforts made me think upon and understand a number of problems and solve them sincerely, her keen interest and encouragement serves as a constant support and inspiration during the period of this work.

I would like to express heartily thankful to **Dr. Heba Adel Abd El-Hamid Barakat** Assistant Prof. of Biochemistry and Nutrition, Biochemistry and Nutrition Department, Faculty of Women for Arts, Science and Education, Ain Shams University, for helping me, sincere advice and supporting me during all steps of this work.

I would like to express my sincere gratitude to the staff members of Biochemistry and Nutrition Department, Faculty of Women for Arts, Science and Education, Ain Shams University, for their encouragement to carry out this work.

Abstract

The aim of the present study was to determine the amount and anti-free radical activity of Deoxynojirimycin (DNJ) in Egyptian white and black mulberry fruits and leaves water extracts. Moreover, the study aimed to evaluate the water extracts effect of Egyptian white and black mulberry fruits and leaves, on improving hyperglycemic condition and renal complications in diabetic rats.

Seventy-two adult male albino rats Sprague-Dawley strain were equally divided into six groups: group (1) healthy control and group (2) diabetic control were given tap water by gavage tube, groups (3-6) diabetic treated with different water extracts of mulberry white fruits (WMF), white leaves (WML), black fruits (BMF) and black leaves (BML) (containing 50 mg DNJ/kg body weight) by gavages' tube daily for 28 days. At the end of the experiment, rats were sacrificed under ether anesthesia. Blood samples were collected to separate serum; also kidneys were removed for biochemical parameters estimation.

The High Performance Liquid Chromatography (HPLC) analysis of the mulberry extracts showed that water extracts contained higher amount of DNJ than methanol or ethanol extracts. Also mulberry leaves had higher amount of DNJ than fruits and the amount of DNJ in black mulberry was more than the white one.

The treatment with mulberry water extracts effectively decreased the blood glucose level, glycated hemoglobin percentage, renal aldose reductase activity and renal protein level as compared to untreated diabetic control rats; on the other hand there was an elevation of insulin level. Also mulberry extracts ameliorate the oxidative stress status with significant elevation in nonenzymatic antioxidant with significant improvement in kidney function parameters. Based on the presented data in this study, it can be concluded that mulberry extracts possess antidiabetic effect and improving properties of nephropathy complications.

List of abbreviations

4-AAP	4-Aminoantipyrine
AGEs	Advanced glycation end-products
AOPPs	Advanced oxidation protein products
AR	Aldose reductase
ARI	Aldose reductase inhibitor
BMF	Black mulberry fruits
BML	Black mulberry leaves
CML	N (carboxymethyl) lysine
Cox-2	Cyclooxygenase-2
CTGF	Connective tissue growth factor
DAG	Diacylglycerol
DCCT	Diabetes control and complications trial
DHBS	3,5-Dichloro-2-hydroxybenzene sulfonic acid
DMJ	1-Deoxymannojirimycin
DN	Diabetic nephropathy
DNJ	1-Deoxynojirimycin
DPPH	1, 1-Diphenyl-2-picrylhydrazyl radical
DTNB	5.5-Dithio-bis-2-nitrobenzoic acid
ECM	Extracellular matrix
EDTA	Ethylene diamine tetra acetic acid
EDTA Na ₂	Disodium ethylene diamine tetra acetic acid
EMT	Epithelial-mesenchymal transformation
ESRD	End- stage renal disease
ET-L	Endothlin-1
FER	Feed efficiency ratio
FGF	Fibroblast growth factor
GBM	Glomerular basement membrane
GFAT	Glutamine: fructose-6-phosphate-amidotransferase
GFR	Glomerular filtration rate
Glu	Glucose

Glu NAc	N-acetyl glucosamine
GlucN-6-P	Glucosamine-6- phosphate
GLUT-1	Glucose transporter 1
GSH	Reduced glutathione
HbA1C	Glycohemoglobin
HBP	Hexosamine biosynthetic pathway
HIV	Human Immunodeficiency Virus
HPLC	High performance liquid chromatography
INOs	Inducible nitric oxide synthase
LDL-C	Low-density lipoprotein--cholesterol
MAPKs	Mitogen-activated protein kinases
MC	Mesangial cells
MDA	Malondialdehyde
NaClO	Sodium hypochlorite
NAD ⁺	Oxidized nicotinamide adenine dinucleotide
NADH	Reduced nicotinamide adenine dinucleotide
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
NDF	Neutral dietary fiber
NEDA	N-(1-naphthyl) ethylenediamine
NF-B	Necrosis factor B
NO	Nitric oxide
NT	Nitrotyrosine
O ⁻²	Superoxide radicals
PARP	Poly-ADP-ribosyl polymerase
PBS	Phosphate buffered saline
PCO	Protein carbonyl products
PDGF	Platelet-Derived Growth Factor
PFF	Protein free filtrate
PKC	Protein kinase C
POD	Peroxidase
Pkα ^{-/-}	Protein kinase c-alpha
RAGEs	Receptors of advanced glycation end-products

