INVESTIGATION OF PHYTOCHEMICAL AND BIOLOGICAL PROPERTIES ON TRADITIONAL MEDICINAL PLANTS FOR HYPOGLYCEMIC ACTIVITY

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DOCTOR OF PHILOSOPHY

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DECLARATION

I hereby declare that the thesis entitled "**Investigation of Phytochemical and Biological Properties on Traditional Medicinal Plants for Hypoglycemic Activity**" submitted by me to The Tamilnadu Dr. M.G.R. Medical University, Chennai, for the award of Degree of Doctor of Philosophy in Pharmacology, is the result of my original and independent research work carried out at JKKMMRF's- Annai JKK Sampoorani Ammal College of Pharmacy, B. Komarapalayam, under the supervision of **Dr. N. Senthilkumar,** Principal, JKKMMRF's- Annai JKK Sampoorani Ammal College of Pharmacy, B. Komarapalayam. The thesis or any part thereof has not been previously formed the basis for the award of any degree, diploma, associateship, fellowship, or any other similar title, of this or any other University.

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LIST OF ABBREVIATIONS

ABTS	2, 2'-Azino-Bis (3-ethylbenz-Thiazoline-6 Sulfonic acid) diammonium salt
AEBN	Aqueous extraction of *Barleria noctiflora*
AGEs	Advanced Glycation End Products
ALP	Alkaline Phosphatase
ALT	Alanine Aminotransaminase
AST	Aspartate Aminotransaminase
ATP	Adenosine Triphosphate
BUN	Blood Urea Nitrogen
CEBN	Chloroform extract of *Barleria noctiflora*
CEEBN	Crude ethanolic extract of *Barleria noctiflora*
CHD	Coronary Heart Disease
CPCSEA	Committee for the purpose of Control and Supervision of Experiments on Animals
CVD	Cardio Vascular Disease
DAG	Diacyl glycerol
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DPPH	2,2-Diphenyl-1-Picryl Hydrazyl
EAFBN	Ethyl acetate fraction of *Barleria noctiflora*
EEBN	Ethanolic extract of *Barleria noctiflora*
EGF	Epidermal Growth Factor
ELISA	Enzyme Linked Immunosorbant Assay
FFA	Free Fatty Acid
FGF	Fibroblast Growth Factor
FT-IR	Fourier Transform Infrared Spectroscopy

GDM	Gestational Diabetes Mellitus
GLUT	Glucose Transporter
Hb	Haemoglobin
HbA1c	Glycated haemoglobin
HDL- C	High Density Lipoprotein Cholesterol
HFD	High Fat Diet
IDDM	Insulin Dependent Diabetes Mellitus
IGT	Impaired Glucose Tolerance
LADA	Latent Autoimmune Diabetes in Adults
LDL –C	Low Density Lipoprotein Cholesterol
MODY	Maturity Onset Diabetes of the Young
MRDM	Malnutrition Related Diabetes Mellitus
MS	Mass Spectroscopy
NBFBN	n-butanol fraction of *Barleria noctiflora*
NBT	Nitro Blue Tetrazolium
NDDG	National Diabetes Data Group
NIDDM	Non-Insulin Dependent Diabetes Mellitus
NMR	Nuclear Magnetic Resonance
OECD	Organization of Economic Co operation Development
OFR	Oxygen Free Radicals
OGTT	Oral Glucose Tolerance Test
PCV	Packed Cell Volume
PDGF	Platelet Derived Growth Factor
PEBN	Petroleum ether extraction of *Barleria noctiflora*
PKC	Protein Kinase C
p-NDA	p-Nitroso Dimethyl Aniline

PNDM	Permanent Neonatal Diabetes Mellitus
PPAR	Peroxisome Proliferator Activated receptor
PRFO	Partially Reduced Form of Oxygen
RBC	Red Blood Cells
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
SOD	Superoxide Dismutase
STZ	Streptozotocin
TBA	Tertiary Butyl Alcohol
TC	Total Cholesterol
TEF-B	Transforming Growth Factor B
TG	Triglycerides
TLC	Thin Layer Chromatography
TNDM	Transient Neonatal Diabetes Mellitus
UV	Ultraviolet
WBC	White Blood Cells
WHO	World Health Organization

1. INTRODUCTION

1.1. HERBAL MEDICINES

During the life, human being have depended on plants for their fundamental needs like foodstuff, shelter, clothing, transportation, fertilizers, flavors, fragrances and not the least, medicines etc. The plants have produced a beginning for traditional system of medicine, which has survive for more than hundred years in countries such as India[1] & China.[2] These systems of plant maintain to play a fundamental responsibility in health care and 80 percent of the world's population continue to rely on traditional medicine system for their healthcare needs. The natural plant product is playing a significant role or part in the health care system of enduring 20 percent of the people, mostly in residential countries. These drugs have approach from different sources of materials including earthly plants, earthly microorganisms, sea organisms and earthly vertebrates and invertebrates.

