EXPLORATION OF ANTIDIABETIC ACTIVITY OF INDIGENOUS PLANT, NEEM

Thesis submitted in partial fulfillment of the requirements of the degree of

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Under the guidance of

DR. SAIKAT DEWANJEE

SUBMITTED

BY

MS. Sujata Barma
Class Roll Number: 001411402022
Examination Roll Number: M4PHA1618
Registration Number: 112025 of 2010-2011

DEPARTMENT OF PHARMACEUTICAL TECHNOLOGY
FACULTY OF ENGINEERING AND TECHNOLOGY
JADAVPUR UNIVERSITY
KOLKATA- 700032
INDIA

CERTIFICATE OF APPROVAL

This is to certify that the thesis entitled ***"Exploration of Antidiabetic activity of indigenous plant, Neem"*** *has been carried out by* ***Ms. Sujata Barma*** *under my supervision in Advanced Pharmacognosy Research Laboratory at the Department of Pharmaceutical Technology, Jadavpur University, Kolkata-700032. She has incorporated her findings into this thesis of the same title, being submitted by her, in partial fulfillment of the requirements for the degree of* ***"Master of Pharmacy"*** *of this university. She has pursued this research work independently and with proper care and attention to my entire satisfaction.*

……………………………………

Dr. Saikat Dewanjee

Project supervisor
Assistant Professor
Department of Pharmaceutical Technology
Jadavpur University
Kolkata 700032, India

…………………………….

Prof. (Dr.) Biswajit Mukharjee
Head of the Department
Department of Pharmaceutical Technology
Jadavpur University
Kolkata 700032, India

………………………………..

DEAN
Faculty of Engg. & Tech.
Jadavpur University
Kolkata-700032, India.

DECLARATION OF ORIGINALITY AND COMPLIANCE OF ACADEMIC ETHICS

I hereby declare that this thesis contains literature survey and original research work pursed by me. As a part of my thesis work entitled **"Exploration of Antidiabetic activity of indigenous plant, Neem".**

All the information in this document have obtained and presented in accordance with academic rules and ethical conduct.

I also declare that as required by thesis rules and conduct, I have fully cited and referenced all the materials and results that are not original to this work.

Name: **Sujata Barma**

Examination Roll No.: **M4PHA1618**

Registration No.: **112025 0f 2010-2011**

Thesis title: **"Exploration of Antidiabetic activity of indigenous plant, Neem".**

Date :

Place:

……………………………………………

Sujata Barma

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Date: ***Ms. Sujata Barma***

PREFACE

Natural products, including plants, animals and minerals have been the basis of treatment of human diseases. Herbal medicine has been used throughout history and within every culture to prevent and treat diseases. Many drugs available for treatment of human diseases are obtained from the plant sources. Natural products are important sources for new drugs and are also good lead compounds suitable for further modification during drug development. The starting point for plant-based new drug discovery should be identification of the right candidate plants. It is achieved by the use of traditional documentation, ayurvedic wisdom and exhaustive literature search. The reasons for this were that pure compounds were easily obtained, structural modifications to produce potentially more active. Herbs contain many compounds with powerful antioxidant properties.

My work focuses on presenting the current scientific evidence of biomolecular effects of selected herbs in relation to clinical outcomes and therapy for promotion of human health. There are following chapters in my thesis work:

Chapter 1 is an introductory part which deals with tradition medicine used by the different culture and steps of drug discovery from plant source. It also gives concept and research scheme about mechanism of diabetes, oxidative stress and objective of this work.

Chapter 2 deals with the literature review of the research work.

Chapter 3 highlighted the methodology of extraction, fraction and isolation of experimental plant.

Chapter 4 demonstrated the antidiabetic activity of *Melia azadirachta* leaf extract through *in vitro* and *in vivo* experiment with respect to streptozotocin induced diabetes.

Chapter 5 contain the conclusion of the total thesis work.

Chapter 6 contain reference of my total project work. It contains list of all the article and book that I have been used in my research work as reference.

During the whole project work principles of laboratory animal care were followed and instructions given by our institutional animal ethical committee (Registration No. 0367/01/C/CPCSEA) were followed throughout the experiment.

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CHAPTER 1

INTRODUCTION

1. INTRODUCTION

The herbal medicines occupy distinct position right from the primitive period to present day. In recent years, herbs are being effectively tried in variety pathophysiological states. India has more than one fourth of the world's known medicinal plant species. *Melia azadirachta* (*M. azadirachta*) is one of them. It is a large evergreen tree which freely grows all over India. Ethnomedicinal literature survey revealed that the leaf of *M. azadirachta* possess significant antidiabetic effect in experimental animal model (Patil *et al.,* 2013). *M. azadirachta* has several medicinal properties. It is used in traditional medicine. Leaf extract of *M. azadirachta* have anti-inflammatory and immunostimulant effect other then hypoglycemic effect. Seed oil shows antifertility, hypoglycemic and antibacterial properties. Present investigation is aimed to explore scientifically the phytotherapeutic efficacy of *M. azadirachta* leaves on diabetes. Diabetes is one of the several increasing health problem of modern civilization. Despite great strides have been made to understand the management of diabetes, the disease and its related complications are increasingly unabated throughout the word. According to World Health Organization (WHO) fact sheet on diabetes, the global burden of diabetics would be projected to increase to 438 million in 2030. Similarly, for India this increase is estimated to be 87 million in 2030 (Huang, 1999 and McChesney, 2007).

Diabetes is a group of metabolic disease in which a person has high blood sugar, either because the pancreas does not produce enough insulin or because cells do not respond to the insulin that is produce. There are two types of diabetes mellitus i.e. insulin dependent diabetes mellitus (IDDM) and non insulin dependent diabetes mellitus (NIDDM).

Type 1 or Insulin dependent diabetes mellitus also called Juvenile onset diabetes mellitus probably an autoimmune disorder. Insulin in circulation is low or absent in blood. B-

cells which produced from islets of Langerhans in pancreas are destroyed by antibodies present in the blood.

Type 2 or Non insulin dependent diabetes mellitus also called maturity onset diabetes mellitus. In these diabetes insulin present in blood circulation is low, normal or even high. There is no present anti-β-cell antibody. It has a high degree of genetic predisposition; generally has a late onset. Gluco-receptor of β cells act abnormal so that they need higher glucose concentration for their respond. Sensitivity of peripheral tissues to insulin are reduced due to reduction in number of insulin receptor, 'down regulation' of insulin receptors. Excess of hyperglycemic hormones (glucagon etc.) / obesity: cause relative insulin deficiency- the β-cell lag behind (K.D.Tripathi, 1956).

Diabetes mellitus especially type 2 diabetes is a most metabolic problem now a day's which progress to most serious complications of the organs damaged in future. Diabetic nephropathy is one of the single most frequent causes of end stage renal disease. This complication was result from the interaction of hemodynamic and metabolic factors. However, diabetic complications are not merely due to hyperglycemia. Oxidative stress is a key factor which causes diabetic complications like cardiomyopathy and nephropathy, investigated these previously. Reactive property of the ROS directly oxidize and damage DNA, protein, lipid and carbohydrate which are major role of pathogenesis of diabetic nephropathy. Therefore, overproduction of ROS is directly related with hyperglycemia and at this condition different cells like endothelial, vascular smooth muscle, messangial and tubular epithelial cells are capable of developed ROS.

However, the source of oxidative free radicals is yet to be recognized, some part of its known to us, such as mitochondrial dysfunction, advanced glycation end processes and others

(A.K. Das, B.K. Dutta and G.D. Sharma, *et al*., 2011). Generation of oxidative free radicals results in oxidative disruption of structural proteins and degradation of membrane–bound phospholipids. Moreover, reactive free radicals cause fragmentation of DNA, which results apoptotic cell death (J. Das, J. Ghosh, P. Manna, *et al.,* 2010). Oxidative stress also leads to impairment of endogenous antioxidant enzymes due to their non enzymatic glycation and auto-oxidation (S. Dewanjee, A. Maiti, R. Sahu, *et al.,* 2011). These super active radicals may also disturb the expressions of several transcription proteins like NF-κB, PKCs, caspases etc (S. Dewanjee, A. Maiti, R. Sahu, *et al.,* 2011).

