ANTIDIABETIC ACTIVITY OF *Mahonia nepaulensis* DC IN NEONATAL STREPTOZOTOCIN INDUCED TYPE-II DIABETIC MODEL RATS

A DISSERTATION

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BOARD OF EXAMINER AND CERTIFICATE OF APPROVAL

This dissertation work entitled- "**ANTIDIABETIC ACTIVITY OF *Mahonia nepaulensis* DC IN NEONATAL STREPTOZOTOCIN INDUCED TYPE-II DIABETIC MODEL RATS**" submitted by Mr. Durga Bahadur Basnet (Exam Roll No.: CHE.721/073 and T.U.Reg.No.: 5-2-37-145-2012) under the supervision of Dr. Achyut Adhikari, Associate Professor of Central Department of Chemistry, Tribhuvan University, Nepal, is approved for the partial fulfillment for course CHE 662 required for Master's of science in chemistry.

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This is to certify that the dissertation work entitled "**ANTIDIABETIC ACTIVITY OF *Mahonia nepaulensis* DC IN NEONATAL STREPTOZOTOCIN INDUCED TYPE-II DIABETIC MODEL RATS.**" has been carried out by Mr. Durga Bahadur Basnet as partial fulfillment of the master's of science in chemistry under the course CHE 662. The results presented here are his original findings. I hereby recommend this thesis for final evaluation.

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DECLARATION

I declare that this dissertation work entitled "**ANTIDIABETIC ACTIVITY OF *Mahonia nepaulensis* DC IN NEONATAL STREPTOZOTOCIN INDUCED TYPE-II DIABETIC MODEL RATS**" was carried out by me in the Bangladesh University of Health and Science, Mirpur, Dhaka and Central Department of Chemistry, Tribhuvan University, Kirtipur, Kathmandu under the supervision of Prof. Dr. Begum Rokeya and Assoc. Prof. Dr. Achyut Adhikari. The information taken from literatures has been duly acknowledged in the text and a list of references is provided. No part of this dissertation was previously presented for another degree.

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LIST OF ABBREVATION

- ANOVA- Analysis of Variance
- BW- Body Weight
- DWC- Diabetic Water Control
- ELISA- Enzyme Linked Immunosorbent Assay
- Ext1- Extract-1
- FSG- Fasting Serum Glucose
- GA- Glacial Acetic Acid
- GT- Glibenclamide
- HDL- High Density Lipoprotein
- IDF- International Diabetes Federation
- ip- Intraperitoneal
- LMICs- Low-Middle Income Countries
- MN- *Mahonia nepaulensis*
- MNB- *Mahonia nepaulensis* Bark
- MNR- *Mahonia nepaulensis* Root
- µl- MicroLiter
- mg/dl- Milligram per deciliter
- n-STZ - neonatal streptozotocin
- NWC- Normal Water Control
- NCDs- Non-Communicable Diseases
- OGTT- Oral Glucose Tolerance Test
- Std- Standard
- SEM- Standard Error of Mean
- SPSS- Statistical Package for Social Sciences
- TCA- Trichloro Acetic Acid
- TC- Total Cholesterol
- T2DM- Type-II Diabetic Mellitus
- WHO- World Health Organization

LIST OF TABLES

LIST OF FIGURES

Pages

TABLE OF CONTENTS

ABSTRACT

Diabetes mellitus, is a group of metabolic disorders characterized by a high blood level over a prolonged period of time. It is due to either the pancreas not producing enough insulin, or the cells of the body not responding properly to the insulin produced. Symptoms often include frequent urination, increased thirst and increased appetite. Serious long-term complication include cardiovascular disease, stroke, chronic kidney disease, foot ulcers, damage to the nerves, damage to the eyes, and cognitive impairment. Diabetes management concentrates on keeping blood sugar level as close to normal, without causing low blood sugar. This can usually be accomplished with dietary changes, excessive weight loss and use of appropriate medication (insulin, oral medication)

The main aim of this research work is to evaluate the antidiabetic activity of methanolic extract from *Mahonia nepaulensis* bark in n-STZ induced type-II diabetic model rats. The oral glucose tolerance test was performed in normal as well as diabetic rats. Experimental rats were divided into four groups (n=6) as group-I control received normal water (10 mL/kg/bw), Group-II control received diabetic water (10 mL/kg/bw), Group-III standard received Gliclazide (20 mg/kg/5 mL/bw) and Group-IV received methanol extract (80%) of stem bark of *Mahonia nepaulensis* (MNB) at a dose of 1.25 g/kg/10 mL/bw. The blood was withdrawn from tail the vein at a different time intervals of 0, 30, 60, and 90 minutes again glucose levels were measured using glucose oxidase-peroxidase reactive strips and glucometer.

In the oral glucose tolerance test MNB group showed significant decrease in blood glucose level. In STZ induced diabetic rats fasting blood glucose levels of the treatment group were significantly reduced by 28 days of treatment with MNB extract.

The isolated compounds from *M. nepaulensis* include β-Sitosterol, Berberine, and 5,6-Dihydro-9,10-dimethoxy benzo-1,3-benzodioxolo 5,6-quinolizinium.

The crude extracts of *Mahonia nepaulensis* bark have potent antidiabetic activity and antilipidemic activity in streptozotocin-induced type-II diabetic rats.

Keywords: *Mahonia nepaulensis* bark, OGTT, Gliclazide, Streptozotocin, Glucometer

CHAPTER ONE
INTRODUCTION

1.1 General Background

Diabetes mellitus has become a growing problem in the contemporary world.[1] According to the World Health Organization (WHO), an estimated 422 million of the world's population have diabetes mostly in low and middle-income countries (LMICs), whereas about 16 million deaths are attributed to diabetes per year.[2] Such diabetes related diabetes mellitus (DM) are also dreadfully projected to increase by more than 50% over the few next decades.[3] WHO has also projected that by 2030 diabetes would become the 7th leading cause of death globally.[4] However in developing countries, DM is considered the 5th main cause of death.[5] Over 387 million people worldwide have been affected by diabetes and 4.9 million were dead due to diabetes in 2014 only. Currently one in every twelve people has diabetes.[6] Latest diabetes burden research by the International Diabetes Federation (IDF) indicated that there were 463 million people with diabetes worldwide in 2019 and data were expected to increase to 700 million by 2045.[7] Reasons for this rise include an increase in sedentary lifestyle, consumption of energy-rich diet, obesity, higher life span and others.[8] Regions with the greatest potential are Asia and Africa, where diabetes mellitus rates could rise to 2-3 folds than the present rates.[9]

In South Asia, greater population-level affluence is associated with an increase in health comprising behaviors related to chronic diseases such as cardiovascular disease, cancer and diabetes.[10] With prevalence greater than 6% and growing rapidly in South Asia, the expected prevalence increase in India is 171% from 2007 to 2025.[11] India leads the way with its the largest number of diabetic subjects in any given country. It has been estimated the number of diabetes in India is expected to increase 57.2 million by the year 2025. [12]Currently, South Asia is experiencing an increasing burden of type-II diabetes and its complications.[13] Type-II diabetes is mainly associated with several lifestyle behaviors including daily smoking, heavy alcohol drinking, obesity and reduced physical activity.[14] There is an increasing trend in the prevalence of type-II diabetes in low and middle-income countries (LMICs) and more than 75% of adults with type-II diabetes are now living in developing countries.[15]

Nepal is a low-income country in South Asia. While communicable diseases remain an important public health issue in Nepal, there is also a rapidly increasing burden of non-communicable diseases (NCDs), including type-II diabetes, posing an additional burden on a resource-poor health system. However, there is limited demographic knowledge of type-II diabetes and its risk factors in Nepal. The prevalence of diabetes in Nepal is estimated to be 4,36,000 (2%) in 2000 and it is projected to affect 13,28,000 persons (10 %prevalence) in 2030.[16] Prevalence rates of were highest among middle-aged and older men.[17] Other studies in Nepal have suggested prevalence rates of 15% among persons 20-39 years old and 19% among 40 years and older.[18] In Bir hospital, the central government hospital in Kathmandu, diabetes was reported to be the seventh most common reason for medical admissions with 2.5% prevalence,[19] whereas, in Tribhuvan University Teaching Hospital in Kathmandu, diabetes comprised 9.5% of total medical admissions per year.[20]

Diabetes can lead to increased morbidity and mortality.[21]The type-II DM has been revealed that behavioral risk factors are responsible for a large number of premature mortality due to cardiovascular disease followed by stroke.[22] More than 70% of diabetes patients die of cardiovascular events, leading to an epidemic of diabetes-related cardiovascular diseases.[23] In Nepal risk factors for type-II diabetes have so far rarely been investigated. However, our recent review found several modifiable risk factors for type-II diabetes in Nepal such as high socioeconomic status, high body mass index (BMI), lack of physical activity, hypertension alcohol and tobacco use.[24] Statistical analysis of the predictors of type-II diabetes in Nepal have been lacking and further research is hence needed. Moreover, diabetes awareness, treatment and control in Nepal has not received attention.[24] There is improper guidance about the diseases due to a lack of public awareness regarding DM in Nepal, where medical services are poor.[25] Several interventions have been carried out to improve the knowledge level of diabetes patients.[26-28]Obtaining information about diabetes in the population is the first step-in formulating a prevention program for diabetes.[29] A study from Pakistan highlight the fact that a proper education and awareness program can change the attitude of the public regarding diabetes[30] as a large gap between knowledge regarding various aspects of health, education program can improve the knowledge of patients and change their attitude.[31]Another study showed that intensive diabetes education and care management

can improve patient outcomes, glycemic control, and quality of life in patients with diabetes mellitus. [32]

1.2 Diabetes

According to WHO, diabetes is characterized by hyperglycemia due to an absolute or relative deficiency of insulin.[33] Hyperglycemia is often instigated by relative or complete insulin insufficiency or by insulin resistance.[34] It is evident that insulin resistance and relative insulin deficiency lead to a chronic elevation of plasma free fatty acid levels which contribute to the dysfunction of pancreatic beta cells and lipotoxicity.[35] Diabetes mellitus is also a complex metabolic disorder manifested by irregular metabolism in carbohydrates, fats, and proteins causing hyperglycemia and hyperlipidaemia.[36] The hyperglycemia condition is characterized by the body's inability to transfer sugar into energy and it can cause retinopathy, nephropathy and cardiovascular damage.[37] Hyperglycemic-induced oxidative stress is assumed to escalate the pro-inflammatory proteins along with infiltrated macrophages ultimately leading to local and systemic inflammation in a vicious cycle. Moreover, oxidative stress leads to protein and/or enzyme inactivation such as superoxide dismutase (SOD) and reduced glutathione (GSH) which promotes further oxidative stress. Hyperglycemia generates reactive oxygen (ROS), which in turn causes lipid peroxidation and membrane damage. Consistently elevated blood glucose levels result in the production of ROS that leads to oxidative stress, causing beta-cell damage and reduced insulin sensitivity within the pancreas. The excessive generation of ROS under the condition of hyperglycemia regulates expression levels of growth/transcription factors and cytokines in diabetes nephropathy (DN). Oxidative stress could furtherly induce chronic tubulointerstitial fibrosis and inflammation.[38]

Hyperlipidemia is an umbrella term that refers to an acquired or genetic disorder that results in high levels of lipids (fats, cholesterol, or triglycerides) circulating in the blood. This disease is usually chronic and requires ongoing medication to control blood lipid levels. Treatment of hyperlipidemia in diabetes involves improving glycemic control, exercise, and the use of lipid-lowering diets and drugs.[39] the relationship between diabetes and hyperlipidemia is a well-recognized phenomenon. Hypercholesterol is a feature observed in diabetes that contributes to the high prevalence of accelerated atherosclerosis. Many compositional abnormalities of the

lipoproteins very low-density lipoprotein (VLDL), low-density lipoprotein (LDL) and high-density lipoprotein (HDL) have been found in diabetic patients. These alterations may be relevant in explaining at least in part the increased predisposition of diabetics to atherosclerosis.[40]

Recently, attention has been focused on the relationship between the production of activated oxygen species such as hydrogen peroxide, superoxide anions, singlet oxygen and hydroxyl radicals can be formed in cells not only during ionizing radiation, but also during-aerobic metabolism of either endogenous and exogenous substances. Cells have enzymatic and non-enzymatic scavenger systems against these free radicals. Nevertheless, if free radical production and scavenger system somehow become unbalance cells are exposed to oxidative damage resulting in cell injury.[41] Diabetes is a state of increased free radical production.[42] Mechanisms that contribute to the formation of free radicals in diabetes may include not only increased non-enzymatic and auto-oxidative glycosylation, but also metabolic, levels of inflammatory mediators, and the status of antioxidant defense.[43]

Based on etiology and clinical presentation diabetes mellitus is classified into two classes.

a. Type-I DM

   It is also known as insulin-dependent diabetes (IDDM). It is caused by the immunological destruction of pancreatic beta cells resulting in insulin deficiency.[44] It's pathogenesis involves environmental triggers that may activate autoimmune mechanisms in genetically susceptible individuals, leading to progressive loss of pancreatic beta cells.[45] Many of the acute effects on blood vessels, nerves, and other organs of the body.[46]

   Insulin therapy affords glycemic control in IDDM yet, it's short-comings include ineffectiveness on oral administration, short life, need for preservation in refrigeration, fatal hypoglycemia in the event of excess dosage, reluctance to take an injection, and above all, the resistance due to prolonged administration, limits it's usage.[47] On different types of diabetes mellitus like type-I or IDDM or severe diabetics (SD) where functional beta cells are not present or are present in very few.

b. Type-II DM

It is also known as non insulin-dependent diabetic mellitus (NIDDM),which is characterized by both impaired insulin secretion and insulin resistance which is often associated with obesity and hereditary disposition.[48] Of the known forms of diabetes type-II diabetes occurs when the body is unable to produce enough insulin or to use it properly. It is the most common type and occurs for 90% of the case around the world.[49] this non insulin-dependent type was formerly more largely known in middle age or older adults. However, with increasing obesity in children, it is now being witnessed among young people too, leading to negative health and economic impacts.[50]

Treatment of NIDDM patients with sulfonylurea and biguanides is always associated with side effects. In type-II or non insulin-dependent diabetes mellitus (NIDDM) or mild diabetic (MD) where functional beta cells are present in a remarkable number.