Throughout the last 4 decades, at least a effective drugs consequent from flowering plant including *Dioscorea* plant derived diosgenin, which an ovulatory cause of contraceptive has been derived; anti-hypertensive like reserpine and alkaloids from *Rauwolfia* plant species; Pilocarpine to treat 'dry mouth' and glaucoma, resulting from (*Pilocarpus spp*.) the family of citrus; the powerful cytotoxic agents from the *Catharanthus roseus*; laxative agent from *Cassia* plant species and Digoxin as a cardiac stimulate to treat cardiac failure from *Digitalis* species. Examples of some important drugs obtained from plants like quinine and quinidine from *Cinchona* species, morphine and codeine from *Papaver somniferum* and atropine from *Atropa belladonna* and etc.

In totaling to this historical success in drug finding, rapid sales have seen in the herbal drug market in modern years. A growing number of consumers are

convinced of the benefits of plant medicament as an alternative to medicinal substance with chemically consequent active pharmaceutical constituents.[3] While the pharmaceutical manufacturing industry developed in the world will persist toward scrutinize promising lead from natural substances in their effort to produce new molecule, the production of new molecules in the world may have relatively different precedence.

1.1.1. The Role of Herbal Medicine in Traditional System

The WHO has newly stated that traditional drug as a comprising practice of therapeutic that have been in continuation, often for more then hundred years ago, the spread and development of drug are at rest used today. The synthesis of traditional medicine is the therapeutic experience, generations of indigenous practicing systems of medicine. The preparations of traditional form medicinal plants, organic matter, minerals etc. The elements play a dynamic role in the Chinese system of medicine like in making groups of herbal tastes and parts of the body.[4] The treatment disease at pharmacologically start long ago with the use of herbs. Methods of folk curative throughout the world used herbs and their part of tradition.

Traditional system of medicine refers to a range of ancient and natural health care practices, including tribal and folk medical practices as well as organization of medicine such as herbal systems. These originate of medical practices much before the application of modern scientific method. Further the herbal medicines are also used in self medication in all cultures. The Indian subcontinent is endowed with rich and diverse local health traditions which matched with an equally rich and diverse plant genetic resource. The resource base of local health traditions is mainly the plants.

1.1.2. Indian System of Medicine (Ayurveda)

Ayurveda the ancient science of life believed to prevalent for last five thousand years, most noted systems of medicine in the world. Ayurveda is based on the hypothesis, everything in the universe is composed of air, space, energy, liquid and solid. Homeostasis is expressed by a number of biological constituent's i.e. stable quantitative indices characteristics of the normal state of the organism. Thus, the principle plays a vital role in maintaining internal environment, buffer system (blood and tissue fluids) and in homeostasis of the body.[5]

1.1.3. Difference of Herbal and Traditional Drugs

While many drugs or their precursors derived from plants, there is a basic difference between administering chemical substance and the same chemical in a plant matrix. It is merits of chemical complexity, which is both refused by orthodoxy as having no fundamental, in fact and avoided by most researchers as beginning too many variables for comfortable research. Contrast with well-defined chemical synthetic medicines, natural medicines reveal some noticeable differences.[6]

1.1.4. Standardization of natural drugs

In original or traditional systems of drugs are distribute as polar solvents extract. Fresh natural plant fraction or powder crude drug is infrequency slightly than a regulation of rule. Thus parts of therapeutic plant should be reliable and free from injurious materials like heavy metals, pesticides, infectious or radio active pollution, etc. The natural plant is focused to a particular extraction of solvent repeatedly, or water decoction or as expressed in earliest texts. The extract should be checked for natural activity indicated in an investigational animal model. The extract of bioactive substance should be consistent on the active principle or major complex along with finger prints. Clinical trials are establishing beneficial potential to promote scientific

use. The herbal drugs developed should be distribute as treatment drugs or even OTC drugs rely upon pathological consideration and beneath no circumstance as a nutraceuticals or health foods.[7]