Numerous synthetic drugs were developed to combat against diabetes but diabetes and its relation complications still remain uncontrolled herbal drugs which comes from traditional medicinal plants are prescribed widely even when their biologically active compounds are unknown, because of their effectiveness, less side effect and relatively low cost.

Literature survey suggests that *M. azadirachta* leaves have antidiabetic activity. Little initial research has been done on this field. In our current study, we would emphasize on scientific exploration of *M. azadirachta* leaves to authenticate folklore claims. The most significant mechanism of diabetes is over production of reactive oxygen species (ROS) and reactive nitrogen species (RNS).The chronic hyperglycemia resulting from diabetes brings about a rise in oxidative stress due to overproduction of reactive oxygen species (ROS) as a result of glucose auto oxidation and protein glycation. Generation of ROS leads to oxidative damage of the structural components (such as lipids, DNA and proteins) of cells and potentiate diabetes related complications. Investigating the signalling pathways, streptozotocin administration caused the activation of phospho-ERK1/2, phospho-p38, phosphorylated NF-κB and destruction of mitochondrial transmembrane potential. One of the important pathogenic mechanisms of

pancreatic cell damage during experimental streptozotocin-induced diabetes is associated with increased expression of pro-inflammatory cytokines, increased expression of the inducible NO synthase (iNOS) gene and increased NO production in the pancreatic islets (Haluzik and Nedvidkova, 2000). Streptozotocin itself is a NO donor and liberates this reactive species during its intracellular metabolization. Besides nitrosative stress, streptozotocin-induced cell death is also associated with the oxidative stress caused by the production of excess intracellular reactive release of cytochrome c as well as activation of caspase 3 and caspase 9 in the pancreas tissue keeping the levels of total ERK1/2 and p38 significantly unchanged. Apoptosis is known to be a delicately controlled programmed cell death pathway (Singh and Anand, 1994). Several lines of earlier investigations suggest a change in mitochondrial transmembrane potential is able to 3 switch the committed cells to apoptotic death with oxidative stress as the mediator (Keeble and Gilmore, 2007).Oxidative stress can increase the permeability of the mitochondrial membrane, resulting in the disruption of the mitochondrial transmembrane potential and release cytochrome c, which in turn could activate caspase 3 in the cytosol and cause the cleavage of a variety of important molecules, like PARP, an endogenous caspase substrate (Jurgensmeier *et al.*, 1998). The established biological roles of niacin are attributable to the function of its active metabolites, NAD and NADP, as redox coenzymes. NAD^+ is synthesized from tryptophan, in addition to its coenzyme role can be further metabolized to poly (ADP-ribose) and cyclic ADP-ribose by poly (ADP-ribose) synthetase/polymerase (PARP) and ADP-ribosyl cyclase/cyclic ADP-ribose hydrolase (CD38), respectively. NAD^+ metabolites and enzymes are crucially involved in the death, regeneration, and functioning of the insulin-producing β-cells of the pancreatic islets of Langerhans.

CHAPTER 2

LITERATURE REVIEW

2. Literature review

2.1. Description of the plant to be investigated

Under the name of Neem it grows luxuriantly in throughout the world including India, China, Pakistan, south eastern Asia, France, Spain, US and Latin America. It is found growing in avenues. It grows 30-50 feet high. The bark should be new and is a rusty grey colour, inside yellow and foliated, coarsely fibrous, no odour, powerfully bitter and less astringent than the outer coarser bark, if taken from old roots the outer crust must be taken off. It is evergreen plant. The branches are wide and spreading. Every branch has new leaves which are slightly curved and are shiny from top and rough from below the surface. Leaves are shiny, dark green in colour. Tree bears flowers from March to May; Flowers are white and small with typical odour. Fruits 4 are small, egg shaped, greenish yellow in colour. Fruits contain only one seed which is embedded in brownish pulp (Chattopadhyay *et al.*, 1992).

2.2. Distribution

Melia azadirachta (*M. azadirachta*) is native to India and Indian subcontinent including Nepal, Pakistan, Bangladesh and Sri Lanka typically growing in tropical and semi- tropical regions. Neem trees now also grow in islands in the Southern part of Iran. Others Thialand, Malaysia, Mauritius, Figi, South Africa and East Africa here also Neem tree found (Dewanjee *et al.*, 2008).

2.3. Scientific classification (Liu *et al.*, 2001)

Kingdom: Plantae

Order: Sapindales

Family: Meliaceae

Genu : Azadirachta

Species: *Melia azadirachta*

Fig. 2.1. ***Melia azadirachta***

2.4. Synonyms

Bead tree, Pride of china, Nim, Margosa, Neem, Holy Tree, Indiar, Lilac Tree.

2.5. Vernacular names (Dewanjee *et al.*, 2008)

Bengal: Nim

Hindi: Nim, Neem

Kannada: Bevu

Malayalam: Arya, veppu

Punjabi: Nimm

Sanskrit, Marathi,

Oriya: Nimba

Sinhala: Kohomba

Telugu: Vepa

Tamil: Vembu

Yoruba: Dongoyaro

Gujarati: Limado

2.6. Ethnomedicinal uses

Various parts of these plants are used in different purpose. Leaves of this plant are used for prevent insects eating the clothes (Chattopadhyay *et al.*, 1992; Dewanjee *et al.*, 2008); dried leaves are burnt in the tropical regions to keep away mosquitoes. The flowers are also used in many Indian festivals like Ugadi. The tender shoots and flowers of the neem tree are eatenas a vegetable in India. Products made from neem trees have been used in India. Neem products are believed by Siddha and Ayurvedic practitioners to be anthelmintic, antifungal, antidiabetic, antibacterial, antiviral, contraceptive and sedative (Liu *et al.*, 2001; Dewanjee *et al.*, 2011). It is 6 considered a major component in Siddha medicine, Ayurvedic and Unani medicine mainly prescribed for skin diseases. Neem oil is used for liver disease as well as hair growth improver, blood detoxifier and balance blood sugar level. Neem is a key ingredient in non-pesticidal management, providing a natural alternative to synthetic pesticides. Neem seeds are ground into power then soaked into water overnight and sprayed onto the crop. Neem acts as an anti-feedant, repellent, and egg laying deterrent, protecting the crop from damage; it does not directly kill the insects.

CHAPTER 3

PHYTOCHEMISTRY

3. Phytochemistry

3.1. Introduction

Phytochemicals can be defined, in the strictest sense, as chemicals produced by plants. These are naturally occurring in the medicinal plants, leaves, vegetables and roots that have defense mechanism and protect from various diseases. Phytochemicals are primary and secondary compounds. It contains complex mixture of many plant metabolites. The isolation and use of natural products such as digoxin, morphine and quinine has resulted in replacing the plant extracts used with single chemical entities. There is a basic supposition that any plant possessing clinical effectiveness must contain an active principle which can completely replace the extract.

Different extracts of *Melia azadirachta* (*M. azadirachta*) have been tested for the presence or absence of primary and secondary bioactive compounds like carbohydrates, proteins, oils, alkaloids, flavonoids to name a few. The ethanolic leaf extract of *M. azadirachta* contains highest amount of phenolic compounds (Dewanjee *et al.*, 2013). The phytochemical screening was carried on the leaves extracts of *M. azadirachta,* revealed the presence of some active ingredients such as Alkaloids, Tannins, Saponins, Phenols, Glycosides, Steroids, Terpenoids and Flavonoids. The quantitative phytochemical analysis would be performed to quantify for total flavonoids (Dewanjee *et al.*, 2009). Based on the evidences my aim is to isolate more active compounds from *M. azadirachta.*

3.2. Collection and identification of selected plants

Matured leaves of *M. azadirachta* would be collected in the month of June from Sikkim Himalayan range, Sikkim. . The plants were authenticated by the Taxonomists, Botanical Survey of India, Howrah, India. The voucher specimens JU/PT/PC/07/12 have been deposited at Advanced Pharmacognosy Research Labor1atory, Pharmaceutical Technology, Jadavpur

University for future references. It should be ensured that the plant is healthy and uninfected. The leaves would be washed under running tap water to eliminate dust and other foreign particles and to clean the leaves thoroughly. The whole project would be divided into two simultaneous tasks.