Diabetics have long been treated orally with several medicinal plants extracts, based on folkloric claims.[50] Synthetic hypoglycemic agents can produce serious side effects, including hematological coma and disturbances of liver and kidney. In addition, they are not suitable for use during pregnancy.[51] Therefore, the search for more effective and safer hypoglycemic agents has continued to be an important area for research. Following the recommendations made by the WHO on the beneficial uses of medicinal plants for the treatment of diabetes mellitus.[52] Investigation of hypoglycemic agents from medicinal plants have also become more important. In traditional practice, medicinal plants are widely used in many countries for the treatment of diabetes mellitus. The antihyperglycemic effect of several plant extracts and herbal formulations that are used as antidiabetics remedies has been confirmed. Plant drugs are frequently considered to be less toxic and freer from side effects than a synthetic one. Combined extracts of herbs are used as the drug of choice rather than individual plant extracts. Herbal formulations such as D-400 and Trasina exhibit antidiabetic effects. Such Diamed have antidiabetic and antioxidative effects. Ethnobotanical information indicates that more than 800 plants are used as traditional remedies for the treatment of diabetes,[52] but many plants do not have a scientific scrutiny. Herbal medicines are used for primary health care, by about 80% of the world population particularly in

developing countries because of better cultural acceptability, safety, efficacy potent inexpensive, and lesser side effects. Plant drugs are frequently considered to be less toxic when compared to synthetic drugs. More than 1,123 plant species have been used to treat diabetes and more than 200 pure compounds have shown lowering blood-glucose activity.

The WHO expert committee recommended that the important to investigate the hyperglycemic agents from plant origin, which were used in traditional medicine for the treatment of diabetes mellitus. The antihyperglycemic agents have been focused on plants used in traditional medicine because that may be a better treatment than currently used synthetic drugs. The modern oral hyperglycemic agents showed undesirable side effects thus in recent years considerable attention has been directed towards the antidiabetic potential of medicinal plants and their herbal formulation in the management of disease. The concept of polyherbal is peculiar to Ayurveda although it is difficult to explain in term of modern parameters. There are many herbal formulations of varying potency. Since these preparation act by different combinations of these extracts will do better job in reducing blood glucose. In the traditional system of plant medicine, it is usual to use plant formulation and combined extract of the plant are used as a drug.[53]

The present aim of this work is to explore the scientific basis of the utility of this methanolic bark extract of *M. nepaulensis* for the correction of hyperglycemia and hyperlipidemia in diabetes. Moreover,the mode of action of this plant extract for it's antidiabetic activity is another part of researching that has been performed here by conducting an intravenous oral glucose tolerance test (OGTT), serum insulin assay and 28 days extract feeding process.

1.3 Plant introduction

Mahonia nepaulensis DC, is an evergreen or semi-evergreen shrub or small tree with yellow flowers in winter belonging to the family Berberidaceae, is widely distributed in the high Himalayan region at an altitude of about 1000-2000 m in Nepal, Sikkim, Bhutan, China, Vietnam[54] etc and is known vernacularly as "Jamanemandro" in Nepali and "Michiki swan" in Newari. It is a medium-sized fully hardy perennial tree of about 6m/19.7ft in height with plane brown bark and dull green leaves. The leaves are elliptical, obovate, and glabrous, measuring 7-9 cm in length. When crushed bark, emit

chocolate like odour. Flowers cluster as yellowish trichatomous panicles. The blossoms have 4 petals, 7-12 cm diameter fruits are avoided with a concave tip and a wrinkled texture. The fruits turn purplish upon ripening. Among various species of the genus *Mahonia* is the less known and traditionally used medicinal plant and origin the of these shrub is Nepal.

The principal chemical constituents reported for the genus *Mahonia* consists of isoquinoline alkaloids mainly aporphine groups, palmatine, magnoflorine, isocorydine, and oxyacanthine. These constitute possess array of biological activites such as anti-oxidant, anti-hyperglycaemic, anti-inflammatory, hepato-protective, and hypotensive properties.

Mahonia nepaulenssis DC has a major component called Berberine. This plant also contains other alkaloids which belong to class protoberberine, bisbenzylisoquinolines,[54] jattrrozhine, o-methyl puljabine,[55] isotetradine, homoaromaline[56] etc. These isoquinoline alkaloids have a yellowish color that is easily seen in the stem and leaves materials that contain a significant amount of these compounds. It is believed that plants that are rich in a wide variety of secondary metabolites belonging to chemical classes such as tannins, terpenoids, alkaloids, polyphenols are generally superior in their anti-microbial activities.[57]

The root and stem of this plant are a good source of berberine having anti-tumor activity and used as anti-mutagenic, stomachic, diaphoretic, astringent, gentle aperients, curative of piles, and periodic neuralgia. This plant is also used for antifungal textile dyeing based on their antifungal and antibacterial activity.[58]

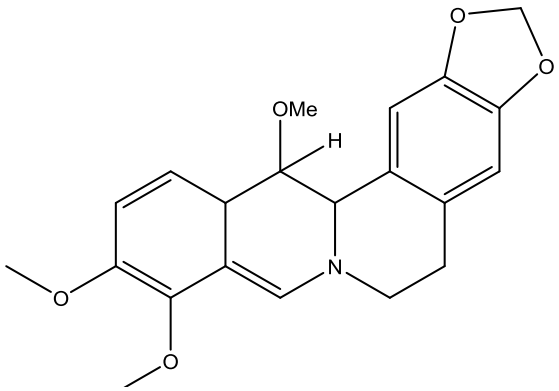
In India, this plant is commonly used for skin diseases, fever, eye cataracts, diuretic and eczema.[59]

In Nepal, it is useful in architectural and security barriers in the garden, traditional essential flower for conducting Bel Bibaha and Bratabhanda in Newar community but the scientific base medicinal value of this plant is not determined yet. This plant is completely new for antidiabeitc and antihyperlipidemic activity.

Figure 1: *Mahonia nepaulensis* flowers

Figure 2: *Mahonia nepaulensis* tree

Isolation and identification of compounds from the stem of *Mahonia nepaulensis*



5, 6–Dihydro-9, 10–dimethoxy benzo-1, 3–benzodioxolo 5, 6-quinolizinium (Berberine Derivatives)

1.4 Phytochemical

Phytochemicals are chemicals produced by plants through primary or secondary metabolism. They generally have biological activity in the plant host and play a role in plant growth or defense against competitor's pathogens or predators. Phytochemicals generally are regarded as research compounds rather than essential nutrients because proof of their possible health effects has not been established yet.

Phytochemicals under research can be classified into major categories such as carotenoids and polyphenols which include phenolic acids, flavonoids, and stilbenes/Lignans. Flavonoids can be further divided into groups based on their similar chemical structure such as anthocyanins, flavones, flavanones and isoflavones, and flavanols. Flavanols further are classified as catechins, epicatechins, and

proanthocyanidins. In total there have been over 25000 phytochemicals discovered and in most cases, these phytochemicals are concentrated in colorful parts of the plants like fruit, vegetable, nuts, legumes, whole grains etc.

1.5 Glibenclamide and Streptozotocin

Gliclazide is a second-generation sulfonylurea that acts as an insulin secretagogue drug for the management of type-II diabetes mellitus. Gliclazide selectively binds to sulfonylurea receptors on the pancreatic beta-cells surface thus shutting the ATP-dependent potassium channels on beta-cells. Subsequent depolarization of beta-cells leads to successive calcium influx that in turn leads to exocytosis and insulin release from vesicles.[60,61] Although treatment with sulfonylurea drugs resulted in a satisfactory decrease in fasting blood glucose and glycated hemoglobin via stimulation of insulin secretion from beta-cells, a progressive linear fall of beta-cells function occurs with sulfonylurea treatment over time and the reserve of insulin decreases as well which lead to a decline in treatment over time and the reserve of insulin decreases as well which lead to a decline in treatment efficacy so combination therapy should be used.[62,63] Before the discovery of insulin in the 1920s and the development of oral hypoglycemic agents, diabetes mellitus was treated mainly by a combination of fasting, diet control and plant therapeutics.

Streptozotocin

Streptozotocin (STZ) is an antidiabetic and anticancer agent that has been widely used for inducing type-I as well as type-II diabetes. Streptozotocin was initially isolated from streptomyces chromogenes in the 1960s, with its diabetogenic properties not described until 1963.[64] In the experiments of hypoglycemic effects evaluation animals are made diabetic usually by injecting alloxan or streptozotocin intraperitoneally (IP) or intravenously (IV). The diabetogenic effects are due to the selective destruction of pancreatic beta-cells. As a result of this action, the animal experiences insulin deficiency, hyperglycemia, polydipsia and polyuria all of which are characteristic of human type-I diabetes mellitus.[65] Several animal species, including the mouse, rat and monkey are sensitive to the pancreatic beta-cell cytotoxic effect of STZ. Free radicals play a crucial role in the streptozotocin-induced diabetes that produced oxidative stress and depletion of antioxidant systems in both blood and tissues, particularly the liver.[66]

The most common substances inducing diabetes in rat are alloxan and streptozotocin. STZ is taken up by pancreatic beta-cells via glucose transporter GLUT2. The main cause of STZ-induced beta-cell death is the alkylation of DNA by the nitrosourea moiety of this compound. However, the production of $NO^{\cdot}$ and reactive oxygen species may also be involved in DNA fragmentation and other deleterious effects of STZ.

1.6 In-vivo animal experiment

In-vivo is Latin for "within the living". It refers to tests, experiments and procedures that researchers perform in or on a whole living organism, such as a person, laboratory animal or plants. In vivo studies using animals provide invaluable information about various disease processes and help in the development of treatment strategies. Virtually every medical achievement in the twentieth century relied on the use of animals in some way. [67]

The Institute for Laboratory Animal Research of the U.S. National Academy of Science argues that even sophisticated computers are unable to model interactions between molecules, cells, tissues, organs, organisms, and the environment, making animal research necessary in many areas. Human testing cannot be done for any novel treatment unless adequate safety has been ensured. Any new implant materials should match various safety standards in terms of biocompatibility, mechanical stability lack of local and systemic toxicity. In vitro studies are the quintessential initial step in testing new implant materials but results from in-vitro studies are hard to apply to the in-vivo situation. Many aspects of biocompatibility and safety like tissue reaction to implant, systemic toxicity, long-term safety, late carcinogenicity, the effect of controlled physiologic loading, etc. cannot be tested in in-vitro situations.[68]

Today, a great deal about the physiology and anatomy of the human body is known by testing on animals that are biologically similar to humans. Non-human animal models usually serve as the penultimate testing method for a drug before it progresses to clinical trials in humans. It is common for animal research to involve mice, rats, birds and fish among others. According to the office of Technology Assessment approximately 17 to 20 million animals were used in 1986 for research in laboratories across the United States. These figures sparked outrage and garnered criticism from animal welfare activities around the world as well as the general public with regards to the pain and

suffering animals were being subject to. In a survey that reported the general public's views on animal testing, 73% of the questioned individuals agreed to ban animal testing due to it being unethical. There have been several arguments made by activists that it is morally wrong to let such a huge number of animals suffer and die because animals possess the same inherent living rights as human and the human benefits from animal testing remain intangible.[69]

Scientists are now much more aware of finding ways to overcome the ethical constraints associated with animal testing and acknowledge that it should be carried out not only humanly and responsibly, but the number and duration of animals used in drug screening are also reduced in addition to the need for non-animal alternatives to animal research. To achieve this goal, modern science has been developing and implementing various types of computerized modelling, biokinetic-modelling and invitro systems involving tissue cultures as testing strategies.

The benefits of in-vivo research have been enormous, especially in drug discovery and development and it is because of advances resulting from animal testing that people all over the world benefits by having a prolonged and improved quality of life. It would have dire consequences for public health and pharmaceutical research if in-vivo research were to be completely abandoned. Although animal use cannot yet be completely replaced, scientists must prioritize animal well being through reduction and refinement. With this sentiment the modern alternatives and stimulations that have been discussed so far have undoubtedly reduced the number of animals used in research. However, despite having a pivotal role in early preclinical testing, a successful in vitro outcome is only the first criterion for progression into in-vivo testing. Therefore, in vitro testing doesnot eliminate the need for animal testing but minimizes animal use due to the early detection of poor drug candidates.[70]

1.7 ELISA

It is the basic assay technique known as enzyme-linked immunosorbent assay used to detect and quantify peptides, antibodies, protein and hormones. It works on the principle that "specific antibodies bind the target antigen and detect the presence and quantity of antigens binding."