Formulation of important guidelines in defining the extent and type of our participation is important while discussing the acceptance of the role of traditional system of medicine and their practitioners in the primary health care. Some of the practices of traditional systems of medicine have already been explored and some require special attention. In this respect the following parameters can be considered:

- Traditional scholarly systems may be accepted as such
- Traditional popular practices may be assessed and then accepted
- The specific areas, which have gained prominence in traditional practices, may be allowed to constitute the specialized component of the total medical care.
- The specialties of traditional system of medicine wherever this can offer a solution to the areas where modern medicine is unable to make any headway may be given importance.

Therefore, to establish the potentiality of traditional medicine, research needs to be simultaneously conducted on important aspects of these disciplines to meet the requirement of society to serve. The importance of such requirements is enormous and the ancient Ayurvedic medicine from traditional and folklore system need the following points as guiding forces to bring more objective conclusions, to explore their applicability:

- To standardize material, method and measures for preparation, preservation, presentation and administration of Ayurveda drugs

- To give new scientific meaning and significance of the fundamental principles of the system to the extent possible, so that they can be accepted within the scientific framework
- To rationalize the utility of positive and judicious use of modern scientific methods that pertains to the development of Ayurveda.

1.1.5. Natural Products for Modern Medicine

Natural plants over all as other agents are used for the drug from occasion immemorial because they have fitted instant personal require are easily available and economical. There are roughly more then thousands of Indian medicinal plants, which were used in formulating therapeutic research according to Ayurveda and other traditional systems of medicines. The activity is not completely dismissed in scientific societies and plants are valued

need of steady refrigeration, and event excess dosages fatal decrease of glucose limits its tradition. The drugs of biguanid and sulfonylurea's are treatments associated in pharmaceutical research as the major resource, new medicines and developing bodies of medical literature sustain the clinical efficacy of herbal treatments.

Traditional uses of herbal medicine are the very basis and integral part of various cultures, which was developed within an ethnic group before the development and spread of modern science. In totaling to this past success in drug finding, rapid growth has been seen in the natural drug promote in current years. An growing number of consumers are convinced of the advantages of plant extracts an substitute to medicinal products with synthetically derived lively pharmaceutical ingredients.[3] The pharmaceutical industries are developed country will investigate to talented leads

as of natural palnt products in their endeavor to create latest being, the manufacture of new drugs in the growing country.

A recent study of herbal products as source of recent drugs over the time period of 1980-2003 shows that 68% of the 879 small molecules and new drug entities, but, 16.8% communicate to chemical molecules include a pharmacophore resultant directly from natural substances.[8] Natural drugs supply a early point for recent synthetic compounds with varied structures and multiple stereo center that can be synthetically challenged.[9, 10, 11]

Several structural features frequent to herbal products are shown to be extremely relevant to drug finding efforts.[12, 13] The new chemical substance allied with natural substance is advanced than any other source: 45% of the chemical gallows in available database of natural substance are not present from synthetic chemistry.[14]

In the order of 1/3 of top promoting drugs in the world are natural substance and their derivatives[15] of 22 most excellent non-protein drugs in 2000, some of the drugs was moreover derived from developed in the result of guide produced by natural substances. Some of drugs like *simvastatin, enalapril, atorvastatin, ciprofloxacin and cyclosporin* had a joint annual sale approx United States of 16 billion dolar.[16] So the used natural substance are the most successful approach for the detection of new medicines.[17]

A steady are beginning of new natural substance and usual product was derived drugs in different countries of world. *Capsaicin* based creams and patches were available for topical use to relieve pain in the condition such as osteoarthritis, post hepatic neuralgias, psoriasis and diabetic neuropathy. The mesylate salt of

ruboxistaurin was evaluated in Phase III clinical trials for diabetic retinopathy and diabetic edema was undergoing Phase II trials for diabetic peripheral neuropathy. *Biostratum* is investigating vitamin B_6 derivative, pyridoxamine dihydrochloride were no adverse effects experimental in Phase I and Phase II diabetic kidney disease. Anti diabetic botanical extract of proprietary derived from *Artemisia dracunculus* study with type 2 diabetes mellitus in 30 patients. *Ruboxistaurin* is a synthetic analogue *staurosporine* is a competitive inhibitor of ATP binding to protein kinase is being evaluated in Phase III clinical trials for diabetic retinopathy.