3.3. Extraction

The leaves were dried in an incubator (40 ± 5 ºC, for 72 h) and pulverized in an electrical grinder into fine powder. The powdered plant materials were macerated with methanol with occasional shaking and stirring. The extract were filtered to remove particulate matters and dried in vacuum in a rotary vacuum evaporator at 50ºC to powdered extract for further experiments. The extract was stored in desiccator for use in subsequent experiments. Qualitative analysis of phytoconstituents was performed through preliminary phytochemical analysis .

3.4. Fractionation

Solvent-solvent extraction (fractionation) is one of the most popular methods for partial purification of crude plant extract. At first, the dry crude methanolic extracts were suspended in distilled water in separating funnel (1 liter) and successively partitioned with hexane and ethyl acetate according to increasing polarity. At last, aqueous residue is kept as a fraction after partitioned with ethyl acetate. The fractions were concentrated to give a rest, which was subjected to preliminary phytochemical analysis. Finally, individual fractions were chromatographed using column chromatography (CC)

Figure 3.1. Fractionation by separating funnel

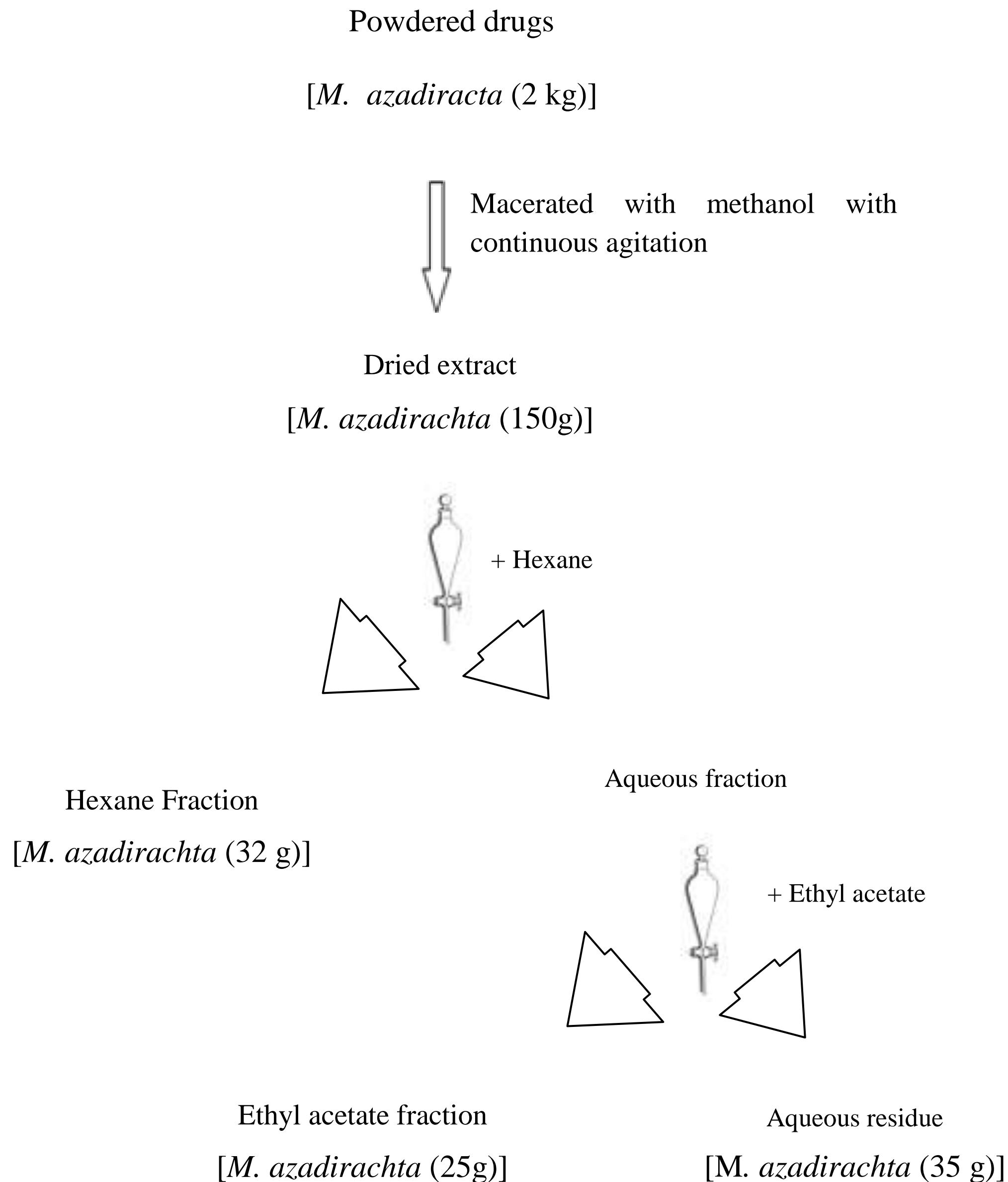

Figure 3.2. Schematic presentation of Fractionation of methanol extract of *M. azadirachta.*

3.5. Preliminary phytochemical analysis

3.5.1. Detection of alkaloids

Fractions were dissolved individually in dilute Hydrochloric acid and the solutions were filtered.

Mayer's Test

Filtrates were treated with Mayer's reagent (Potassium Mercuric Iodide). Formation of a yellow colored precipitate marked the presence of alkaloids.

Hager's Test

Filtered solutions were taken in a test tube and Hager's reagent (saturated picric acid solution) was added with it. Presence of alkaloids was confirmed by the formation of yellow colored precipitate.

Dragendroff test

A small quantity of test sample was treated with few drop of dilute hydrochloric acid and filtered. The filtrate immediately treated with Dragendroff"s reagent. Presence of orange brown precipitate indicated the presence of alkaloids.

Wagner test

A small quantity of test sample was treated with few drop of dilute hydrochloric acid and filtered. The filtrate was treated with Wagner's reagent. Presence of reddish brown precipitate suggested the presence of alkaloids.

3.5.2. Detection of carbohydrates

Fractions were dissolved individually in 5 ml distilled water and filtered. The filtrates were evaluated for the presence of carbohydrates.

Benedict's test

Filtrates were treated with Benedict's reagent and heated gently. Orange red precipitate pointed the presence of reducing sugars.

Fehling's Test

Filtered solutions were hydrolyzed with dil. HCl, neutralized with alkali and heated with Fehling's A & B solutions. Formation of red precipitate specified the presence of reducing sugars.

3.5.3. Detection of glycosides

Fractions were hydrolyzed with dil. HCl, and then subjected to test for glycosides.

Legal's Test

Fractions were mixed with sodium nitropruside in pyridine and sodium hydroxide. Formation of pink to blood red color indicated the presence of glycosides.

Modified Borntrager's Test

Fractions were treated with Ferric Chloride solution administered in boiling water for about 5 minutes. The mixture was cooled and extracted with equal volumes of benzene. The benzene layer was separated and treated with ammonia solution. Formation of rose-pink color in the ammoniacal layer showed the presence of glycosides.

3.5.4. Detection of saponins

Froth Test

Fractions were diluted with distilled water to 20 ml and this was shaken in a graduated cylinder for 15 minutes. Formation of 1 cm layer of foam expressed the presence of saponins.

Foam Test

0.5 gm of fractions was shaken with 2 ml of water. Stable foam was produced which remained for 10 minutes and pointed the presence of saponins.

3.5.5. Detection of phytosterols

Salkowski's Test

Fractions were treated with chloroform and filtered. The filtrates were treated with few drops of conc. sulphuric acid, shaken and allowed to stand. Appearance of golden yellow color showed the presence of triterpenes.

Libermann-Burchard's test

Fractions were mixed with chloroform and filtered. The filtrates were treated with few drops of acetic anhydride, boiled and cooled and then conc. sulphuric acid was added. Formation of brown ring at the junction confirmed the presence of phytosterols.

3.5.6. Detection of phenols

Ferric Chloride Test

Extract solutions were taken in test tubes and 3-4 drops of ferric chloride solution were added to them. Formation of bluish black color indicated the presence of phenols.