ELISA tests can be classified into three types. Toxicity depends upon the different methods used for binding between antigen and antibodies namely;

a) Indirect ELISA: Antigen is coated to the microtiter well

b) Sandwich ELISA: Antibody is coated on the microtiter well

c) Competitive ELISA: Antigen-coated Microtiter well is filled with the antigen-antibody mixture.

ELISA Assays: Antibody- Antigen interaction;

The fundamental molecular compounds of an ELISA typically include the use of antibodies conjugated to enzyme an immobilized molecules of interest and a detection substrate. A critical aspect that determines the success and quality of data obtained from an ELISA is dependent on the affinity and specificity of antibody-antigen interactions. Antigen-antibody interactions are influenced by numerous factors, including P^H, temperature, and ionic strength.

ELISAs that use direct detection methods require an immobilized antigen that is bound directly to the surface of an assay plate or indirectly by a capture antibody followed by an antigen-specific primary antibody conjugated to an enzyme and the detection substrate. The more commonly used direct detection format incorporates both an unconjugated primary antibody followed by a scope conjugated secondary antibody that is specific to the detection of the primary antibody. Indirect detection benefits from increased immunoreactivtiy with the target antigen as the conjugated enzyme element is only present on the secondary antibody. In addition to direct and indirect detection methods, capture or 'sandwich' assay use an additional antigen-capturing antibody that is first attached to the microplate surface, followed by the use of both a primary and an enzyme-conjugated secondary antibody, similar to the indirect method previously described.[71]

1.8 Toxicity

Toxicity is defined as the amount or degree of a substance needed to be poisonous. Toxicity is dependent on the amount and concentration used frequency of user interactions of the person. Toxicity can be systemic or local. It can be reversible or non-

reversible. It can be acute, subacute, or subchronic. Systemic toxicity means toxicity at a cell level that causes the organ to fail with the possible death of the organism. Local toxicity means the organ responsible for absorption and elimination may be severely affected, for example stomach, liver, skin, lungs or kidney. The effects may be reversible (when the toxic agent has been removed) or irreversible. Toxicity can be measured by its effects on the target (organisms, organ tissue, or coil) Because individuals typically have different levels of response to the same dose of a toxic substance, a population level measure of toxicity is often used which relates the probabilities of an outcome for a given individual in a population. One such measure is the LD_{50}. When such data does not exist, estimates are made by comparison to know similar organisms. Then specific factors are added to account for uncertainties in data and evaluation processes. For example, if a dose of a toxic substance is safe for a laboratory rat, one might assume that one-tenth that dose would be safe for a human.[72]

The toxicity of a substance can be affected by many different factors, such as the pathway of administration (whether the toxicant is applied to the skin, ingested inhaled, injected), the time of exposure (a brief encounter or long term) the number of exposures(a single dose or multiple doses over time), the physical form of the toxicant (solid, liquid, gas) the genetic makeup of an individual, an individual's overall health, and many others several of the terms used to describe these factors has been included here ;

Acute exposure; A single exposure to a toxic substance that may result in serve biological harm or death; acute exposures are usually characterized as lasting no longer than a day. Chronic exposure: Continuous exposure to a toxicant over an extended period of time, often measured in months or years, can cause irreversible side effects.

1.9 Research plan and design

1.9.1 Research hypothesis

Extract of *Mahonia nepulensis* might be a useful candidate for manage inhibition against diabetes mellitus.

1.10 Justification of study

Nepal is ranked 9th among the Asian countries for its floral wealth with an estimated 9000 species of flowering plants. So far 653 species of flowering plants have been reported. Plants are important for local livelihoods and income generation and they do fetch higher market prices.[73]

Numerous drugs have been introduced to international markets through validation of traditional medicines, indigenous therapies, and ethno pharmacological practices.

Many commercially available drugs have shown significant importance in preventing diabetic complications, but they are still not successful due to their detrimental side effect and low penetration power to the target tissue. Therefore, introducing natural resource that will contribute to the rapid, safer and effective formula for the prevention against the diseases like diabetic complication.[74]

This research is focused on screening the compounds that are present in the medicinally important plant *M. nepaulensis* these compound will be responsible for minimizing the increased level of blood glucose. Medicinal plants have been a valuable source of therapeutic agents, and still many of today's drugs are plant-derived natural products or their derivatives. Medicinal plants are being used to treat diseases from history and are still included under various systems such as Ayurvedic Unani , Tibetan, and Siddha etc. At present several organizations have been prioritizing the research and evaluation of herbal treatments for HIV/AIDS, Malaria, sickle cell anemia and Diabetes mellitus revealing the good potential for herbal drugs. Bioassay technique is one of the feasible techniques to evaluate the therapeutics potential of the plant extracts and fully characterized compounds. Various isolated compounds and their derivatives of *M. nepaulensis* could be used to discover drugs by pharmaceutical company.[75]

1.11 Work plan

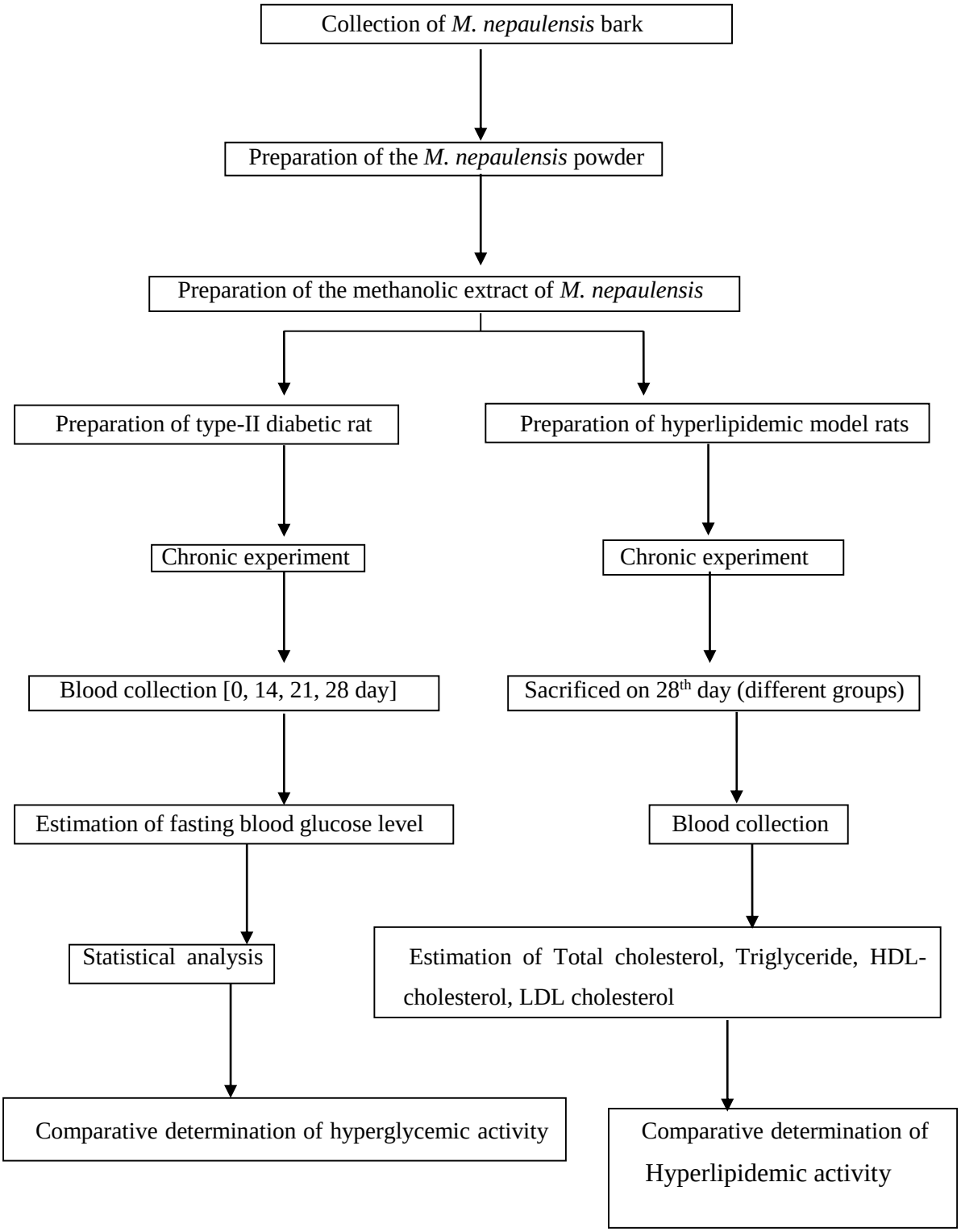


Figure 3: Flow chart for work plan

CHAPTER TWO

OBJECTIVES OF THE STUDY

2.1 General Objectives

- To evaluate the anti-diabetic activity of methanolic extract of *Mahonia nepaulensis* DC bark in neonatal streptozotocin induced type-II diabetic model rats.

2.2 Specific Objectives

- To prepare methanolic extract of *M. nepaulensis* bark.
- To perform phytochemical screening of methanolic extract *of M. nepaulensis* bark.
- To induce diabetes in rats.
- To handle, feeding and body weight measurement of normal and diabetic rats.
- To evaluate the effect of *M. nepaulensis* bark extracts on the rat body weight.
- To determine the effect of *M. nepaulensis* bark extract on fasting serum glucose level of normal and diabetic rats by performing Oral Glucose Tolerance Test (OGTT).
- To evaluate the OGTT on normal and type-II diabetic model rats on 21st day.
- To evaluate the chronic effect of *M. nepaulensis* extract on lipid profiles in n-STZ induced type-II diabetic rats.
- To find an effect of *M. nepaulensis* on the ratio of total cholesterol (TC)& high-density lipoprotein (HDL) and total triglyceride (TG) & HDL of normal and diabetic rats.
- To determine the chronic effect of *M. nepaulensis* extract on liver glycogen of normal and type-II diabetic model rats.
- To evaluate the chronic effect of *M. nepaulensis* bark extract in different organs of n-STZ induced type-II diabetic rats.

CHAPTER THREE

LITERATURE REVIEW

The different sources of information like some books, journal articles, websites (Sci-hub.tw, Google scholar.com, Research gate) and discussed on a first come first basis , the literature review was done for selected plants.

Govindachari et.al (1957) suggested that the presence of alkaloids as the major compound of which belong to class protoberberines and bisbenzylisoquinolines whereas, it is reported that *M. nepulensis* belongs to the family Berberidaceae , which is small shrub tree distributed in the high mountainous areas at an altitude of about 1000 m-2000 m in Nepal.[76]

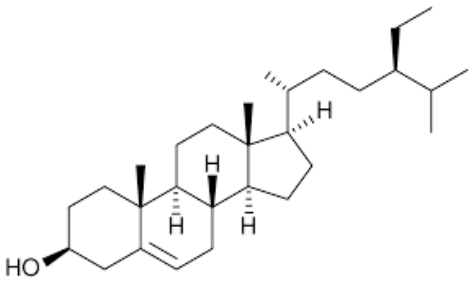
Nguyen et. al (2009) isolated alkaloids like Berberine, Jatrrorzhine , O-methyl puljabine, Isotetradine, homoaromaline etc from the different parts of *M. nepaulensis.* Their structures were determined based on spectroscopic analyses (UV, IR, ^{1}H, $^{13}CNMR$ and HMBC)[77]

Thusa and Mulmi (2017) isolated four compounds from the methanolic extract of the bark of *M. nepaulensis*. The four compounds are MN_1 compound, MN_2 compound, MN_3 compound and MN_4 compound respectively. These compound structures were determined based on melting point, column chromatography, TLC and spectroscopic analyses (UV and IR)

Out of four compounds, the compound MN_1 was white crystalline having M.pt. 135 ℃ and showed single spot on TLC in 20% ethylacetate in hexane solvent system with R.F. value 0.43. It gave greenish red test in Liberman Burchard Test which indicate compound was sterol. It was identified as β-sterol with the help of co-TLC and melting point.