RAS	Renin–angiotensin system
ROS	Reactive oxygen species
SDH	Sorbitol dehydrogenase
SOD	Superoxide dismutase
STZ	Streptozotocin
TBA	Thiobarbituric acid
TBM	Tubular basement membrane
TCA	Trichloroacetic acid
TGF	Transforming growth factor
TGF-1	Transforming growth factor-1
TMB	Tetra methyl benzidine
TMG	Tetramethyleneglutaric acid
UDP-GlucNAc	Uridine 5-diphosphate-N-acetylglucosamine
VEGF	Vascular endothelial growth factor
VLDL-C	Very Low density lipoprotein-cholesterol
WMF	White mulberry fruits
WML	White mulberry leaves

Contents

Title	Page No.
Introduction	1
Aim of the work	4
Review of literature	5
1. Diabetes mellitus	5
2. Diabetic nephropathy	7
2.1. Pathogenesis of diabetic nephropathy	9
3. Mechanisms of hyperglycaemia-induced micro-and macro-vascular damage	11
3.1. Increased polyol pathway flux (sorbitol pathway)	12
3.2 Advanced glycation end products	14
3.2.1. Advanced glycation end products biochemistry	15
3.3. Protein kinase C activation	19
3.4. The hexosamine biosynthetic pathway	23
4 . Oxidative stress	26
5. Aldose reductase	28
5.1. Localization of enzyme in tissues	31
5.2. Structure properties of aldose reductase	32
5.3. Physiological significance of aldose reductase	34
5.3.1. Osmoregulatory role in the kidney	34
5.3.2. Detoxification action of aldose reductase	34
5.4. Aldose reductase inhibitors	36
6. Mulberry	38
6.1. Antioxidant and nutritive value of mulberry	40
6.2. Commercial applications of mulberry in medicine	45
6.3. Black mulberry (<i>Morus nigra</i> Linn.)	47
6.4. White mulberry (<i>Morus alba</i> L.)	48
7. Deoxynojirimycin	50
Materials and Methods	54
1. Materials	54
2. Methods	59
2.1. Selection of the best solvent for extraction	59
2.2. Determination of DNJ in the different extracts by high performance liquid chromatography	59
2.3. Preparation of the different water extracts	61

2.4. Determination of anti-free radical activity	61
2.5. Experimental design	62
3. Biochemical analyses	66
3.1. Diagnosis of diabetes	66
3.1.1. Determination of blood glucose concentration	66
3.1.2. Determination of insulin level in serum	66
3.2. Indices of diabetic complications	69
3.2.1. Determination of glycohemoglobin in blood	69
3.2.2. Determination of renal aldose reductase (AR) activity	72
3.2.3. Determination of renal total protein content	74
3.3. Oxidative stress and antioxidant biomarkers	75
3.3.1. Determination of renal nitric oxide (NO) level	75
3.3.2. Determination of renal malondialdehyde (MDA) level	77
3.3.3. Determination of renal advanced oxidation protein products (AOPPs)	79
3.3.4. Determination of renal reduced glutathione (GSH) content	80
3.4. Renal biomarkers	83
3.4.1. Determination of serum creatinine	83
3.4.2. Determination of serum urea	84
3.4.3. Determination of serum uric acid	86
3.4.4. Determination of serum total protein	88
4. Statistical analysis	88
Results and Discussion	89
1. DNJ content and the best extract	89
2. Anti-free radical activity of standard DNJ and water extracts	94
3. Effect of oral doses of the different water extracts on the biological parameters	96
4. Effect of oral doses of the different water extracts on the progression of diabetes and its renal complications	103
4.1. Blood glucose concentration and serum insulin level	103
4.2. Glycohemoglobin percent in blood and renal aldose	109

reductase activity and total protein level	
4.3. Oxidative stress and antioxidant biomarkers	118
Renal nitric oxide, malondialdehyde, advanced oxidation protein products and reduced glutathione levels	118
4.4. Renal biomarkers	127
Serum creatinine, urea, uric acid and total protein levels	127
Summary	133
Conclusion and recommendations	138
References	139
Arabic summary	

List of tables

Table No.	Title	Page No.
1	Active biomolecules of different <i>Morus species</i>	42
2	Composition of the balanced diet	56
3	Composition of mineral mixture (AIN-93 M-MX)	57
4	Composition of vitamin mixture (AIN-93M-VX)	58
5	DNJ contents of different tested extracts	90
6	Scavenging effect of standard DNJ solution and different mulberry water extracts as % of inhibition.	95
7	Effect of four different mulberry water extracts on food intake, change in body weight, feed efficiency ratio and relative kidneys weight.	97
8	Effect of four different mulberry water extracts on blood glucose and serum insulin levels.	105
9	Effect of four different mulberry water extracts on blood glycated hemoglobin % and renal aldose reductase activity and total protein level.	111
10	Effect of different mulberry water extracts on renal nitric oxide, malondialdehyde, advanced oxidation protein products levels and reduced glutathione content.	120
11	Effect of different mulberry water extracts on serum creatinine, urea, uric acid and total protein.	128