3.5.7. Detection of tannins

Small quantities of test samples were dissolved in minimum amount of distilled water and filtered. The filtrate was treated with 10% aqueous potassium dichromate solution. Development of yellowish brown precipitate demonstrated the presence of tannins.

Small quantities of test samples were dissolved in minimum amount of distilled water and filtered. The filtrate was allowed to react with 10% lead acetate solution. Formation of yellow colour precipitate indicated the positive test for tannins.

Small quantities of test samples were in minimum amount of distilled water and filtered. The filtrates were then allowed to react with 1.0 ml of 5% ferric chloride solution. Formation of greenish black coloration demonstrated the presence of tannins.

Small quantities of test samples were dissolved in minimum amount of distilled water and filtered. The filtrates are then allowed to react with 1.0 ml of 1% gelatin and 1.0 ml of 10% sodium chloride solution. Formation of white buff colored precipitate demonstrated the presence of tannins.

3.5.8. Detection of flavonoids

Alkaline Reagent Test

Fractions were treated with 4-5 drops of sodium hydroxide solution. Formation of intense yellow color, which becomes colorless on addition of dilute acid, indicated the presence of flavonoids.

Lead acetate Test

4-5 drops of lead acetate solution was added to the fractions' solutions. Formation of yellow color precipitate marked the presence of flavonoids.

3.5.9. Detection of proteins and amino acids

Xanthoproteic Test

The fractions were treated with 4-5 drops of conc. Nitric acid. Formation of yellow color indicated the presence of proteins.

Ninhydrin Test

To the fractions, 0.25% w/v ninhydrin reagent was added and boiled for few minutes. Formation of blue color indicated the presence of amino acid.

3.5.10. Detection of fixed oils and fats

A few drops of 0.5N alcoholic potassium hydroxide were added to a small quantity of extract along with a drop of phenolphthalein. The mixture was heated on a water bath for 1 -2 hours. Formation of soap or partial neutralization of alkali pointed the presence of fixed oils and fats.

3.5.11. Detection of gums and mucilages

1 ml of the extract was hydrolyzed using dil. HCl (3 ml). Then Fehling's solution was added drop by drop till the appearance of red. Test for mucilages were carried out by treating 1 ml of extract with 2 ml of ruthenium red solution to get red coloured solution.

3.6. Isolation of compounds from *Melia azadirachta*

After solvent-solvent extraction we get three major fractions according to polarity hexane fraction, ethyl acetate fraction and water residue. Hexane fraction was subjected to silica gel (100-120 mesh) column chromatography using n-hexane, mixtures of *n*-hexane-ethyl acetate and ethyl acetate-methanol of increasing polarity to yield 8 major fractions. Fraction 1 was subjected to silica gel (100-200 mesh) column chromatography using n-hexane, mixture of n-hexane-ethyl acetate and ethyl acetate-methanol according to polarity. We get very little amount of compound which was not properly purified by purification method due to very low amount.

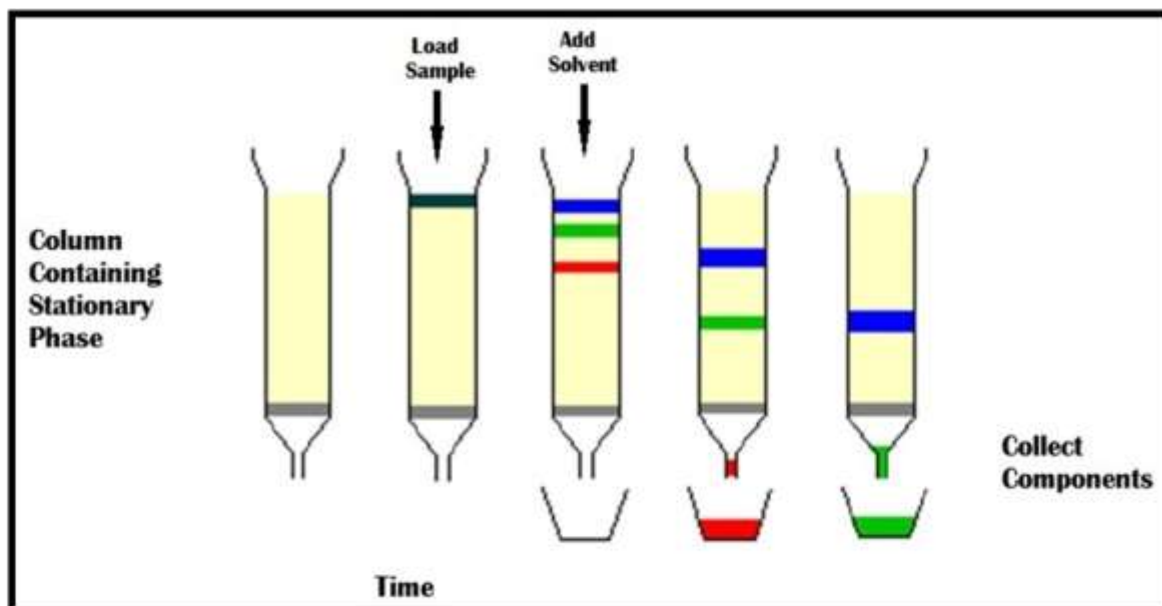

Figure 3.3. Technical view of column chromatography

Fraction4 (0.35g) was chromatographed with hexane, hexane-ethyl acetate and ethyl acetate-methanol, to yield compound 1 (12 mg).). Compound was purified by direct crystallization method. To the total fraction about 100 ml of n-hexane was added with heating at 40 ± 2°C few drops of ethyl acetate was added to facilitate the solubilization. The resulting solution was allowed to stand at room temperature which resulted white needle shapped crystals. The crystals were separated and washed with excess cold hexane (3 times) and cold methanol (minimum quantity). The crystals were dried under vacuum at 35 ± 2°C. From the rest of major fractions (2-8) we do not get any significant amount of compounds. The dried compounds were subjected to NMR analysis for structure interpretation.

3.7. Results and discussion

Qualitative analysis of *M. azadirachta* suggests the presence of various phytochemical constituents such as such as alkaloids, carbohydrates, glycosides, phytosterols, proteins, flavonoids, tannins, saponins, phenols, gums and mucilages, fats & fixed oils in individual fractions have been shown in table 3.1.

Table 3.1. Results of preliminary phytochemical analysis

Metabolites	**Fractions**		
	Hexane	**Ethyl Acetate**	**Aqueous**
Alkaloides	+	+	+
Sterols	–	–	–
Triterpenoids	+	+	–
Carbohydrates	–	–	–
Glycosides	+	+	–
Saponins	–	–	–
Phenols	+	+	–
Tannins	–	–	–
Flavonoids	+	+	–
Proteins and amino acids	–	–	–
Fixed oils and fats	–	–	–
Gums and mucilages	–	–	–