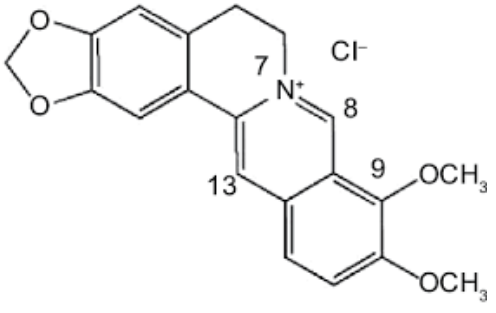
The compound MN_2 was needle-shaped yellow colored crystalline compound having MP 200-205℃. IR spectra showed peaks of 3549cm^{-1}, 3402cm^{-1}, 3317cm^{-1}, 3224cm^{-1}(N-H stretch), 3055cm^{-1}(C-H stretch in aromatic functional group), 2908 cm^{-1}, 2846cm^{-1}(CH stretch of alkenes), 2121cm^{-1}(C triple bond N stretch), 1605cm^{-1}(C-C stretch n ring aromatic), 1396cm^{-1},1365-60cm^{-1}(C-H bending), 1293.02cm^{-1}, 1204.44cm^{-1}(C-O stretch C-N stretch in aromatic amines) 1111cm^{-1} , 1041cm^{-1}(C-O-C bending), 970cm^{-1}(O-H bending), 887cm^{-1},825cm^{-1}, 640cm^{-1},524cm^{-1}, 424cm^{-1},

393cm^{-1}(C-H out of plane bending) which identical with that of authentic sample of *Berberine.*



Berberine

The compound MN_3 was needle-shaped orange red colored crystalline compound having MP 140℃. It showed a clear spot, which was observed in 30% methanol in $CHCl_3$ with RF value 0.81. Meyer's test and Dragendroff's test indicate compound was alkaloid. The authentic sample was 7,8-dihydro methoxyberberine which gives the peaks at 1605cm^{-1}, 1510cm^{-1}, 1050cm$^{-1,}$ 975cm^{-1} and 850cm^{-1}. The fingerprint region peaks of the isolated compound were very identical with that of the authentic sample 7,8-dihydro methoxy berberine for IR spectrum of MN_3. The UV spectral data λmax /n (EtOH) of the authentic sample were 285 and 365. The UV spectral data of 7,8-dihyro-8-mehoxy berberine were very much identical with that of authentic sample 7,8-Dihydro-8- methoxy berberine were very much identical with that of the authentic sample 7,8-dihydro-8-methoxy berberine whereas the compound MN_4 was needle-shaped dark orange-brown colored crystalline compound MN_4 was obtained in a pure and dry state. A sharp melting point was found to be 140℃ and decomposed 160℃. A clear spot was observed in 30% methanol in $CHCl_3$ with RF value 0.86. Furthermore, Meyer's test and Drayendroff's test indicated the compound was alkaloid. The exact structure and name of the compound will be confirmed after analysis of NMR, Mass and UV spectra.



β-sitosterol

Das and Chhetry (2016) investigated that the NMR(root of *Mahonia nepaulensis*) and MNB(stem bark of *Mahonia nepaulensis*) extract treated group at the doses of 200 and 400 mg/kg b.w. respectively, which were observed that there are significant increases in body weight compared to diabetic control group values are expressed as mean ± S.E.M. whereas, Kumar et al (2011) suggested that STZ include diabetic, is associated with a characteristics loss of body weight due to excessive catabolism of protein to provide amino acids for gluconeogenesis during insulin deficiency results in muscle wasting and weight loss in diabetic untreated rats.

Itankar et al., (2011) suggested that the animals were overnight fasted and diabetes was induced experimentally in rats by a single intraperitoneal(i.p.) dose of a freshly prepared solution of streptozotocin (STZ) at a dose of 50 mg/kg b.w. in 0.1 M cold citrate buffer pH 4.5. The animals were allowed to drink 5% glucose solution overnight to overcome the drug induced hyperglycemia. Whereas, Huang et al (2011) suggested that after 72h of the STZ injection, blood was withdrawn from tail vein of the animals and fasting blood glucose (FBG) level was estimated by glucometer. The rat with a blood glucose level was above 250 mg/dL were selected for the experiment as diabetic rats.[80]

Das and Chhetry (2016) suggested that, the blood glucose levels of the normal control, MNR and MNB extract groups (at dose 200 and 400 mg/kg b.w.) were measured after oral administration of glucose (2 g/kg b.w.). All groups had shown increased blood glucose level was observed after 30 min. Its level remained high over next 60 min. MNR (200 and 400 mg/kg) and MNB (400 mg/kg) group showed significant ($p<0.05$) decrease in blood glucose level at 60 and 120 min. compared to the normal control group. Fasting blood glucose level of the treatment group significantly ($p<0.05$) reduced by the 14^{th} days treatment with MNR and MNB extract, fasting blood glucose level reduced from 5^{th} day. At the end of the experiment (15^{th} day) fasting blood glucose level was found to be 176.64±1.50, 144±2.12, 188.52±1.66 and 161.07±2.53 mg/dl for MNR 200 and 400 mg/kg, MNB 200 and 400 mg/kg comparable with glibenclamide 0.5 mg/kg group.

American Diabetes Association (ADA) has been recommended that 6.5% HbA1c as the cut point for diagnosing diabetes. HbA1c is formed throughout the circulatory life of red blood cells (RBCs) by the addition of glucose to the N-terminal of the

hemoglobin β-chain. This non-enzymatic process reflects the average exposure of hemoglobin to glucose over an extended period.[81] HbA1c was found to increase in patients with diabetes mellitus due to glycosylation of a number of proteins haemoglobin, β-crystalline of the eye lens and increase HbA1c level directly proportional to the fasting blood glucose level.[82] However, Das and Chhetry (2016) shows that MNB extract treatment group significantly decline in HbA1c level compared to the diabetic control.

Naskar et al., (2011) was observed that there was an elevated level of serum TG, Cholesterol, LDL cholesterol and decreased levels of HDL cholesterol in diabetic rats. This might be attributed due to lack of insulin which activates the lipase enzymes, hydrolyzing the stored TG and releasing large amounts of fatty acids and glycerol in the circulating blood.[83] However, Wang et al (2010) was suggested that high levels of total cholesterol and LDL cholesterol are major risk factors for cardiovascular diseases, whereas increased HDL cholesterol is associated with a decrease in cardiovascular risk. The post treatment results for MNR and MNB extract treated diabetic rat has showed declined in serum TG cholesterol, LDL cholesterol, level with marked increased in HDL levels. These results indicate that the MNR and MNB extract has a lipid lowering effect on the diabetic rats. Treatment with MNR and MNB extract at dose 200 and 400 mg/kg b.w. in diabetic induced rats significantly ($p<0.05$, $p<0.001$) decreased the triglycerides, cholesterol, LDL cholesterol level with a significant ($p<0.005$, $p<0.001$) increase in HDL cholesterol level in a dose dependent manner as compared to the diabetic control group.[84]

Thusa and Mulmi (2017) suggested that hexane extracts and ethyl acetate extract of stem and leaves of *M. nepaulensis* were resistant to all of the bacteria species tested while methanol extract of this plant species were found to be strongly active towards the gram positive bacteria *Staphylococcus aureus.* Similarly, from brine shrimp bioassay the cytotoxicity LC_{50} values (μg/ml) for stem and leaves of *M. nepaulensis* have 8.3 (μg/ml) 389.04 (μg/ml) which indicate that stem of *M. nepaulensis* highly cytotoxicity and leaves of M. nepaulensis moderately cytotoxic. (less than 1000 μg/mL) is bioactive)

The stem and wood of this plant have anti-inflammatory, anti-bacterial, anti-fungal activity. It is particularly used for the treatment of skin diseases like eczema, psoriasis etc. This plant contains alkaloids as a major compound.

CHAPTER FOUR
MATERIALS AND METHODS

4.1 Materials

4.1.1 Chemicals

In the extraction process, 80% methanol solvent was used which was an analytical grade. Reagents and solvents used during phytochemical analysis were prepared with the chemicals provided in the laboratory. For the induction of type-II diabetes 0.1 M citric acid and streptozotocin were used. An oral glucose tolerance test (OGTT), GOD-PAP reagent and anhydrous glucose solution were used for standard preparation. In hypolipidaemia test, also GOD-PAP reagent and anhydrous glucose solution were used for making standard by serial dilution method.

For evaluation of liver glycogen, 5% Trichloro Acetic Acid (TCA), 10N potassium hydroxide, Paraffin solution, Anthron, Sulphuric acid, Glacial Acetic Acid (GA) were used. For the short-time anesthesia diethyl ether was used for rats. Whatman no.1 filter paper, saline, ice all were provided in the laboratory. To control the elevated blood glucose standard glibenclamide drug was used. Distilled water and pettles were provided during the experiment.

4.1.2 Apparatus/ Equipment

Grinding Machine

Rota Evaporator: EYELA^{SB-11}, Heating: 40^{o}C ± 1 Speed: 2, 7

Weighing machine: SHIMADZU

Dessiccator

Freeze Dryer with Shell Freezer (Ilshine Biobase, Temperature: -47^{o}C, Vac. Gauge: 999 mTorr, Temperature: 31^{o}C)

HH-S Digital Thermostatic Water Bath (AC 220 V 50 HZ, Heat power 800 W, Made in China)

POWER SONIC 405 (Microprocess Controlled Benchtop ultrasonic chamber, Bath capacity: 5.7 L, Frequency: 40 KHZ)

ELISA:

96 Well plate Reader, Centrifuge, Vortex, Autoclave, Incubator, Deep Freeze

Syringe, Eppendroff, Mortar and pestle, Test tube, Screw cap test tube, Micropipette etc were used.

4.2 Methods

4.2.1 Collection and Authentication of Plant materials

The bark of *M .nepaulensis* was collected from Godavari in November and December 2019. The selection of part of this plant was based on its reported ethno-medicinal practices by local people and literature review. Collected plant sample was authenticated at National Herberium and plant laboretoris, Godavari, Lalitpur, Nepal. The collected plant materials were dried under shade and grinded to a fine powder and collected in airtight plastic bags.

4.2.2 Preparation of extract from plant materials

The extraction was done by the cold percolation method. In the first time 504 gm of fine powder was dissolved in 3000 mL of 80 % methanol and 222 gm of fine powder was dissolved in 2000 mL of 80% methanol at room temperature (3000 mL×3 days ×3, 2000 mL× 3 days×3). Again, the second time 545.38 gm of fine powder was dissolved in 3500 mL of 80 % methanol at room temperature (3500 mL×3 days×3). Each day obtaining solution was filtered by using filter paper (Whatman no.1) and stored in a glass bottle. The final collection of dissolved parts were evaporated at reduced pressure at 50 ℃ using a Rotary Evaporator to yield the crude extract. Then, the crude extract was subjected to a freeze dryer to get the solid extract. The weight of solid extract was taken, collected in a glass bottle, labeled and stored in a refrigerator until further use.

The yield of different extracts was calculated by using the given formula;

$$\text{Percentage yield} = \frac{\text{weight of extract(gm)}}{\text{weight of sample powder(gm)}} \times 100\%$$

4.2.3 Phytochemical screening of plant extract

The solution of an extract of bark was tested for the presence of major phyto-constituents like alkaloids, steroids, tannins, saponins, glycosides, etc. based on the standard procedure described by Sasidharan *et al.,* 2011; Tamilselvi *et al.,* 2012 and

Yadav *et al.*, 2011.[85,86,87] The qualitative results are expressed as (+) for presence and (-) for the absence of phytochemicals.

4.2.4 Experimental design

Starting Date:

04-02-2020	05-02-2020	07-02-2020	08-02-2020

Ending Date:

02-03-2020	03-03-2020	05-03-2020	06-03-2020

Duration: 28 days

Total number of rats n= 24

Group-1	Group-2	Group-3	Group-4
Normal Water control	Diabetic Water control	Glicazid treated	Extract treated
n= 6	n= 6	n= 6	n= 6
(10 mL/kg/B.W)	(10 mL/kg/B.W)	(20 mg/kg/5 mL)	(1.25 g/kg/10mL)

04-02-2020	05-02-2020	07-02-2020	08-02-2020
On 0^{th}day			
Blood collection at fasting condition for glucose, lipid profile & Insulin			

24-02-2020	25-02-202	27-02-2020	28-02-2020
on 21^{st} day			
Blood withdrawing at time interval of 0 min, 30 min, 60 min & 90 min to perform OGTT			

02-03-2020	03-03-2020	05-03-2020	06-03-2020
on 28^{th}day			
After 12 hours of fasting, sacrifice of rats and collection of blood and organs for glucose, lipid profile, Insulin level estimation & liver glycogen tests			

Date of Body Weight Measurement

Cage-01 (n= 5)	Cage-02 (n= 8)	Cage-03 (n= 5)	Cage-04 (n= 6)
03-02-2020	04-02-2020	06-02-2020	07-02-2020
09-02-2020	10-02-2020	12-02-2020	13-02-2020
16-02-2020	17-02-2020	19-02-2020	20-02-2020
23-02-2020	24-02-2020	26-02-2020	27-02-2020
01-03-2020	02-03-2020	04-03-2020	05-03-2020

Figure 4: Experimental design

4.2.5 Animals

Healthy male and female Long Evans rats of local strain, having around 150 to 290 gm, were taken for the experiment, which was bred in the Animal House, Bangladesh University of Health and Science (BUHS), Dhaka. The animals were fed a standard pellet diet and water, used for the present investigation of hypolipidemic and hypoglycemic effect studies. The proper environmental condition for the experimental rats was confirmed, kept under firm supervision and maintained at a constant room temperature of 25℃ ± 5℃, with humidity of 40 % to 70 % with a natural day-night cycle. For the hypolipidemic effect studies, the rats were divided into eight groups consisting of six in each, maintaining the body weight of experimental rats.[88]

For evaluation of the hypoglycemic activity of *M. nepaulensis* the rats were segregated into four groups comprising of six in each with the congruence of body weight. Before starting the experiments the weight of the rats were taken precisely and the rats was marked on the tail. All the experimental protocols were approved by the ethical committee.