Tropical rain forest continues to carry a huge reservoir of possible drug species. Approximately 50 % (125,000) of the world flowering plant species grow in the tropical forest. It continues to give natural substances with important compounds and develops the preliminary of new molecules.

In modern medicine, special effects on hypoglycemic levels are well recognized; the preventing activity of these drugs against the progressive nature of diabetes. Their micro and macro vascular complication was modest and always effective. The insulin treatments were efficient control of glycemic, so far its shortcomings inefficient on enteral administration, recently with side effects. For the different motive in current years, the reputation of harmonizing drugs has increased. Nutritional measures are traditional herbal plant treatment in natural and original system of medicine in India.

1.2. DIABETES MELLITUS

Diabetes mellitus is recognized as being a syndrome, a collection of disorders that have hyperglycemia and glucose intolerance as their hallmark, due either to insulin deficiency or to impaired effectiveness of insulin action or to a combination of these.[18] The glucose levels of blood are controlled by endogeneoius substance of

insulin. When the systemic blood glucose level was elevated, insulin was released from the endogenious pancreas to regulate the glucose level. The impact of diabetes mellitus can lead to eye blindness, failure of kidney and damage of nerve. These results are injure small vascular, referred to microvascular disease. It is accelerate harden and narrow of the vascular arteries leading to stroke, coronary vascular disease.

1.2.1. Types of Diabetes Mellitus

The classification of diabetes based on the age of recognized onset, which seemed to be the only reliable means of classification for universal use. The specific types of diabetes are including brittle, insulin resistance, gestational, pancreatic, endocrine and iatrogenic diabetes. Since then, several pathogenic mechanisms have been described and long terms learn shown variable courses and result of diabetes type. A revised classification glucose intolerance was formulated by National Diabetes Data Group (NDDG).[19] They proposed two major classes of diabetes mellitus named Insulin Dependent Diabetes Mellitus (IDDM) or type 1 and Non-Insulin Dependent Diabetes Mellitus (NIDDM) or type 2, but the class of juvenel onset and maturity onset were preserve and a latest class of unbalanced lake of nutrients related diabetes mellitus.[20] In 1985 included other type of diabetes Impaired Glucose Tolerance (IGT) as well as Gestational Diabetes Mellitus (GDM).

The presence of islet cell antibodies and antibodies of glutamic acid decarboxilase in a normoglycemic individual indicates the autoimmune process, which underlies type 1 diabetes is present, although the individual may or may not ultimately develop diabetes.[21] Diabetic mellitus type 1 comprise the cases attributable to an autoimmunolgical progression as β-cell damage and etiology nor disease contitions is known as idiopathic. The rate of β-cell destruction is quite variable,

typically being robbed in children and slower in adults. Typically type 1 diabetes requires insulin therapy from the time of presentation in both adults and children, but a slowly progressive form, Latent Autoimmune Diabetes in Adults (LADA).[22] The blood glucose level can initially be controlled by lifestyle change and oral hypoglycemic agents and may therefore masquerade as type 2 diabetes. Some of these patients have endured insulinopenia and are tending to ketoacidosis. This form is more common among individuals of African and Asian origin.[23] Diabetes mellitus type 1 specify the process of β-cell damage that may eventually leading to diabetes mellitus, which is required for the endurance of prevent and the development of diabetic complications.

Diabetes type 2 is commonest form of diabetes mellitus and characterized by diabetes of resistant insulin and secretion of insulin, either of largest feature. For type 2 diabetes, there are few useful highly specific indicators, through the presence of risk factors such as obesity indicates the likelihood of developing type 2 diabetes. In the setting of insulin resistance, these levels are inadequate to maintain normoglycemia. This relative insulin deficiency is differentiated diabetic insulin resistant individuals with normoglycemic insulin resistance individuals. The majority of genes that have been associated with type 2 diabetes are related to insulin secretion, and not to insulin resistance.[24] The type 2 diabetes originally and often all through their life span, these individual doesn't require insulin management to survive. However, such patient is at improved danger of increasing macro and micro vascular complication. The type 2 processes of diabetes are overweight or obese and obesity itself causes insulin resistance. Many of those not obese by traditional criteria, for example body mass index, may have an improved proportion of body fat distributed predominantly in the abdominal area. Ketoacidosis occurs in type 2 diabetes typically happen in