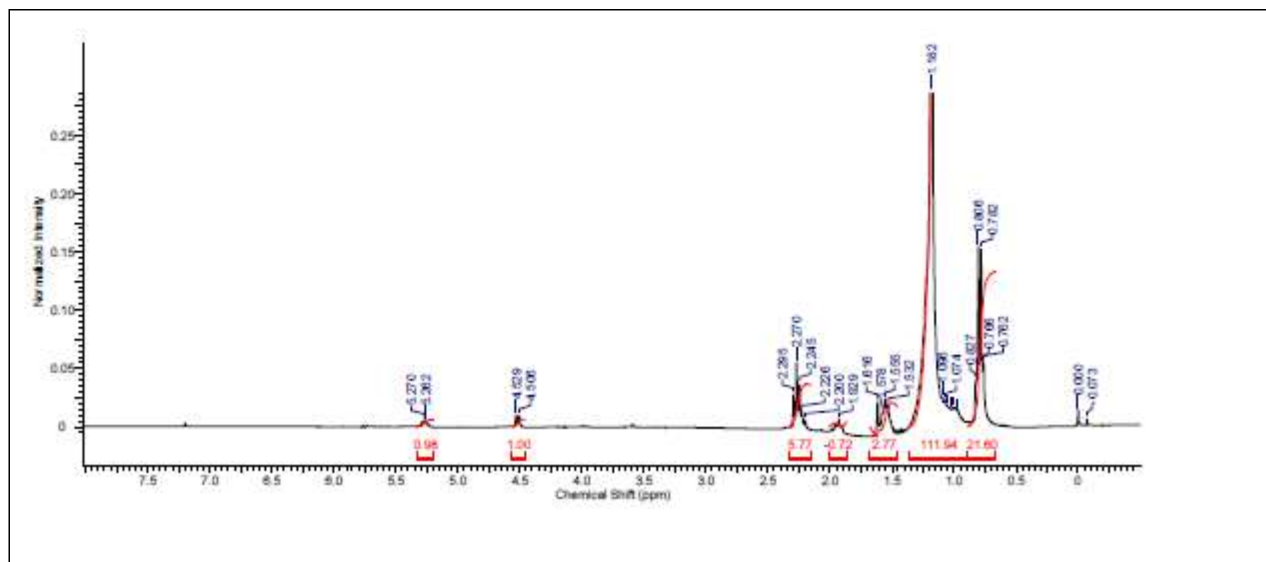

Figure 3.4.^{1}H NMR of compound 1

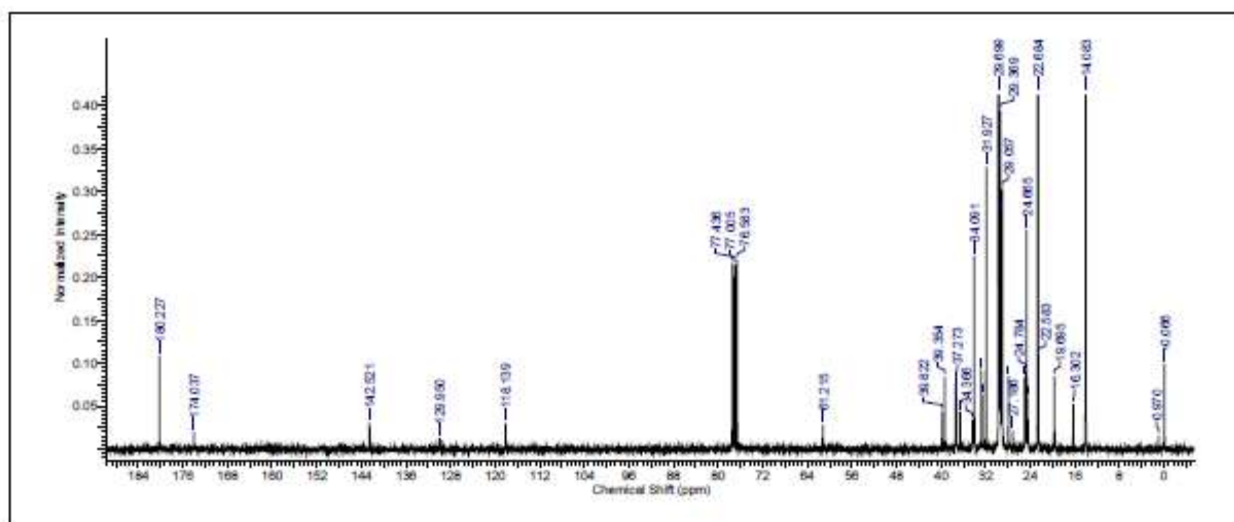

Figure 3.5.^{13}C NMR of compound 1

Compound 1 has been isolated from hexane fraction of *Melia azadirachta*. The chemical structure of isolated compound would not be identified by the combination of data of ^{1}H and ^{13}C NMR (Figure 3.4. and 3.5). Hexane and ethyl acetate fraction will be subjected to column chromatography later.

CHAPTER 4

ANTIDIABETIC ACTIVITY OF *MELIA AZADIRACHTA*

4. Antidiabetic activity of *Melia azadirachta*

4.1. Introduction

India has more than one fourth of the world's known medicinal plant species but interestingly many of traditionally claimed medicinal plants are yet to be explored scientifically i.e. establishment of pharmacological role with respect to phytoconstituents. Ethnomedicinal literature survey revealed that the leaf of *Melia azadirachta* (*M. azadirachta*) possess significant antidiabetic effect in experimental animal model (Patil *et al.*, 2013). Present investigation is aimed to explore scientifically the phyto-therapeutic efficacy of *M. azadirachta* leaves on diabetes as because in modern civilization diabetic mellitus is an increasing health problem. Despite great strides have been made to understand the management of diabetes, the disease and its related complications are increasingly unabated. According to recent estimation the global population is approaching to the midst of pandemic diabetes. India having the highest number of diabetic patients in the world, the sugar disease is posing an enormous health problem in the country. According to a WHO fact sheet on diabetes, the global burden of diabetics would be projected to increase to 438 million in 2030. Similarly, for India this increase is estimated to be 87 million in 2030. Diabetes is a group of metabolic disease in which a person has high blood sugar, either because the pancreas does not produce enough insulin or because cells do not respond to the insulin that is produce. There are two major types of diabetes mellitus i.e. insulin dependent diabetes mellitus (IDDM) or Type 1 diabetes mellitus (T1D) and non insulin dependent diabetes mellitus or Type 2 diabetes mellitus (T2D). Numerous synthetic drugs were developed to combat against diabetes but diabetes and its related complications still remain uncontrolled herbal drugs which comes from traditional medicinal plants are prescribed widely even when their biologically active compounds are unknown, because of their effectiveness, less

side effect and relatively low cost . Plant drugs may act on diabetes through different mechanisms viz. 1) β-cell regeneration and insulin releasing activity, 2) aldose reductase pathway inhibitors, 3) antioxidant mechanism, and 4) affecting glucose absorption. Literature survey suggests that *M. azadirachta* leaves have antidiabetic activity. Little initial research has been done on this field. In our current study, we would emphasize on scientific exploration of *M. azadirachta* leaves to authenticate folklore claims. The most significant mechanism of diabetes is over production of reactive oxygen species (ROS) and reactive nitrogen species (RNS).The chronic hyperglycemia resulting from diabetes brings about a rise in oxidative stress due to overproduction of reactive oxygen species (ROS) as a result of glucose auto oxidation and protein glycosylation. Generation of ROS leads to oxidative damage of the structural components (such as lipids, DNA and proteins) of cells and potentiate diabetes related complications. Investigating the signalling pathways, streptozotocin administration caused the activation of phospho-ERK1/2, phospho-p38, NF-κB and destruction of mitochondrial transmembrane potential. One of the important pathogenic mechanisms of pancreatic cell damage during experimental streptozotocin-induced diabetes is associated with increased expression of pro-inflammatory cytokines, increased expression of the inducible NO synthase (iNOS) gene and increased NO production in the pancreatic islets (Haluzik and Nedvidkova, 2000). Streptozotocin itself is a NO donor and liberates this reactive species during its intracellular metabolization. Besides nitrosative stress, streptozotocin-induced cell death is also associated with the oxidative stress caused by the production of excess intracellular reactive release of cytochrome c as well as activation of caspase 3 and caspase 9 in the pancreas tissue keeping the levels of total ERK1/2 and p38 significantly unchanged. Apoptosis is known to be a delicately controlled programmed cell death pathway (Singh and Anand, 1994). Several lines of

earlier investigations suggest a change in mitochondrial transmembrane potential is able to switch the committed cells to apoptotic death with oxidative stress as the mediator (Keeble and Gilmore, 2007).Oxidative stress can increase the permeability of the mitochondrial membrane, resulting in the disruption of the mitochondrial transmembrane potential and release cytochrome c, which in turn could activate caspase 3 in the cytosol and cause the cleavage of a variety of important molecules, like PARP, an endogenous caspase substrate (Jurgensmeier *et al.*, 1998). The established biological roles of niacin are attributable to the function of its active metabolites, NAD and NADP, as redox coenzymes. NAD+ is synthesized from tryptophan, in addition to its coenzyme role can be further metabolized to poly (ADP-ribose) and cyclic ADP-ribose by poly (ADP-ribose) synthetase/polymerase (PARP) and ADP-ribosyl cyclase/cyclic ADP-ribose hydrolase (CD38), respectively. NAD+ metabolites and enzymes are crucially involved in the death, regeneration, and functioning of the insulin-producing β-cells of the pancreatic islets of Langerhans.