4.2.6 Induction of T2DM and body weight measurement

The animal was overnight fasted and diabetes was induced experimentally in rats by a single intraperitoneal (i.p.) dose of a freshly prepared solution of streptozotocin (STZ) at a dose of 90 mg/kg b.w. in 0.1 M (1.0507 gm citric acid dissolve in 50 mLdion-water) cold citrate buffer (p^H4.5). For the preparation of type-II model rats 48 hours rat's pup (Average body wt. 7gm) was used at a dose of 90 mg/kg body wt. in citrate

buffer (90mg/10mL). The solution was injected intraperitoneal at dose 10 µL/g of STZ solution and 1 pup gets 10 µL STZ solution. Three months of injection rats were checked for type-II diabetes with an OGTT test (dose 2.5g/kg b.w.)

Day zero was the day on which experimental design. During the experimental period the body weights were monitored weekly and the change in body weight of the rats was estimated as follows;

$$\text{Change in body weight} = \frac{\text{final body weight} - \text{initial body weight}}{\text{intial body weight}} \times 100\%$$

4.2.7 Glucose measurement for checking T2DM and separation of group

The glucose measurement for checking T2DM was performed in overnight fasted (18h) streptozotocin-induced rats. Rats was an average weight about 140-200 gm. Glucose (2.5/kg/10mL b.w.) was fed all streptozotocin induced rats, and blood was withdrawn from tail vein at 0, 30, 60, and 90 min of glucose administration. After that glucose levels were compared at 0, 30, 60 and 90 min. The glucose level was reduced at 90 min as compared to 60 min or before, then rats were considered to be T2DM and selected for the experiment.

For evaluation of the hyperglycemic activity of *M. nepaulensis* bark, the rats were segregated into four groups comprising of six in each with the congruence of body weight. These included

1. Group-A Normal control rats fed with 100% lab diet.
2. Group-B Streptozotocin-induced diabetic rats fed with 100% lab diet, diabetic control
3. Group-C Streptozotocin-induced diabetic rats fed with lab diet plus glibenclamide, drug control)
4. Group-D Streptozotocin-induced diabetic rats fed with 100% lab diet plus extract of *M. nepaulensis*

Before starting the experiment, the weights of the rats were taken precisely and the rats were marked on the tail, right-front, right-back, left-front, left-back and kept unmark which was subsequently applied as identification purpose for a particular rat, so that the

reaction of a specific rat before and after the drug administration could be observed individually. The rat were kept in four cages, in which cage first contains all female rat cage second third and fourth contains all male rats. Treatment rats were mixed in all cages and they are identified by their tail marking.

4.2.8 Treatment and measuring of fasting serum glucose level (OGTT)

Normal and experimentally diabetic rats were randomly divided into four groups (each group consist of 6 rats ; n=6), except Group A, which served as non-diabetic and was received normal water(10 ml/kg b.w.) all other groups were comparised of diabetic control were received normal water (10 ml/kg b.w,) Group C received reference drug glibenclamide (20 mg/kg/5 mL b.w. po) and group D were received MN extract (1.25 g/kg/10 mL b.w.) daily for 28 days.[89]

The oral glucose tolerance test was performed in overnight fasted 18 hours normal rats. Rats were divided into four groups. Group A served as normal control, Group B served as diabetic control, Group C served as drug control and Group D received methanol extract of stem bark (MNB) of *M. nepaulensis*. Blood was withdrawn from tail vein approximate 300 µL in eppendroff tube. After 25 min. later collected blood sample was centrifuged for 10 minutes at 3700 rpm and serum was separated. The 20 mM (milimolar) glucose standard solution was prepared by dissolving 36 mg anhydrous glucose in 10 mL dion water. Then, serial dilution was done at different concentrations. After standard preparation 5µL standard solution and 250 µL GOD-PAP reagents, similarly 5 µL samples and 250 µL GOD-PAP reagents were pipette and kept into 96 well plates. After incubation for 15 min. at 37℃, the plate was kept for absorbance reading at 490 to 510 wL in ELISA. Finally, the concentration of sample the was calculated from absorbance using a kinetic calculation program.

Table 1: Preparation of standard solution by serial dilution method

Concentration (mM/L)	Water (µL)+ Glucose 20 mM/L (µL)	Total Volume (mL)
1	950+50	1
2	900+100	1
4	800+200	1
8	600+400	1
12	400+600	1
16	200+800	1
20	0+1000	1

4.2.9 Estimation of fasting blood glucose level

The fasting blood glucose level was measured using glucometer (Bayer's contour TS) on the 0, 21, and 28 days. Day zero was the day on which experimentally diabetic rats selected for the experimental design. The blood sample was collected by nicking the lateral tail vein using sterile scalpel scissors under ether anesthesia. Just before cutting the tail was immersed into warm water 40℃ for around 25 seconds for vasodilation. Blood glucose level estimated by the above procedure.

4.2.10 Estimation of the lipid profile

The rats were made hyperlipidemic by feeding a high-fat diet for 28 days containing Lab Diet. For evaluation of hypolipidemic activity, the extract of the *M. nepaulensis* was administered orally with the regular diet for 28 days. Blood samples were collected on the 28th day by sacrificing the rats after making anesthesia using diethyl ether. After sacrificing about 2 mL of blood was taken cautiously and the blood samples were then centrifuged.

After 20 minutes at 4000 rpm for 10 minutes and recentrifuged at 2000 rpm for 5 minutes. After that, the serums samples were separated and taken into Eppendorf tubes. Then the serum triglyceride, total cholesterol, high density lipoprotein cholesterol (HDL-C) and low-density lipoprotein cholesterol (LDL-C), were measured. One milliliter of serum was aliquoted and kept frozen at -20°C until analysis of serum for lipid profile. Serum total cholesterol was measured by enzymatic colorimetric (Cholesterol Oxidase /Peroxidase, CHODPAP) method (Randox Laboratories Ltd.,

UK) using autoanalyzer, AutoLab. Serum HDL-cholesterol was estimated by enzymatic colorimetric (Cholesterol CHODPAP) method (Randox Laboratories Ltd., UK) using microplate reader (Bio-Tec, ELISA) and Serum triglyceride (TG) was examined by enzymatic colorimetric (GPOPAP) method (Randox Laboratories Ltd., UK) using auto analyzer, Auto Lab. Then, serum LDL cholesterol was calculated manually. The calculated formula was

$$\text{LDL-C}=\text{TC-}\left(\frac{\text{TG}}{5}+\text{HDL-C}\right)$$

All the groups of rats were remained under similar environmental conditions and provided with the measured food and water throughout the experiment. The body weight of each rat was measured and compared with the controls.

4.2.11 Hepatic glycogen content measurement

In the last day (28^{th} day) of experiment all the rats were sacrificed and hepatic glycogen content was measured accordingly.

In the first step, 200mg liver rat was mixed with 5% trichloro acetic acid (TCA) to make homogenized solution. The homogenized suspension was filtered and a watery-like sample solution was obtained. Then, a standard solution was prepared in following ways;

Table 2: Preparation of standard solution for glycogen test

Tubes	5% TCA(ml)	10N KOH(ml)	Sample(s)
S_0	1	2	-
S_1	1	2	-
S_2	1	2	-
S_3	1	2	-
S_4	1	2	-
S_5	1	2	-
S_6	1	2	-
S_7	1	2	-
Sample 1	-	2	1

All the test tubes were incubated for 1 hr in paraffin solution at 100℃.

In, the second step, anthrone sulphuric acid solution was prepared by dissolving 4 mg anthrone in 2 mL H_2SO_4 solution.

In the third step, glucose standard solution was prepared by dissolving 0.0094 gm glucose stock in 50 mL distilled water and serial dilution from glucose stock was carried out in following ways;

Table 3: Preparation of serial dilution from glucose stock

Concentration of glucose (mg/mL)	Glucose (µl & mL)	Water (mL)
0.3375/100	200 µL	5.8 mL
0.75/100	400 µL	5.6 mL
1.5/100	800 µL	5.2 mL
3.0/100	1.6 mL	4.4 mL
4.5/100	2.4 mL	3.6 mL
6.0/100	3.2 mL	2.8 mL
7.5/100	4.0 mL	2.0 mL

In the fourth step, after 1 hr incubation, all the test tubes were kept on ice cool water and 1 ml Glacial acetic acid was added to all the test tubes.

The sample and blank test tubes were made 10 mL volume by adding 6 mL deionised water and for standard solution glucose solution (6 mL) was added sequentially. All the sample test tubes were mixed by vortex, then 2 mL anthrone-H_2SO_4 solution was taken in new test tubes. After that 1ml solution was taken from 10 mL sets serially and shakes in ice cool water. All the test tube was incubated in paraffin solution for 10 min at 100℃ and again they are kept into ice-cool water for few minutes. And finally 200 µL was pipette on ELISA plate and reading was taken with 650 nm or 492 nm frequencies.

4.2.12 Organ weight measurement and toxicity

All the experimental rats were sacrificed on the 28th day. The different organs like kidney, spleen, heart, liver, and pancreas were separated and there was no toxicity detected.

4.2.13 Data analysis

The data analysis was performed using SPSS 16.0. Windows program $P<0.05$ was considered significant. For charts and a graphical representation, Microsoft Word and Microsoft Excel were used. All the values of body weight, fasting blood sugar level and biochemical estimations were expressed as mean ± standard error of the mean (mean ± SEM) and analyzed for ANOVA and post hoc Dun net's t-test. Differences between groups were considered significant at $P<0.001$ levels.

CHAPTER FIVE

RESULTS AND DISCUSSION

5.1. Percentage yield of plant extract and their physical characteristics

The percentage yield of *M. nepaulesis* extract and their physical characteristics are demonstrated in the table;

Table 4: Percentage yield of plant extract

Plant	Part	Extract	% Yield	Color	Consistency
Mahonia nepaulensis	stem bark	methanol	7.39	dark orange	Sticky

It is clear that the dark orange color of the extract was obtained and the percentage yield of plant extract (bark) was found to be 7.39% which is a slightly lower yield compared to that of previous reports.

5.2. Phytochemical Screening

The obtained results from phytochemical screening of bark of *M. nepaulensis* are presented below in the table.

Table 5: Phytochemical Screening of plant extract

SN	Phytochemicals	*M. nepaulensis (bark)*
1	**Alkaloid**	++
2	**Saponin**	-
3	**Coumarin**	-
4	**Glyco-side**	+
5	**Tanin and Polyphenol**	++
6	**Reducing Sugar**	+
7	**Flavonoid**	+
8	**Steroid**	-
9	**Triterphenoid**	+
10	**Anthra-quione**	+
11	**Cartenoids**	-

Where, (+) indicates the presence and (-) indicates the absence of phytochemicals.

5.3 Results for antidiabetic effect of *Mahonia nepaulensis* bark extracts

In the first part of the study, the antidiabetic effect of *Mahonia nepaulensis* was investigated on type 2 diabetic model rats. Rats (total 24 rats) were designated and selected according to their body weight and glucose level for making the group in the experiment. Rats were divided into four groups with six rats in each group. One extract-treated i.e Extract-1 was *Mahonia nepaulensis* methanolic extract, one Positive control i.e Gliclazide treated, and two negative control group i.e Diabetic water control and Normal water control was taken for comparing with each other's.

5.4 Effect of *M. nepaulensis* bark extracts on the body weight

The effect of *M nepaulensis* extracts on body weight of type-II diabetic model rats during 28 days of chronic administration was observed. Body weight of each rat was taken at seven days intervals. Gradually increased Body weight was observed in all the groups at the end point of the study period. (**Table 6& Figure 5**)

Table 6: Effect of *Mahonia nepaulensis* extract on the body weight of normal and type-II diabetic model rats.

Group	Body weight (gm) (M±S.D)				
	0 day	7 days	14 days	21 days	28 days
NWC (n=6)	187.66±16.56 (100%)	211.00±14.22 (112%)	226.00±24.19 (120%)	246.16±22.85 (131%)	241.66±19.10 (128%)
DWC(n=6)	193.50±29.36 (100%)	213.66±21.48 (110%)	224.83±24.50 (116%)	237.33±27.81 (122%)	246.16±32.92 (127%)
GT(n=6)	186.50±32.28 (100%)	201.00±19.63 (107%)	209.66±30.36 (112%)	221.00±33.23 (118%)	232.66±35.38 (124%)
Ext-1(n=6)	201.16±27.09 (100%)	210.00±23.63 (104%)	226.33±22.99 (112%)	225.83±26.38 (112%)	240.16±27.92 (119%)

Group NWC, DWC, GT, Ext-1 represent normal water control diabetic water control rat, Gliclazide treated diabetic rat and *Mahonia nepaulensis* treated diabetic rat respectively. Data presented as mean ± standard deviation (M±SD).