relationship with the strain of a further illness such as infection. The diabetes type 2 is characterized by severe hyperglycemia and ketoacidosis requiring immediate insulin therapy.[25] The risk of developing type 2 diabetes, enhance with age, obesity, and lack of physical action. It has been more frequently in woman with prior GDM, in those with hypertension or dyslipidemia. It's associated with strong families, likely genetic, predisposition but the genetics of type 2 diabetes. The disease process can cause various degrees of impaired glucose metabolism, such as impaired fasting glycemia and impaired glucose tolerance without satisfying the criterion for the diagnosis of diabetes. Weight reduction, exercise and oral hypoglycemic therapy can achieve satisfactory glycemic control in type 2 diabetes.

The gestational diabetes is a state of glucose intolerance first recognized during pregnancy, which usually resolves after delivery, but is associated with later increased long term risk of type 2 diabetes. The other types of diabetes are less common and can be broadly classed as genetic, exocrine pancreatic, endocrine and drug induced. The genetic based syndromes associate with diabetes. The genetic defects of β-cell function. These forms are characterized by the onset of mild hyperglycemia during childhood or early adulthood and include Maturity Onset Diabetes of the Young (MODY), Permanent Neonatal Diabetes Mellitus (PNDM), Transient Neonatal Diabetes Mellitus (TNDM) and many other insulin deficient syndromes with a myriad of other clinical features. The genetic irregularities that result in incapability to convert proinsulin to insulin have been recognized in a new family. Such character is inherited in an autosomal leading pattern and the resulting carbohydrate intolerance is mild.

Genetic defects in insulin action are rare and the associated metabolic abnormalities may range from hyper insulinemia and modest hyperglycemia to severe

symptomatic diabetes resulting in death.[26] The syndromes have alteration in the insulin receptor gene with consequent alterations in insulin receptor function and extreme insulin resistances are called leprechaunism and Rabson – Mendenhall syndrome.

The acquired processes of the pancreas may reason diabetes are pancreatitis, infective disease, pancreatic carcinoma and pancreatectomy.[27] This involve mechanism are decrease in β–cell accumulation.[28] The drug or chemical induced diabetes are β–cell demolition may occur with use of convinced poison such as vacor, pentamidine and some immune suppressive drugs like tacrolimus and cyclosporine. There are lots of drugs and hormones to can impair insulin action. There are several commonly used diabetes inducing drugs include HMG CoA reductase agents,[29] glucocorticoide steroids, anti-HIV agents and antipsychotic drugs.

1.2.2. Symptoms of Diabetes Mellitus

Common symptoms include the following:

- Urination frequency
- Extreme thirst
- Affordable weight loss
- Excessive changeg of vision
- Heal sore is slow

1.2.3. Common sequences of Diabetes mellitus

- Over time, diabetes can injure the heart, eyes, blood vessels, kidney and nerves,
- Diabetic retinopathy is an imperative cause of blindness and chances as a result of lasting accumulated damage to the small blood vessels in the retina. Subsequent to

15 years of the diabetes mellitus approximately 3% of people become blind and about 11% develop severe visual impairment.

- Diabetic neuropathy is injuring to the nerves as an effect of diabetes. Although many diverse problems can occur as an effect of diabetes neuropathy, common symptoms are pain, tingling, numbness, or weakness in the feet and heads.
- Combined with reduced blood flow, neuropathy foot increases the changes of foot sore and ultimate limb amputation.
- Diabetes is among the important causes of kidney failure. 10-18% of people with diabetes die of renal failure.
- Diabetes augments the risk of heart disease and stroke. 50% of the population with diabetes, expire of cardiovascular disease (primarily heart disease and stroke)
- The overall hazard of dying among people through diabetes is at least twice the risk of their peers without diabetes.