4.2. Materials and methods

4.2.1. Collection and identification of selected plants

Matured leaves of *M. azadirachta* were collected from Sikkim Himalayan range, Sikkim. The plants were authenticated by the Taxonomists, Botanical Survey of India, Howrah, India. The voucher specimens JU/PT/PC/07/12 have been deposited at Advanced Pharmacognosy Research Labor1atory, Pharmaceutical Technology, Jadavpur University for future reference. It should be ensured that the plant is healthy and uninfected.

4.2.2. Extraction

The leaves were dried in an incubator (40 ± 5 ℃, for 72 h) and pulverized into fine powder. The powdered plant materials would be macerated with double distilled water

containing 1 % of chloroform for 48 h at 30 ± 5 ºC with continuous stirring. The aqueous extract of *M. azadirachta* (AEMA) was filtered to remove particulate cellular debris and lyophilized (Heto FD 3 Drywinner) to yield the powdered crude extracts. The testing samples were prepared by dissolving lyophilized powder in distilled water containing 1% tween 80 prior to in vivo experiment, whereas for in vitro assay, the extracts were solubilize in DMSO and diluted with autoclaved water into desired concentration (resultant ≤ 0.4% DMSO in contact to cells to avoid DMSO induced cytotoxicity).

4.2.3. Animals

Swiss albino male rats, weighing approximately 120-130 g were acclimatized under laboratory conditions of temperature (30 ± 2 ºC) relative humidity (50 ± 15%) 12 h light-dark cycle, standard diet and water ad libitum for two weeks prior to the experiments. All the experiments with animals were carried out according to the guidelines of the institutional animal ethical committee (Reg. No. 0367/01/C/CPCSEA).

4.2.4. Chemicals used

Streptozotocin (STZ) was purchased from Sisco Research Laboratories Pvt. Ltd. (India). All other chemical used in the study were of analytical reagent and obtained from Sisco Research Laboratories (India), Qualigens (India/Germany), SD fine chemicals (India), Himedia (India) and Central Drug House (India). Kits for measurement of serum biochemical parameters were purchased from Span diagnostic Ltd., India. 1-Chloro-2, 4-dinitrobenzene, ammonium sulphate, 2,4-dinitrophenylhydrazine, ethylene diaminetetraacetic acid, 5,5-dithiobis(2-nitrobenzoic acid), N-ethylmaleimide, nitro blue tetrazolium, reduced nicotinamide adenine dinucleotide, potassium dihydrogen phosphate, phenazine methosulphate, sodium pyrophosphate, reduced glutathione,

sodium azide, thiobarbituric acid, 5-thio-2-nitrobenzoic acid and trichloro acetic acid were purchased from Sisco Research Laboratory, Mumbai, India.

4.2.5. Induction and selection of diabetic animals

Diabetes was induced in overnight fasted rats by a single intraperitoneal injection (i. p.) of streptozotocin (65 mg/kg) in citrate buffer (pH 4.5), 15 min later the administration of nicotinamide (110 mg/kg, i. p.). After 1 week, animals exhibiting fasting glucose levels between 140– 200 mg/dl were screened as type 2 diabetic (T2D) rats and were used for the antidiabetic assay (Masiello *et al.*, 1998).

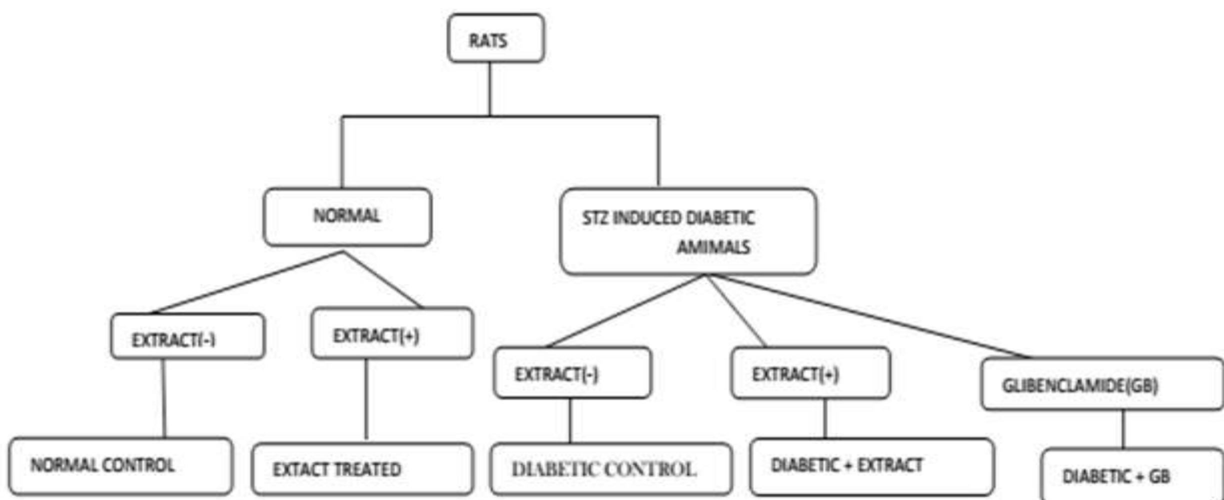

Figure 4.1. Schematic diagram of in vivo experimental protocol

The animals were divided into five groups of six rats each and they were treated as follows.

Gr I: Normal rats were given double distilled water (2 ml/kg, p.o.) daily for four weeks;

Gr II: T2D control rats were given distilled water (2 ml/kg, p.o.) daily for four weeks;

Gr III: T2D rats were given AEMA (100 mg/kg , p.o.) daily for four weeks;

Gr IV: T2D diabetic rats were given AEMA (200 mg/kg , p.o.) daily for four weeks;

Gr V: T2D rats were given standard drug glibenclamide (1 mg/kg, p.o.) daily for four weeks;

At the end of the experiments, all the animals would be euthanized after 12 h fasting. Blood and liver tissue were collected and used for the various experiments.

4.2.6. Determination of serum parameters

The serum was prepared according to the method described by Fischer and co-workers (Fisher et al., 1982). Serum biochemical parameters viz. alanine aminotransferase (ALT) and aspartate aminotransferase (AST), cholesterol and triglycerides were estimated by standard kits (Span Diagnostic Limited, India).

4.2.7. Assessment of fasting blood glucose level

The fasting blood glucose level in the experimental animals was monitored periodically during the treatment via tail prick method using an Accu Check glucometer.

4.2.8. Determination of intracellular ROS production, lipid peroxidationend products and protein carbonyl content

Intra cellular ROS production was estimated by using 2, 7-dichlorofluorescein di acetate as a probe. Intracellular ROS production (Kim *et al.*, 1996), lipid peroxidation i.e. thiobarbituric acid reactive substances (TBARSs) (Ohkawa *et al.*, 1979), protein carbonyl contents (Uchida and Stadtman , 1993) were determined as per protocols.

4.2.9. Assay of antioxidant enzymes

The activities of antioxidant enzymes, superoxide dismutase (Nishikimi *et al.*, 1972), catalase (Bonaventura *et al.*, 1972), glutathione-S-tranferase (Habit *et al.*, 1974) glutathione

reductase (Smith and Vierheller, 1988), glutathione per oxidase (Flohe and Gunzler 1984) and glucose -6- phosphate dehydrogenase (Lee *et al.*, 1982) were measured as per protocol.

4.2.10. Histological analysis

Tissues from the normal and experimental animals were fixed in 10% formalin and processed for paraffin sectioning. Sections of about 5 μm thickness were stained with hematoxylin and eosin to evaluate the patho-physiological changes under a light microscope.

4.2.11. Statistical analysis

Data were statistically examined by one way ANOVA and reported as mean ± SE followed by Dunnett's *t*-test using Graph Pad InStat software, version 3.05, U.S.A. The values were considered significant when $p < 0.05$.