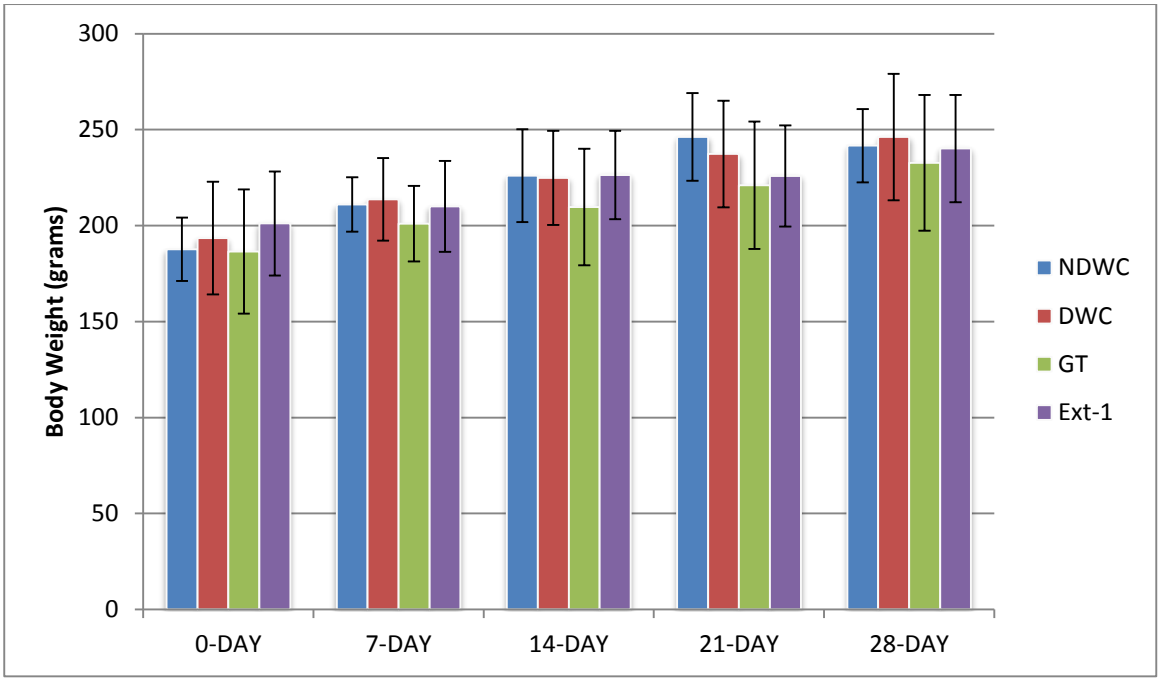


Figure 5: Effect of *M. nepaulensis* on the body weight of normal and type –II diabetic model rats

There was a tendency to decline the body weight in all sample fed rat groups whereas normal control rats displayed an increase in body weights at the end of thestudy period (Table 6). Mathematically around a maximum 0.90% body weight reduction was observed in comparison to the body weights measured on the first day considered as 100%. It is noticed from the data presented in Table 2 that there was a raise in body weight in each group. But the sample administered groups displayed slower enhancement in body weight than high-fat diet-fed groups. Data also suggest that after feeding of only high fat diet for 28 days, body weight augmentation was almost a maximum of 10% if the initial body weight was considered as 100%. But, when along with the high-fat diet, the sample at different percentages was given; the body weight increase was only a maximum of 0.20% considering the initial body weight as 100%.

STZ induces activation of poly adenosine diphosphate ribosylation and nitric oxide release. As a result of STZ action, pancreatic β-cells are destroyed by necrosis. STZ induced diabetes is associated with a characteristic loss of body weight due to excessive catabolism of protein to provide amino acids for gluconeogenesis during insulin deficiency resulting in muscle wasting and weight loss in diabetic untreated rats. Diabetic rats treated with the MNB extract showed significant improvement in body weight as compared to the diabetic control animals.

5.5 Effects of *M. nepaulensis* on fasting serum glucose level of type 2 diabetic model rats

Results of fasting serum glucose (FSG) levels of the studied rats at baseline (before the onset of feeding i.e 0 day) and 28 day of feeding is presented in **table 7.** At baseline FSG levels ofT2DM water control, Gliclazide and extract-1 treated group was almost similar (M±SD, mmol/l;12.34±2.48; 13.60±2.59; and 11.70±1.32 respectively).

After oral administration of respective treatment for 28 days of the experimental period, FSG level of all the groups of rats decreased except for normal water control group. However, there were 36 % and 19 % deceased were found in Gliclazide control and Ext-1 treated group respectively.

Table 7: Effect of *Mahonia nepaulensis* extract on fasting serum glucose level of normal and type-II diabetic rats

Group	Fasting Serum Glucose Level (mmol/L)		
	0 day	21 day	28day
NWC (n=6)	7.21±0.79 (100%)	5.03±0.81 (69%)	7.45±1.03 (103%)
DWC(n=6)	10.53±0.74 (100%)	6.87±1.11 (65%)	9.80±0.41 (93%)
GT(n=6)	13.60±2.59 (100%)	7.93±0.61 (58%)	8.73±0.73 (64%)
Ext-1 (n=6)	11.40±1.19 (100%)	10.99±0.95 (96%)	9.25±0.65 (81%)
One Way ANOVA			
NDWC Vs DWC	0.03	0.00	0.00
NDWC Vs GT	0.00	0.00	0.21
NDWC Vs EXT-1	0.00	0.00	0.02
DWC Vs GT	0.05	0.36	0.49
DWC Vs EXT-1	1.00	0.00	1.00
GT Vs EXT-1	0.40	0.00	1.00
Paired sample t test (0 day Vs 28 day)			
Group	0day Vs 21day	0day Vs 28day	
NDWC (n=6)	0.000	0.642	
DWC(n=6)	0.000	0.012	
GT(n=6)	0.003	0.002	
Ext-1 (n=6)	0.200	0.018	

Group NWC, DWC, GT, Ext 1 represent normal water control rat, diabetic water control rat, Gliclazide treated diabetic rat, M. nepaulensis treated diabetic rat respectively. Data represented as mean ± standard deviation (M ± SD). Statistical comparison between groups was performed using one way ANOVA and paired sample t test.

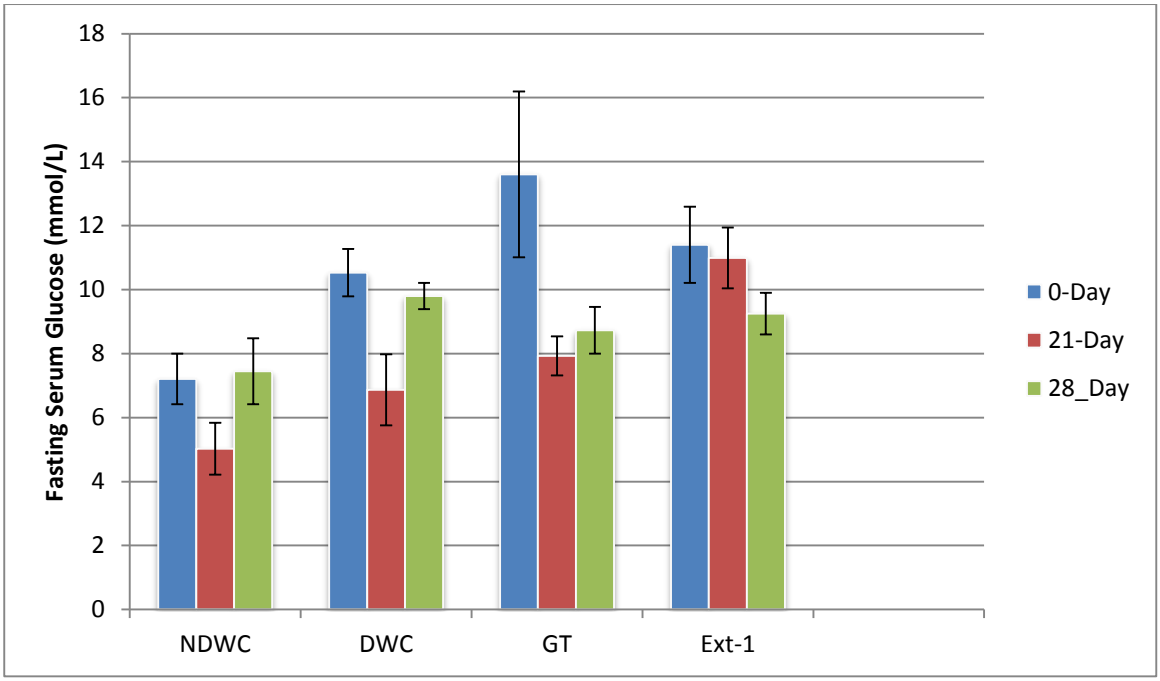


Figure 6: Effect of *M. nepaulensis* extract on fasting serum glucose level of normal and type-II diabetic rats.

In Table 7, blood glucose levels were depicted and more significant ($p < 0.05$) anti-diabetic activity was observed on the 28th day in STZ induced type 2 diabetic model rats. In a study it has been suggested that a 19 % reduction in blood glucose level is considered a significant hypoglycemic effect. The results of the study were satisfactory. *M. nepaulensis* extract exhibited significant ($p<0.05$) hypoglycemic activity.

The fasting blood glucose level of the treatment group significantly ($p < 0.05$) was reduced by the 28th days treatment with MNB extract, fasting blood glucose level reduced from the 5th day. At the end of the experiment (28th day) Fasting blood glucose level was found 12.34±2.48, 13.60±2.59 and 11.70±1.32 for MNB comparable with the Glibenclamide 0.5 mg/kg group shown in Table-1. Values are expressed as mean ± S.E.M. (n = 6). Diabetic control vs normal group, #$p<0.01$; b treated group vs diabetic control, ($p<0.05$, $p<0.01$) Treatment with MNB extract in diabetic induced rats significantly ($p<0.05$, $p<0.01$) decreased the triglycerides, cholesterol, LDL cholesterol level with a significant ($p<0.05$, $p<0.01$) increase in HDL-cholesterol level in a dose-dependent manner as compared to a diabetic control group.

American Diabetes Association (ADA) has been recommended 6.5 % HbA1c as the cut point for diagnosing diabetes. HbA1c is formed throughout the circulatory life of red blood cells (RBCs) by the addition of glucose to the N-terminal of the hemoglobin β

chain. This no enzymatic process reflects the average exposure of hemoglobin to glucose over an extended period. HbA1c was found to increase in patients with diabetes mellitus due to glycosylation of several proteins, hemoglobin, β-crystalline of the eye lens, and increased HbA1c level directly proportional to the fasting blood glucose levels. The present study shows the MNB extract treatment group significantly decline in HbA1c level compared to the diabetic control.

5.6 Acute effect of *M. nepaulensis* extracts on the blood glucose levels of type-II Diabetic Model rats when fed simultaneously with glucose load:

Effect of oral administration of *M. nepaulensis* extract on type-II model rats with simultaneous glucose load on 21stday was depicted in Fig-7 in the time of 0 min, 30 min, 60 min and 90 minutes respectively. On 21stday after treatment, the serum glucose level of extract-1 treated groups was comparatively lower at 90 min comparatively at 60 min. At 0 min, the initial glucose level in the extract-1 treated group was higher than T2DM water control and positive control (Gliclazide).

At 30 min after glucose load, an almost similar rise in serum glucose level was found in normal and diabetic water control groups, gliclazide and extract treated groups. At 60 min further increase in glucose levels were noticed in extract-1 treated group but decrease in glucose level was noticed in all other treated groups respectively. Finally at 90 min, the gradual fall in glucose level was found in extract-1, Gliclazide and Diabetic water control treated groups but increase of glucose level in normal water control. Extract-1 treated group decreased the glucose value significantly at 90 min.

The blood glucose levels of the normal control, MNB extract groups (1.25 g/kg/10 mL.) were measured after oral administration of glucose (2.5 g/kg b.w.). All groups had shown increased blood glucose level was observed after 30 min. Its level remained high over the next 60 min.) Showed significantly ($p< 0.05$, decrease in blood glucose level at 60 and 120 min compared to the normal control group shown in figure-7. Significant increases in body weight were observed in treatment groups (MNB) compared diabetic control group. Values are expressed as mean ± S.E.M. (n = 6).*$p<0.05$ @$p < 0.01$ when compared with control group at corresponding time.

Table 8: Oral glucose tolerance test on normal and type-II diabetic model rats on 21st day

Group	OGTT (mmol/L) (21 Day)			
	0 Min	30 Min	60 Min	90 Min
NDWC (n=6)	5.03±0.81 (100%)	7.94±1.68 (157%)	7.02±1.56 (139%)	7.75±3.16 (154%)
DWC(n=6)	6.92±1.03 (100%)	14.65±1.97 (211%)	12.23±3.44 (176%)	11.72±3.71 (169%)
GT(n=6)	7.87±0.70 (100%)	12.96±1.32 (164%)	10.78±1.51 (136%)	10.73±1.46 (136%)
Ext-1(n=6)	10.44±1.60 (100%)	18.93±3.33 (181%)	20.31±2.18 (194%)	18.40±0.19 (176%)
One Way ANOVA				
NDWC Vs DWC	0.03	0.00	0.00	0.20
NDWC Vs GT	0.00	0.01	0.05	0.76
NDWC Vs Ext-1	0.00	0.00	0.00	0.00
DWC Vs GT	1.00	0.49	1.00	1.00
DWC Vs EXT-1	0.00	0.01	0.00	0.00
GT Vs EXT-1	0.00	0.00	0.00	0.00
Paired sample t test (0 min Vs 90 min)				
NDWC (n=6)	0.078			
DWC (n=6)	0.009			
GT (n=6)	0.013			
Ext-1 (n=6)	0.000			

Group DWC, GT, Ext-1 represent diabetic water control rat, Gliclazide treated diabetic rat, M. nepaulensis treated diabetic rat respectively. Data presented as mean ± standard deviation (M±SD). Statistical comparison between groups was performed using one way ANOVA and Paired sample t test.