1.2.4. Precise prevalence of diabetes mellitus

The diabetes prevalence varies markedly among Asian populations. The type 2 diabetes mellitus were established relatively younger age in India & Pakistan. In a community prevalence was over the age of 35-45 years at 14%, increased occurrence of 65-75 years of age at 31% in men, indicating major health threat diabetes in India and Pakistan. The predictable numeral of adults with diabetes mellitus in India 19.41 millions in 1995 and in the year of 2025 is 57.2 million. The age consistent increased from 8.3% to 13.5% in 1989 – 2000 a 65% increase within the two decades.[30]

In Europe, a revise of WHO 1998 measure showed that the frequency of diabetes in 2000 was 16.7% in men and 8.6% in women, at 54-59 years of age and 23.5% in men and 18% in women at 71-74 years of age.[31]

The frequency of diabetes mellitus in 1988-1994 was 5.8% in men and 4.7% in women at 41-48 years of age and expanced peak of 19.1% in men and 16.3% in women at 75 years.[32, 33] The overall percentage of diabetes mellitus in US was 13.9 million in 1995; it was increased to 21.9 million in the year of 2025.

1.2.5. Complications of Type 2 Diabetes Mellitus

- Type 2 diabetes the insulin ablity to prevent that usually go along with and the gathering of irregularity that define the metabolic syndrome the entire encourage atherosclerosis and their complications.
- Both Type 2 diabetes mellitus and atherosclerosis involves extended subclinical segment prior to demonstration of clinical syndrome. The metabolic condition include of hazard factors for increasing diabetes mellitus and cardiovascular disease.
- Increase the glucose level exerts pathological property on vascular tissues by multiple complex pathways, including oxidative stress, increased inflammation, lipotoxicity involving free fatty acids, glycation of proteins and cellular insulin resistance. The mechanisms of increasing inflammation, promoting fibrosis and thrombosis and accelerating atherosclerosis with effects on endothelial cells, vascular smooth muscle cells, monocyte, lymphocytes and platelets. The optimal glycemic control may confer a long term CVD benefit in diabetes.
- The lipid accumulations of tissues are negatively influence metabolism of glucose and insulin signaling in diabetes mellitus.
- Depositions of lipids are in particular instinctive adiposity may augment inflammation, promote diabetes and CVD throughout hormonal paracrine effects.

1.2.6. Hemostatic Abnormalities in Diabetes Mellitus

Diabetes is a long time progressive metabolic distinguish by presence of swelling to blood clot in vessels changes to promote damage vessels.[34] The insulin prevent states to inhibition of fibrinolysis due to prominent levels of fibrinolytic inhibitor.[35]

Oxidative stress plays a pathophysiology of diabetic cardiomyopathy. According to recent studies the maladaptation of the heart is based on metabolic dysfunction associated with diabetes. Key factors contributing to oxidative stress in diabetic particular

- Excess formation of ROS induced by hyperglycemia, AGE and elevated free fatty acid;
- Reduction in mitochondrial ROS generation;
- ROS mediated activation of factors that are involved in the disease states of diabetic cardiomyopathy: inflammation mesodermal origin dysfunction, cell death, cardiovascular remodeling;
- The lipid accumulation interrelated mechanisms, increased production of free radicals such as superoxide or decreasing antioxidant status. These mechanisms include glycoxidation and arrangement of higher glycation substances, creation of polyol pathway are altered cell and glutathione redox state and ascorbate metabolism antioxidant enzyme inactivation and perturbation in nitric oxide and prostaglandin metabolism.[36]

Free radicals were extremely reactive molecules an unpaired electron of outer orbits. The constructions of free radicals are arise whichever by the addition or the deletion of electron in an oxidation reduction. Since oxygen has two electrons with

parallel spin in its outermost shell, it is characterized as a biradical which requires four electrons to be completely reduced to water. The addition of electron to O_2 result in the formation of peroxide anion which protonates to form hydrogen peroxide (H_2O_2). The latter is not a radical, its able of reasoning cell damage by cooperate with conversion metals as iron. A reduction of electron H_2O_2 results in the arrangement of the hydroxyl reductive. The first excited state of O_2 is single oxygen (O_2) which can also initiate oxygen radical chain reactions. Superoxide radical reacts with nitric oxide during reperfusion to form peroxynitrite, which also has a harmful effect by opposing the vasodilator effect of nitric oxide. These reactive species is able to relate with large molecules and begin free radical chain reactions ensuing in cellular damage.