4.3. Result

4.3.1. Effect of sugar level and serum parameters

A significantly ($p < 0.01$) high fasting blood glucose level (170–190 mg/dl) was observed in T2DM rats. Maintenance of blood glucose level within normal range is a primary approach during T2DM treatment. AEMA (100 and 200 mg/kg) treatment could significantly reduce fasting blood glucose level in T2D rat on day 28, respectively. The standard drug glibenclamide (1 mg/kg) exhibited maximum reduction on day 28 (Table 4.1).

Table 4.1. Effect of AEMA on fasting blood glucose level of T2D rats

Groups	Fasting blood glucose level (mg/dl) in days						
	0	1	3	7	14	21	28
Group I	74.12 ±4.86	75.35 ±6.33	74.39 ±7.14	76.24 ±5.74	75.33 ±4.88	76.22 ±6.71	77.27 ±5.39
Group II	173.46 ±14.36#	175.97 ±12.22#	180.22 ±14.49#	187.33 ±13.74#	195.35 ±17.43#	203.12 ±18.27#	199.22 ±17.37#
Group III	171.27 ±16.44#	165.25 ±13.21#	147.17 ±10.04*	137.45 ±15.31**	131.30 ±14.24**	126.98 ±11.33**	121.26 ±13.33**

Group IV	172.24 ±14.09$^{\#}$	157.74 ±13.33$^{\#}$	143.27 ±11.47*	129.23 ±12.13**	120.12 ±11.34**	114.76 ±12.61**	107.37 ±8.45**
Group V	171.33 ±13.55$^{\#}$	164.24 ±14.16$^{\#}$	137.19 ±13.27**	127.35 ±14.12**	109.32 ±7.69**	98.28 ±10.06**	90.22 ±8.37**

Data were expressed as mean ± SD (n=6). $^{\#}$p< 0.01 compared with Group I; *p< 0.05 compared with Group II; **p< 0.01 compared with Group II. Group I: Normal control; Group II: T2D control, Group III: T2D rats treated with AEMA (100 mg/kg, p.o.); Group IV: T2D rats treated with AEMA (200 mg/kg, p.o.); Group V: T2D rats treated with glibenclamide (1 mg/kg, p.o.).

The serum biochemical parameters serve as the most primitive indicators of the abnormalities within the system. The effect of serum biochemical parameters were shown in Table 4.2. The levels of AST, ALT, cholesterol and triglyceride were significantly (p < 0.01) increased, whereas, the level of HDL cholesterol was found to significantly (p < 0.01) decreased in the STZ-nicotinamide diabetic animals. On the other hand AEMA treatment could significantly (p < 0.05-0.01) rescue these serum biochemical parameters as compared with Glibenclamide (1mg/kg) treated rat. This progress was distinct in the groups treated with AEMA (200 mg/kg).

Table 4.2. Effect on serum biochemical parameters.

Groups	Parameter				
	ALT (IU/l)	AST (IU/l)	Cholesterol (mg/dl)		Triglyceride (mg/dl)
			Total	HDL	
Group I	68.17±2.30	44.50±1.08	86.13±3.89*	33.12±2.15	78.38±3.36
Group II	101.17±2.06$^{\#}$	75.33±3.58$^{\#}$	133.32±3.25	20.45±1.97	147.00±9.63$^{\#}$
Group III	88.33±4.27*	66.83±2.88	100.56±3.58	25.36±2.78**	98.70±5.83*
Group IV	84.67±4.43**	55.67±1.59**	95.85±5.12**	28.12±3.12	88.17±9.40
Group V	76.83±1.82**	53.17±1.64**	92.46±4.23	32.00±2.56**	88.19±9.68**

Values are expressed as mean ± SE, for six animals in each group. $^{\#}$ Values differ significantly from normal control (p < 0.01). * Values differ significantly from toxic control (p < 0.05). ** Values differ significantly from toxic control (p < 0.01).

4.3.2. Effects on body and organs' weight

In this study, total body weight and heart weight were evaluated (Table 4.3). A significant ($p < 0.01$) increase of body weight was observed in T2D rats. AEMA (200 mg/kg) treatment decreased significantly ($p < 0.05$) the weight gain of T2D rats. T2D rats, though statistically insignificant, but exhibited slight increase in the weight of heart. However, extract treatment could definitely (though statistically insignificant) reinstate the heart weight near to normalcy. However no significant alteration of body weight/heart weight ratio was recorded.

Table 4.3. Effect of AEMA on body weight, liver weight of T2D rats.

Groups	Body weight (g)	Liver weight (g)
Group I	150.54 ± 13.23	3.97±0.76
Group II	189.90 ± 15.34$^{\#}$	4.67±0.97
Group III	177.65 ± 14.33	4.53±0.46
Group IV	171.13 ± 12.64*	4.30±0.35
Group V	166.65 ± 12.24*	4.12±0.65

Data were expressed as mean ± SD (n=6). $^{\#}p < 0.01$ compared with Group I; $^{*}p < 0.05$ compared with Group II. Group I: Normal control; Group II: T2D control, Group III: T2D rats treated with AEMA (100 mg/kg, p.o.); Group IV: T2D rats treated with AEMA (200 mg/kg, p.o.); Group V: T2D rats treated with glibenclamide (1 mg/kg, p.o.).

4.3.3. Effect of intracellular ROS production, lipid peroxidation and protein carbonyl content

The effects of different treatments on ROS production, lipid peroxidation and protein carbonylation in liver were shown in Table 4.4 Intracellular ROS production plays an important role in STZ-nicotinamide oxidative damage. STZ exposure caused significant ($p < 0.01$) increase of intracellular ROS when compared with untreated animals. MAE treatment prior to STZ administration could significantly ($p < 0.01$) prevent the STZ mediated ROS generation and subsequent oxidative stress. Lipid peroxidation and protein carbonylation are two important

markers of oxidative stress. In oxidative stress related organ pathophysiology, lipid peroxidation and protein carbonylation are considered to be the key indicators. Lipid peroxidation has been measured by estimating the concentration of TBARS in liver of experimental animal (Table 4.3.).

Table 4.4. Effect on ROS, Lipid peroxidation and protein carbonylation in Liver.

Groups	**Parameter**		
	ROS production **(nmol DCF/min/ mg of protein)**	Lipid peroxidation **(TBARS level in µg/g of tissue)**	Protein cabonylation **(nmol/mg of protein)**
Group I	35.77±3.21	4.00±0.23	9.23±1.00
Group II	80.10±4.23$^{\#}$	8.13±0.45$^{\#}$	16.11±1.09$^{\#}$
Group III	54.23±2.19	5.41±0.32*	13.35±1.23*
Group IV	48.33±3.12*	5.99±0.41*	10.56±1.56**
Group V	49.14±4.66**	4.72±0.76*	10.19±1.79**

Values are expressed as mean ± SE, for six animals in each group. $^{\#}$ Values differ significantly from normal control ($p < 0.01$). * Values differ significantly from toxic control ($p < 0.05$). ** Values differ significantly from toxic control ($p < 0.01$).

STZ significantly ($p < 0.01$) increased the level of TBARS and protein carbonylation compared with normal control group (group I). AEMA administration could significantly ($p < 0.01$) decrease the TBARS level in the tissues at the dose of 100 mg/kg body weight. Administration of AEMA could significantly ($p <0.01$) attenuate that enhancement in the levels of protein carbonyl content in selected tissues in all doses of AEMA. However, AEMA at the dose of 200 mg/kg body weight assured better protective effect against oxidative stress associated with STZ induced diabetes.

4.3.4. Assay of antioxidant markers

Table 4.4 showed the effects of AEMA on antioxidant enzymes and reduced glutathione in Liver of experimental animal. The antioxidant enzymes protect biological macromolecules

from oxidative damage in oxidative stress-induced organ pathophysiology. Doxorubicin exposure significantly decreased ($p < 0.01$) the CAT levels of rat (group II) liver as compared with normal animal (group I). Treatment of AEMA (100 & 200 mg/kg) could significantly ($p < 0.05$) increase CAT activities in liver of experimental animals at the dose of 100 and 200 mg/kg body weight. SOD levels were significantly ($p < 0.01$) reduced in diabetic animals (Group II) compare to the tissues of experimental rats (Group III & IV) at the all dose of AEMA. The level of the glutathione regulated enzymes like GST, GPx, GR and G6PD were significantly ($p < 0.01$) reduced in diabetic animals (Group II) treated rats as compared with normal rats (group I). GST level of liver significantly ($p < 0.05$) improved after the treatment with AEMA at the dose of 100 mg/kg and 200 body weight significantly and GPx level significantly ($p < 0.05$ and $p < 0.01$) improved after the treatment of AEMA at the dose of 100 and 200 mg/kg body weight. However AEMA treatment could significantly improve GR ($p < 0.05$) and G6PD ($p < 0.01$) levels in liver of experimental animal (Group III & IV) in all doses.