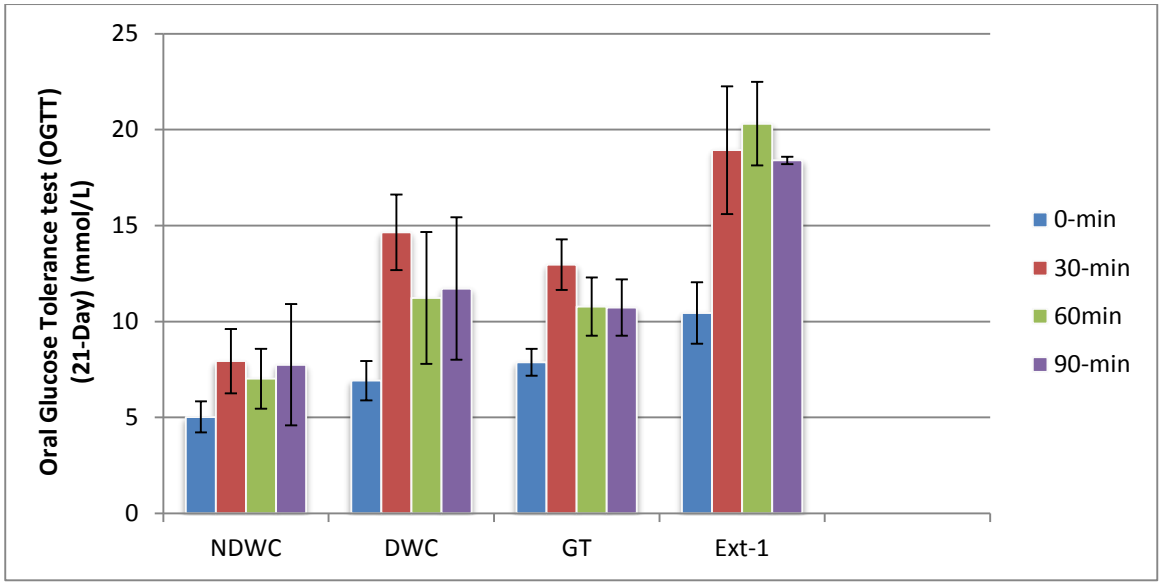


Figure 7: Oral glucose tolerance test on normal and type-II diabetic model rats at 21st day.

5.7 Chronic effect of *M. nepaulensis* extracts on lipid profiles on STZ-induced type-II diabetic model rats

The chronic effect of *M. nepaulensis* extracts on lipid profile presented in **Table 9.** There was only 6 % increment in Gliclazide treated group but 60 %increment by Extract-1 in total cholesterol level on 28thday. In the case of serum triglyceride (TG) level, the decrease was found in extract treated groups, which reduced by 32 % but 15 % increment of TG was seen in gliclazid treated group. HDL-cholesterol level was decreased by 9 % by Gliclazide and 14 % by Extract-1 treated groups and 2 % increment of LDL cholesterol level was shown in gliclazide, 30 % increased by Extract-1 treated group on the final day of the experiment when it was compared with 0 day values respectively.

Table 9: Effects of *M. nepaulensis* extract on lipid profile of normal and type-II diabetic model rats.

Group	TG		Cholesterol		HDL		LDL	
	0 day	28 day	0 day	28 day	0 day	28 day	0 day	28 day
NDWC (n=6)	56.48± 2.20 (100%)	55.66± 1.57 (98%)	138.31±1.47 (100%)	153.21± 3.02 (110%)	48.54± 0.68 (100%)	69.27± 1.60 (142%)	175.56± 1.79 (100%)	211.35± 3.43 (120%)
DWC (n=6)	73.88± 2.66 (100%)	43.94± 1.84 (59%)	80.17± 0.13 (100%)	177.40± 1.38 (221%)	57.24± 1.97 (100%)	76.17± 1.09 (133%)	115.70±9.04 (100%)	244.78±2.20 (211%)
GT (n=6)	60.79± 2.64 (100%)	70.15± 1.29 (115%)	85.59± 3.15 (100%)	91.42± 1.83 (106%)	79.52± 0.83 (100%)	72.55± 1.04 (91%)	146.83±6.42 (100%)	149.94±0.83 (102%)
Ext-1 (n=6)	109.60± 3.58 (100%)	75.14± 1.03 (68%)	93.23± 2.61 (100%)	149.54± 0.62 (160%)	66.90± 1.56 (100%)	58.07± 1.77 (86%)	147.62± 8.12 (100%)	192.58± 2.12 (130%)

Paired sample t test (0 day Vs 28 day)

Group	0 day Vs 28 day(TG)	0 day Vs 28 day (Cholesterol)	0 day Vs 28 day (HDL)	0 day Vs 28 day (LDL)
NDWC (n=6)	0.389	0.000	0.000	0.000
DWC(n=6)	0.000	0.000	0.000	0.000
GT(n=6)	0.000	0.015	0.000	0.315
Ext-1 (n=6)	0.000	0.000	0.000	0.000

Oneway ANOVA

Group	TG		Cholesterol		HDL		LDL	
	0 day	28 day	0 day	28 day	0 day	28 day	0 day	28 day
NDWC Vs DWC	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
NDWC Vs GT	0.14	0.00	0.00	0.00	0.00	0.00	0.00	0.00
NDWC Vs EXT-1	0.00	0.00	0.00	0.03	0.00	0.00	0.00	0.00
DWC Vs GT	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
DWC Vs EXT-1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
GT Vs EXT-1	0.00	0.00	0.00	0.00	0.00	0.00	0.37	0.00

Group DWC, GT & Ext-1 represent diabetic water control rat, Gliclazide treated diabetic rat, M. nepaulensis treated diabetic rat respectively. Data presented as mean±standard deviation (M±SD). Statistical comparison between groups was performed using one way ANOVA and Paired sample t test.

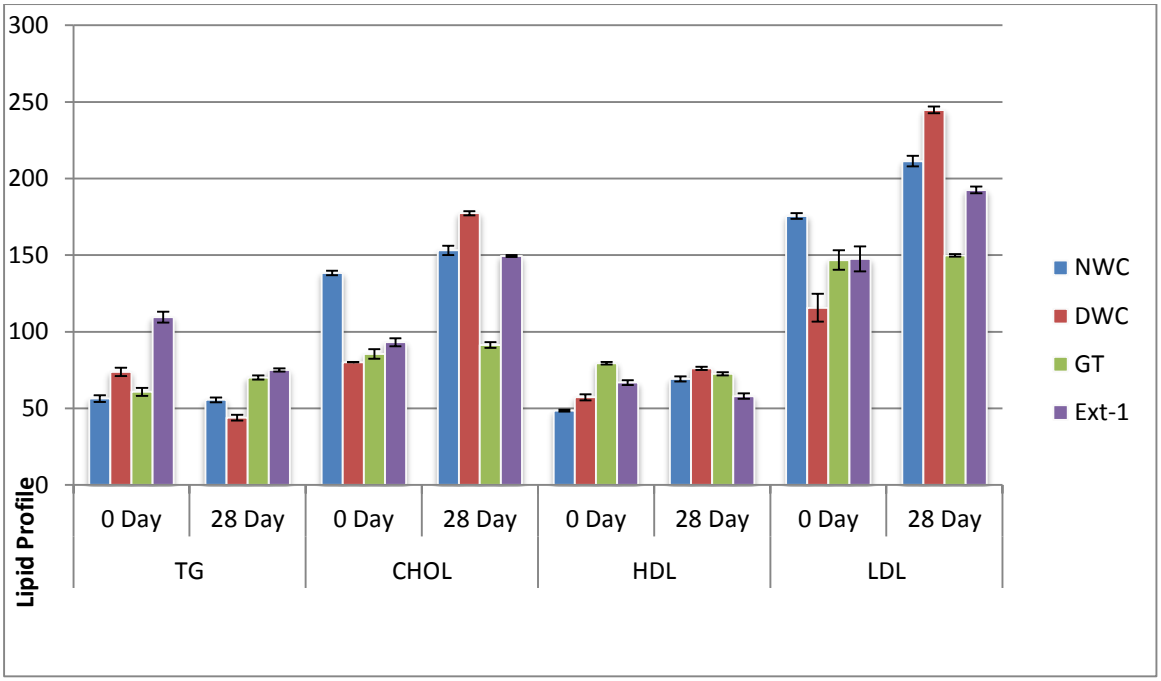


Figure 8: Effects of *M. nepaulensis* extracts on serum triglycerides, total cholesterol level, HDL and LDL of normal and type-II diabetic model rats

In the study, it was observed that there was an elevated level of serum TG, cholesterol, LDL cholesterol, and decreased levels of HDL cholesterol in diabetic rats. This might be attributed due to the lack of insulin which activates the lipase enzymes, hydrolyzing the stored TG and releasing large amounts of fatty acids and glycerol in the circulating blood. High levels of total cholesterol and LDL cholesterol are major risk factors for cardiovascular diseases, whereas increased HDL cholesterol is associated with a decrease in cardiovascular disease risk. The post-treatment results for MNB extract treated diabetic rats have showed a decline in serum TG, cholesterol, LDL cholesterol level with a marked increase in HDL levels. These results indicate that the MNB extract has a lipid-lowering effect on diabetic rats. The liver protein content significantly decreases in the MNB treated groups compared to a diabetic control group. The normal liver can perform its normal function such as protein metabolism. STZ-induced hepatotoxicity in diabetic rats cause damage to the cell membrane, change enzyme activity and finally induce hepatic injury or necrosis, as a result the liver is not capable to perform its normal functions where the protein metabolism is affected. Therefore, deamination of amino acids to breakdown the protein fails to arise resulting in the protein accumulation in the liver. Streptozotocin selectively destroys pancreatic insulin secreting β-cells and causes the enhanced level of ROS in the pancreas, liver, and related

tissues. Increased levels of ROS results in tissue damage and enhanced LPO. GSH antioxidant system plays a fundamental role in first-line cellular defense against reactive free radicals and other oxidant species. Cytosolic free radicals are either removed non-enzymatically or by in vivo antioxidant enzymes such as superoxide dismutase SOD and CAT. SOD, a metalloproteinase, is involved in the antioxidant defense mechanism as the first enzyme by lowering the number of superoxide radicals (O2•), which damages the membrane and biological structures. CAT is a hemoprotein, localized in the peroxisomes and induces the decomposition of H_2O_2 to water and oxygen. In the present study, MNB extract not only increases the decreased levels of antioxidant enzymes (CAT, SOD, and GSH) in the liver but also protects the tissue damage by inhibiting lipid peroxidation (in the terms of MDA) in stressed conditions. In histopathological examination, the pancreatic section of the normal rats showed the clear β cellular architecture in islets of Langerhans which have well preserved cytoplasm and nucleolus. But the pancreatic section of STZ intoxicated rats shows irregular, disarrangement and degradation of cells. Necrosis of the cells was found to be very clear, MNB and Glibenclamide treatment.

5.8 Comparison of the ratio of total cholesterol: HDL cholesterol level and the ratio of triglycerides: HDL cholesterol level

Table 5 was expressed in the ratio of total cholesterol and HDL cholesterol level; and also the ratio of triglycerides and HDL cholesterol level respectively. TC:HDL and TG: HDL decreased by 21 % by Extract-1 at the end of the experiment when we compared with the initial day of the study, and only NWC showed 23 % reduction of CHOL:HDL ratio on the final day in comparison to baseline ratio non-significantly.

Table 10: Effects of *M. nepaulensis* extract on total cholesterol & HDL and triglyceride & HDL ratio of normal and type-II diabetic model rats

Group	TG:HDL		Cholesterol:HDL	
	0 day	28 day	0 day	28 day
NDWC (N=6)	1.16±0.36 (100%)	0.80±0.02 (68%)	2.84±0.04 (100%)	2.21±0.07 (77%)
DWC(n=6)	1.29±0.08 (100%)	0.57±0.02 44%)	1.40±0.04 (100%)	2.32±0.03 (165%)
GT(n=6)	0.76±0.04 (100%)	0.96±0.02 (126%)	1.0±0.04 (100%)	1.26±0.04 (126%)
Ext-1 (n=6)	1.63±0.04 (100%)	1.29±0.04 (79%)	1.39±0.04 (100%)	2.57±0.07 (184%)
Paired sample t test (0day Vs 28day)				
Group	0 day Vs 28 day		0 day Vs 28 day	
NDWC (N=6)	0.000		0.000	
DWC(n=6)	0.000		0.000	
GT(n=6)	0.000		0.001	
Ext-1 (n=6)	0.000		0.000	

Group NWC, DWC, GT, Ext 1 represent normal water control rat, diabetic water control rat, Gliclazide treated diabetic rat, M. nepaulensis treated diabetic rat respectively. TC: HDL= Ratio of Total cholesterol and HDL cholesterol level and TG: HDL= Ratio of Triglycerides and HDL cholesterol.
M Data represented as mean ± standard deviation (M ± SD). Statistical comparison between groups was performed using one way ANOVA and paired sample t test.