1.3. FREE RADICALS AND REACTIVE OXYGEN SPECIES

The recent growth in the knowledge of free radicals in biology is fabricate a medical revolution that assure health and disease management.[37] In the last two decades, there has been an explosive interest in the role of oxygen free radicals, more generally known as “Reactive Oxygen Species” (ROS) and of “Reactive Nitrogen Species” (RNS) in experimental and clinical medicine.[38]

A large amount of oxygen species leads to oxidative stress, which is defined as interruption in pro oxidant balance in favor leading to damage. Reactive oxygen species are capable of chemically altering virtually all major classes of biomolecules (lipids, proteins, nucleic acids) with concomitant changes in structure and function. Evidence implicated oxidative stress in a spectrum of disease such as inflammation, ageing, autoimmune diseas, rheumatoid arthritis and diabetes. Oxidative strain is involved in generation or worsening of more than a hundred pathogenic conditions.

1.3.1. Reactive Oxygen Species and Biochemical Consequences

Oxygen radical is capable of reversibility or irreversibility injurious compounds of all bio-chemical substances and connective tissue macromolecules.

Reactive oxygen classes are created through a multiplicity of events and pathway. It expected that a human cell is uncovered for just about 1.5×10^5 reactive species.[39] The hydroxyl radicals are recognized components respond the DNA molecule, damaging both pyrimidine and purine support and as well deoxyribose backbone.[40]

1.3.2. Antioxidant Defense Mechanisms

The harmful property reactive species are reasonable by antioxidant achievement of nonenzymatic enzymes. These defenses are enormously significant correspond to the straight exclusion of free radicals, thus given that maximal defense for biological sites. The most professional enzymatic antioxidant involves SOD, catalase etc. Non-enzymatic antioxidants are engage vitamin C, E, carotenoids, thiol antioxidants, lipoic acid, natural flavonoids, a hormonal product.[41]

Biological systems cooperate with the outside atmosphere to support an internal atmosphere that favors continued existence, expansion and reproduction. However, the contradiction of aerobic life was that oxidative injure occurs to input biological sites, intimidating their arrangement and function. Oxygenic danger is convening by collection of antioxidants that developed in parallel with our oxygenic atmosphere. The mainly herbal based nutritional antioxidants supposed to significant role in maintaining of diabetes. Hence the investigate of drugs of natural origin hypopglycemic with antioxidant activity turn into a central focus.

1.4. WOUND HEALING

The damage of skin causes restores mechanisms that refresh its purpose in defense the individual next to surrounding factors are harmful. The diabetes injury is slow, non curative wounds can persist for weeks in spite of adequate and approximate care. Such wounds are difficult to manage.[42] The confirmational studies relating both human and animal models reveals several abnormalities in the various stages of the wound healing process. The different stages comprise the physiology arranged of wound-healing are substrate stage, proliferative stage and remodeling stage. All these stages are prohibited manner by mixture of cytokines as well as growth factors. It has been recognized in monochrome wound healing. In extensive duration of wounds the regular healing procedure is fragmented due to several unknown reasons and in such case the exogenous function of several growth promoting mediator or some molecules can improve the generation of these development factors is necessary to addition the process of healing.

1.4.1. Wound Healing in Diabetes

The wound healing is damage by factors such as extrinsic and intrinsic in dfiabetes. The extrinsic factors comprise frequent mechanical pressure applied to a foot provide insensitive due to neuropathy besides the ischemia result of vascular disorder.[43] Thickening of the basement membrane blood vessels in diabetics, resulting in damage wound healing and constant ulcer formation.[44] Intrinsic factors has been attributed the chronic wound in diabetes. Hyperglycemia has a harmful on wound healing effect during arrangement of superior glycation final products induce inflammatory molecule and collagen synthesis.

Hence the present study, plant extracts were experiment for *in vitro* antioxidant property using different methods. The antioxidant activity or production

of free radicals is imported in providing against diabetes. A many plants are shown to possess antioxidant by improving hypoglycemic properties. The obesity is a major complication of diabetes and cardiovascular damage. The diabetes known as chronic metabolic disorder affects many vital organs like heart, liver, kidney, macro and microvascular damage and wound healing. Hence the potent extraction and fraction was tested for antioxidant, diabetic parameters in diabetic induced in obese animals. Isolation of phytoconstituents from the potent extract using column chromatography and their characterization was also aimed.

2. AIMS AND OBJECTIVES

2.1. AIM OF THE STUDY

Though the modern medicines are significant, they remains an ever rising demand for herbal drugs. The successful and potent herbal drugs requires assessment by typical scientific methods, so as to be authenticated for the management of diseases. The present patent laws have increased the necessity to preserve the claims of this time tested folk medicines. Thus, it has become imperative to initiate steps to document components and activity of these medicinal plants.