List of figures

Figure No.	Title	Page No.
1	Aldose reductase and the polyol pathway.	13
2	A scheme showing cyclic form of Amadori products during non-enzymatic glycation of proteins.	18
3	Schematic representation of various biological targets of PKC activation leading to diabetic nephropathy.	23
4	The hexosamine biosynthetic pathway.	25
5	Glucose flux through the polyol pathway has been associated with the pathogenesis of diabetic complications via several potential mechanisms.	29
6	Proposed binding pockets in the active site of AR	33
7	Chemical structures of DNJ (A) and glucose (B).	51
8	Calibration curve for standard DNJ.	60
9	The experimental groups scheme.	64
10	Standard curve for insulin.	68
11-15	Chromatogram of standard DNJ and different mulberry extracts.	91
16	Scavenging effect of standard DNJ solution and different mulberry water extracts as % of inhibition.	95
17	Effect of different mulberry water extracts on food intake.	98
18	Effect of different mulberry water extracts on change in body weight (g).	98
19	Effect of different mulberry water extracts on feed efficiency ratio.	99

20	Effect of different mulberry water extracts on relative kidneys weight.	99
21	Effect of different mulberry water extracts on blood glucose level.	106
22	Effect of different mulberry water extracts on serum insulin level.	106
23	Effect of different mulberry water extracts on blood HbA1C %.	112
24	Effect of different mulberry water extracts on renal AR activity.	112
25	Effect of different mulberry water extracts on renal total Protein level.	113
26	Effect of different mulberry water extracts on renal NO level.	121
27	Effect of different mulberry water extracts on renal MDA level.	121
28	Effect of different mulberry water extracts on renal AOPPs level.	122
29	Effect of different mulberry water extracts on renal GSH content.	122
30	Effect of different mulberry water extracts on serum creatinine level.	129
31	Effect of different mulberry water extracts on serum urea level.	129
32	Effect of different mulberry water extracts on serum uric acid level.	130
33	Effect of different mulberry water extracts on serum total protein level.	130

Introduction

Diabetes mellitus, a metabolic disorder of multiple etiologies is characterized by chronic hyperglycemia with disturbances of carbohydrate, fat and protein metabolism that results from imperfections in insulin secretion or resistance (***American Diabetes Association, 2007***). The disease is found in all parts of the world and is rapidly increasing in most parts of the world. As diabetes aggravates and cell function deteriorates, the insulin level begins to fall below the body's requirements and causes prolonged and more severe hyperglycemia (***Gerich, 2003***). The sustained supraphysiological glucose may induce toxicity to metabolic processes and to cells resulting in the long-term complications of diabetes (***Giaccari et al., 2009***).

Chinese traditional medicines, which consist of several medicinal plants and their extracts, are widely used in many countries for the treatment of diabetes (***Chen et al., 2009 and Wang et al., 2009***). Mulberry tree is widely grown throughout china. Its leaves and their extract have been used in China, Korea, Japan and other Asian countries for the treatment of various diseases, especially in diabetic patients, and the antidiabetic activities of mulberry leaf extract in experimental animals have also been reported by many researchers (***Andallu et al., 2001 and Naowaboot et al., 2009***).

In recent years, 1-deoxynojirimycin (DNJ) extracted from mulberry trees has attracted considerable interest because of its effective and specific inhibition of various carbohydrate-degrading enzymes involved in a wide range of important biological processes, such as intestinal digestion, hepatic glycogen breakdown, lysosomal catabolism of glycoconjugates and maturation of the sugar chains in glycoproteins (*Kuriyama et al., 2008 and Winchester, 2009*).

DNJ is a naturally occurring inhibitor of glucosidases. It is a polyhydroxy alkaloid typical of the six-membered ring piperidine group. It has been shown to be a potent α -glucosidase inhibitor. Its presence has been described in leaves and roots of *Morus sp.* in which its content has been used as antihyperglycemic (*Kim et al., 2003 and Rodríguez-Sánchez et al., 2011*).

DNJ derivatives have found clinical application and have potential in the therapy of Human Immunodeficiency Virus (HIV) and hepatitis C infection. DNJ derivatives also inhibit glucosyl ceramide, and the glycosidase inhibitor 3 is a bifunctional inhibitor of angiogenesis *in vitro*. Moreover, DNJ has potential as a scaffold in peptidomimetic research. In this regard, a novel ligand for somatostatin receptors was obtained when Lysine and Trptophane side chains were grafted to DNJ. Thus, novel synthetic strategies to DNJ