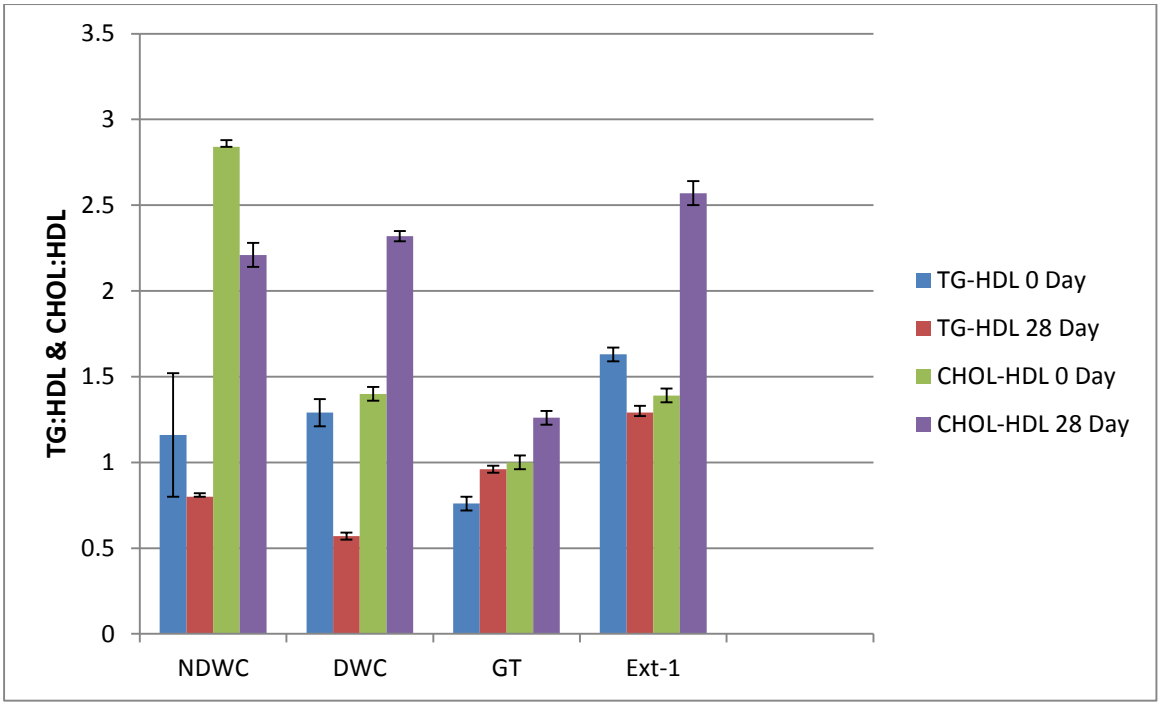


Figure 9: Effects of *M. nepaulensis* extract on total cholesterol & HDL and triglyceride & HDL ratio of normal and type-II diabetic model rats.

5.9 Chronic effect of *M. nepaulensis* extracts on hepatic glycogen content of type-II diabetic model rats:

The chronic effects of methanolic extract of *M. nepaulensis* on hepatic glycogen content of type-II diabetic model rats are presented in Table 11. It is clear from the table that hepatic glycogen content in gliclazide was increased by 11% on final day of the experiment in comparison toT2DM water control group.

Table 11: Effects of *M. nepaulensis* extract on liver glycogen of normal and type-II diabetic model rats.

Group	Liver Glycogen(mg/ml) (28 Day)
NWC (N=6)	8.13±0.67 (100%)
DWC (n=6)	9.85±1.85 (100%
GT (n=6)	6.30±2.08 (77%)
Ext-1 (n=6)	9.07±2.54 (111%)

One Way ANOVA	
NDWC Vs DWC	1.00
NDWC Vs GT	1.00
NDWC Vs EXT-1	1.00
DWC Vs GT	0.05
DWC Vs EXT-1	1.00
GT Vs EXT-1	0.24

Group DWC, GT, Ext-1 represent diabetic water control rat, Gliclazide treated diabetic rat, M. nepaulensis treated diabetic rat respectively. Data presented as mean±standard deviation (M±SD). Statistical comparison between groups was performed using one way ANOVA

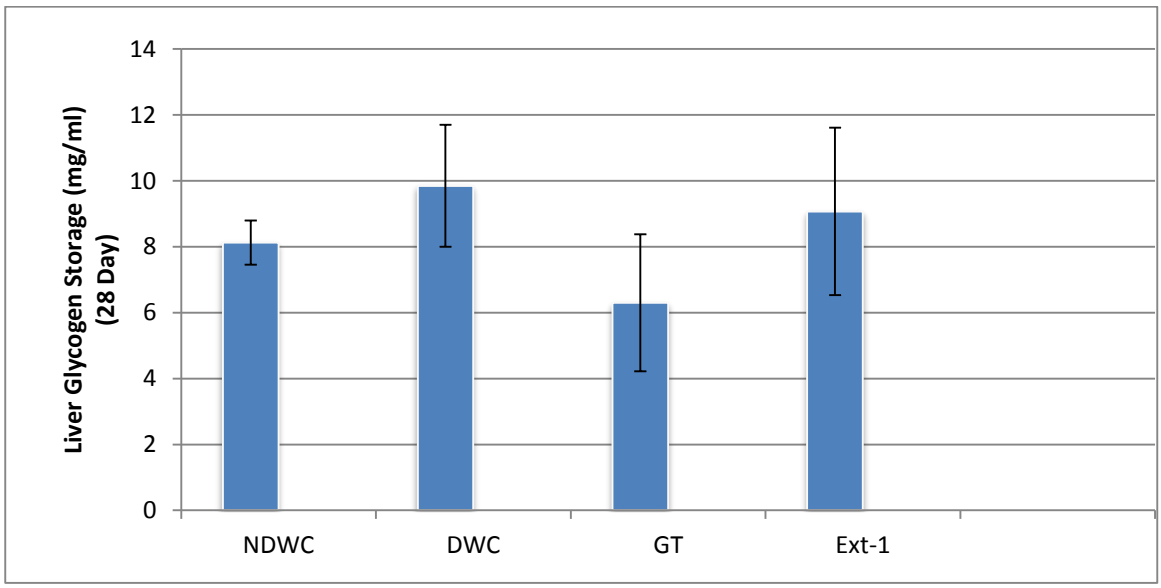


Figure 10: Effects of *M. nepaulensis* extracts on liver glycogen of normal and type-II diabetic model rats.

5.10 Results for organ weight measurement and toxicity

Table 12 : Organ weight for different cages

Cage no-01

S.N.	Kidney	Spleen	Heart	Liver	Pancreas
C_1R_1	1.288	0.638	0.551	5.932	0.620
C_1R_2	1.471	0.590	0.583	6.289	0.868
C_1R_3	1.321	0.654	0.565	7.139	0.861
C_1R_4	1.299	0.730	0.571*	7.412	0.905
C_1R_5	1.268	0.715	0.514	6.586	0.609

Cage no-02

S.N.	Kidney	Spleen	Heart	Liver	Pancreas
C_2R_1	1.712	0.749	0.695	9.84	0.777
C_2R_2	1.810	0.800	0.720	9.654	0.592
C_2R_3	1.766	0.801	0.739	11.05	0.808
C_2R_4	1.673	0.787	0.672	9.018	0.818
C_2R_5	1.857	0.853	0.819	10.694	0.936
C_2R_6	1.564	0.817	0.652	9.069	0.873
C_2R_7	1.580	0.669	0.653	9.118	0.806
C_2R_9	1.722	0.815	0.671	10.425	0.642

Cage no-03

S.N.	Kidney	Spleen	Heart	Liver	Pancreas
C_3R_1	1.659	0.661	0.525	7.982	0.826
C_3R_2	1.576	0.596	0.626	9.578	0.793
C_3R_3	1.744	0.669	0.663	10.548	1.060
C_3R_4	1.311	0.631	0.500	7.067	0.530
C_3R_5	1.659	0.650	0.643	10.108	1.010

Cage no-04

S.N.	Kidney	Spleen	Heart	Liver	Pancreas
C_4R_1	1.781	0.630	0.732	9.351	0.828
C_4R_2	1.680	0.755	0,645	8.716	0,765
C_4R_3	1.584	0.526	0.652	7.72	0,717
C_4R_4	1.768	0.776	0.688	9.158	0,806
C_4R_5	1.516	0.715	0.674	8.673	0.837
C_4R_6	1.478	1.032	0.618	7.224	0.580

During the acute toxicity study, the methanolic extract was administered orally and animals were observed for mortality and behavioral responses. No mortality was observed in rats treated with 1.25 g/kg/10 mL of methanolic extract. All the mice were normal and no gross behavioral changes were observed till the end of the study period.

CHAPTER SIX

CONCLUSIONS

Medicines derived from natural products have played a major role in the development of pharmaceutical treatments for diabetes. Phytochemical analysis of a methanolic extract of *M. nepaulensis* showed the presence of alkaloids, flavonoids polyphenols, terpenoids, glycosides, and reducing sugars whereas, absence of saponins. That is selected plant is rich in phytoconstituents.

The methanol extract of *M. nepaulensis* contained highest quantity of phenolic compounds as compared to the other corresponding phytoconstituents in phytochemical screening. Consequently, the methanol extract of *M. nepaulensis* has the highest amount of phenolics among extracted phytoconstituents in a different solvent. This might be one of the major factors for methanol extract of *M. nepaulensis* exhibiting strong anti-diabetic activity and anti-oxidant activity.

From the overall results, it can be concluded that the methanol extract of *M. nepaulensis* stem bark has a more potent antidiabetic effect against STZ-induced diabetic rat in a dose dependent manner, which may be through increasing insulin expression, beta cell regeneration, reducing the elevated levels of the hepatic enzymes by in vivo antioxidant enzyme activity and decreasing lipid per oxidation.

According to the experimental results, it can be affirmed that methanol extract of *M. nepaulensis* stem bark is endowed with strong hypoglycemic and hypolipidemic properties. Therefore, methanol extract of *M. nepaulensis* stem bark may be utilized for the management of diabetes mellitus and other related complications directly linked to lipid disorders. On the whole, methanol extract of *M. nepaulensis* stem bark is a very promising herbal planthaving enormous medicinal values.

The column chromatography of methanol extract of stem bark of *M. nepalensis* resulted in the isolation of four different compounds MN1, MN2, MN3 and MN4 in which compound MN1 was - identified as β-Sitosterol for the first time and MN2 as Berberine and compound MN3 may be 7, 8-Dihydro-8-methoxy berberine.

CHAPTER SEVEN

SUGGESTIONS FOR FURTHER WORK

This dissertation work has aimed at addressing the medicinal value as well as antidiabetic effect of plant *M. nepaulensis* DC. Although, the results obtained are eye-catching and promising, its great potential is far from achieved. It is desired to prepare the plant extracts in the other major solvents apart from discussed here so that wide varieties of phytochemicals in higher amounts can be extracted. Anti-allergic, anti-helminthic test, antiseptic, antimicrobial test, anticancer test, the anti-inflammatory test can be assessed. Due to lack of enough budget and time factors, only in vivo test or experimental were monitored for the evaluation of antidiabetic activity; in vitro test can be done to further galvanize the results here.

The plant should be examined vigorously to find out for its active ingredients for which hypoglycemic and hypolipidemic activities are being exerted and mode of actions behind the observed effects.

The use of this plant for diabetes treatment is promising but the precise active substance(s), site(s) and mechanism(s) of its pharmacological effects are still to be determined.

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ANNEXS

Detection of tannins

Braemer's test: 10 % alcoholic ferric chloride was added to 2-3 mL of methanolic extracts (1:1). Dark blue or greenish grey coloration of the solution indicated the presence of tannins

Detection of terpenoids

Salkowski test: 2 mL of chloroform and 3 mL of concentrated sulphuric acid were added to 5 mL of methanolic extracts. Reddish brown color of interface indicated the presence of terpenoids.

Alkaline reagent test: To the 5 mL of test solution, 2 mL of 2 % of NaOH was added. Formation of an intense yellow color, which turns to colorless on addition of few drops of diluted acids, indicated the presence of flavonoids.

Detection of phenols

Ferric chloride test: 10 mg of extracts were dissolved in 1 mL of distilled water. To this, few drops of neutral 5% ferric chloride solution was added. A dark green color indicated the presence of phenol compound.

Detection of carbohydrates

Fehling's test: Equal volume of Fehling A and Fehling B reagents are mixed together and 2mL of it was added to crude extracts. It was boiled gently. A brick red precipitate appeared at the bottom of the test tube indicated the presence of reducing sugars.

Detection of fixed oils and fats A drop of concentrated extracts were pressed in between two filter papers and kept undisturbed. Oils stain on the paper indicated the presence of oils and fats.

Detection of saponins Foam test: The extracts of 50 mg were first diluted with distilled water and volume was adjusted to 20 mL. The suspension was then shaken vigorously in a graduated cylinder for 15 minutes. A 2 cm layer of foam indicated the presence of saponins.

Detection of glycosides: 0.5 g of solvent extracts were dissolved in 2 mL of glacial acetic acid containing one drop of ferric chloride solution. It was then under laid with 1 mL of concentrated sulphuric acid. A brown ring at the interface indicated the presence of glycosides

Detection of phenols Ferric chloride test: 50 mg of extracts were dissolved in 5 mL of distilled water. To this, few drops of neutral 5% ferric chloride solution was added. A dark green color indicated the presence of phenol compound.

Detection of alkaloids: Solvent free extracts (50 mg) is stirred with 3 mL of diluted hydrochloric acid and filtered. The filtrate was tested carefully with various alkaloids reagents.

Mayer's test: To a few mL of filtrate, 1 or 2 drops of Mayer's reagent was added by the side of the test tube. Formation of white creamy precipitates indicated the presence of alkaloids

PHOTO PLATES