Table 4.5. Effects on antioxidant enzymes

Groups	**Parameters**					
	CAT (U/mg of protein)	**SOD (U/mg of protein)**	**GST (µmol/h/ mg protein)**	**GPx (nmol/min/ mg of protein)**	**GR (nmol/min/ mg of protein)**	**G6PD (nmol/min/ mg of tissue)**
Group I	260.23 ±16.11	90.45 ±3.23	1.56 ±0.07	74.98 ±3.45	142.78 ±7.34	101.23 ±5.23
Group II	173.00 ±17.32$^{\#}$	64.57 ±4.12$^{\#}$	0.73 ±0.12$^{\#}$	40.45 ±4.12$^{\#}$	76.67 ±3.12	74.54 ±3.12$^{\#}$
Group III	207.25 ±16.99**	77.24 ±.3.76	1.01 ±0.16*	56.76 ±3.76**	108.63 ±5.09*	85.12 ±3.45*
Group IV	236.78 ±18.34*	77.12 ±3.87**	1.29 ±0.07*	58.12 ±4.13**	119.12 ±5.36	89.67 ±3.23**
Group V	251.83 ±18.38**	85.89 ±4.11**	1.31 ±0.19*	59.32 ±4.34*	121.34 ±6.61*	95.23 ±4.19**

Values are expressed as mean ± SE, for six animals in each group. # Values differ significantly from normal control ($p < 0.01$). * Values differ significantly from toxic control ($p < 0.05$). ** Values differ significantly from toxic control ($p < 0.01$).

4.2.4. Histological analysis

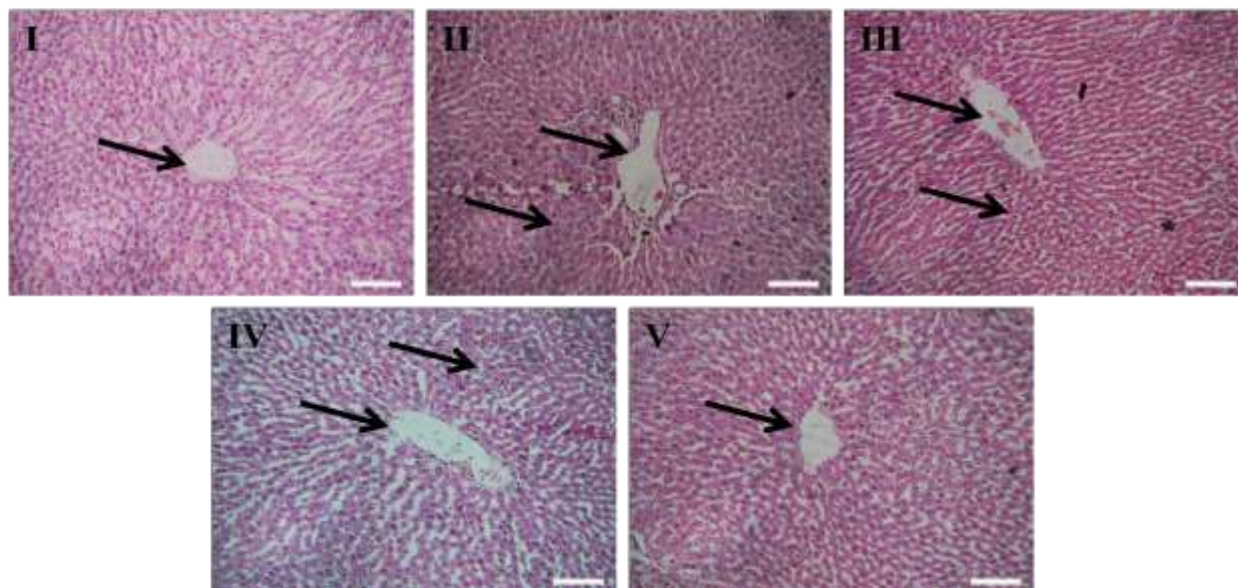

Figure4.2. Diagram shows histological sections of Liver of experimental animals treated with HE- staining. Group I (I) shows normal tissue architecture and normal structure of central canal (black arrows) where group II (II) indicates abnormal tissue architecture and disorganized central canal, Whereas, group III, IV, V shows significant improvement in hepatic tissue sections.

4.4. Discussion

Prophylactic role of *M. azadirachta* in diabetes are claimed by earlier literature. For managing type 2 diabetes mellitus we had used aqueous extracts of *M. azadirachta* leaves, which is beneficial than alcoholic extracts. However, we were used alloxan rats studies for antidiabetic assay. A new animal model of diabetes has been produced through the combined administration of streptozotocin and nicotinamide in adult rats (P. Masiello, C. Broca, R. Gross *et al*., 1998). This is an acceptable model for type 2 diabetes mellitus to screen antidiabetic effect of *M.*

azadirachta leaves in our experiment. Effectiveness of *M.azadirachta* leaves against type 2 diabetes mellitus but the experimental protocol strongly recommended the development of type 1 diabetes mellitus in rats which were subjected for their assay. Supportive data have been presented to show the effect of streptozotocin (singly) and streptozotocin- nicotinamide (combination) on experimental rats to support the experimental model. Streptozotocin, a glucosamide derivative of nitrosourea, was derived from Streptomyces achromogene. This compound can cause the death of beta cells by alkylating or breaking the DNA strands, consequently increasing the activity of poly-ADP-ribose synthetase, an enzyme that depletes nicotinamide, in beta cells, which can produce diabetes mellitus in rats. Along with, neither of these published literatures could substantiate any mechanisms of *M. azadirachta* leaves in diabetic pathophysiology nor to establish a correlation neither between pathophysiology nor to established a correlation between phytochemicals and the observed activity. Significant attempts have made to show the mechanism of action of the test material in diabetic pathophysiology chemicals present within the *M. azadirachta.*

Histological assessment of rat liver section further proves that *M. azadirachta* has a good hepatoprotective nature. AEMA significantly improves hepatic tissue architecture during diabetes and protects liver from oxidative insult during diabetes.

CHAPTER 5

CONCLUSION

Conclusion

The phytochemical investigations, isolation and protective role of *Melia azadirachta* (*M. azadirachta*) against experimentally induced diabetes mellitus has been represented here in this thesis. This thesis is furnished with the characterization and isolation of compounds and exploration of the antidiabetic activity of the plant.

Chapter 1 is an introductory part which deals with traditional medicine and drug discovery. It also gives concept and research scheme about diabetic mellitus, defensive mechanism against diabetes mellitus and basis of plant selection.

Chapter 2 deals with literature review, where previous research work of *M. azadirachta* has been discussed and it discussed about the ethnomedicinal documentation of this plant.

Chapter 3 deals with the methodology of extraction, fractionation, phytochemicals tests and isolation for the presence of phytochemicals in *M. azadirachta.* On the basis of NMR Spectroscopic data the molecular structure of compound 1 is obtained which is not given the satisfactory result.

Chapter 4 is about the study of protective role of *M. azadirachta* against experimentally induced diabetes mellitus. In present study, it was found that aquous extracts of the edible leaves of *M. azadirachta* could attenuate diabetes caused by Streptozotocin. The results suggested that the tested extract could offer protection against Streptozotocin induced diabetes mellitus by counteracting oxidative stress and related inflammatory responses of the heart and kidneys to provide renal protection and cardiac protection in type 2 diabetic rats. In conclusion, augmentation of *M. azadirachta* can be a novel strategy for future use of diabetes mellitus.

CHAPTER 6

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