Natural products play a very vital role in health care systems. The different source of herbal substances, plants has been a foundation of chemical substance, which serves medicine in their input ingredients in formulations include synthetic drugs. The development that leads from the plant to a pharmacologically dynamic, pure ingredient is very extensive and tedious need a multidisciplinary approach. The collection of the plant species are critical factor for the ultimate achievement of the investigation. Though random collection provide targeted collection support on chemotaxonomic associations and ethnomedical in sequence derived from traditional medicine.

Phytochemical investigation will be a useful tool for the identification and authentication of the plants for industrial and further research purpose. Hence, phytochemical tests were carried out on many of the selected plants based on ethnomedical uses. Total phenol content of a tested material is related to the antioxidant activity. Antioxidants are rising as prophylactic and beneficial agents are which search free radicals are prevents the damage reason by them. These properties

are due to many substances, including vitamins, flavonoids, terpenoids, carotenoids, tannins and phytoestrogens.[45] Antioxidant potential of many plants has been studied, such as *Curcuma longa, Capsicum frutescens, Zinziber officinale, Allium cepa* and *Schisangra chinesis,* and their consumption is recommended.[46] Several antioxidants of plant origin are experimentally proved and used as effective protective agents against free radical mediated toxicity. Hence, the selected plant extracts were subjected to *in vitro* antioxidant activity by using various methods.

The aim of this study is to assess the characteristics and care of patients with diabetes in our country. The review shows the significance and the attention on medicinal herbal plants in the force to make obvious their hypoglycemic effects and to separate the bioactive agents. A drug discovered from herbal plants played a vital role in the management of diabetes. *Allium cepa, Allium sativam, Aloe vera, Azadirachta indica, Gymnema sylvestro, Syzygium cumini, Pterocarpus marsupium* etc., are main classes of antidiabetic plants, currently in traditional system.[47] Hence, in the present study *in vivo* antidiabetic action in high fat induced diabetic bearing animals was carried out for the potent fraction. *In vitro* antidiabetic activity and studies related diabetes of wound healing activity of fractions were also carried out. Isolation of phytoconstituents from the active extracts helps in many ways in plant research. These constituents can serve as marker compounds for their standardization. The determination of the biological activities helps in developing these compounds into drugs or lead molecules for further drug development. Hence, the aim was chromatography and isolation of active constituents and their characterization from potent extract. It provides efficient and inexpensive medicine to the society; build up public confidence in indigenous medicine.

2.2. OBJECTIVES OF THE WORK

1. To select plant based on their ethnomedical uses and preparation of their extracts.
2. To screen the extracts for antioxidant activity by using various *in vitro* methods.
3. To screen the fractions for *in vitro* antidiabetic activity.
4. To select the fractions for *in vivo* studies.
5. To screen the acute toxicity and subacute toxicity studies.
6. To screen the potent plant fraction for *in vivo* antidiabetic and wound healing activities.
7. To isolate phytoconstituents from the active plant extracts and characterization by spectral methods.

Based on the objectives of work we have searched and screened several medicinal plants traditionally utilised for the antidiabetic property. Finally we selected *Barleria noctiflora*, used traditionally and claims for this antidiabetic property based on the literature.

3. REVIEW OF LITERATURES

Many herbal products including several metals and minerals were described in the care of diabetes mellitus in ancient literature. In 2000, there were estimated 171 million people with diabetes worldwide and by 2030 the projected estimate is 366 millions.[48, 49]

The prevalence of diabetes varies markedly among Asian populations. The type 2 diabetes was found at a relatively younger age in India and Pakistan. In rural community the prevalence was over 13% at 35-44 years of age, with the highest prevalence of 30% in men at 65-74 years of age, indicating diabetes has already become a major health threat in India and Pakistan. The estimated number of adults with diabetes in India 19.41 millions in 1995 and in the year of 2025 is 57.2 millions prevalence of diabetes is expected to increase to 79.4 million in 2030. A series of studies in the southern Indian city of Chennai showed a steady increase in the prevalence of diabetes in the Indian population. The age standardized increased from 8.2% to 13.5% in 1989 – 2000 a 65% increase within the two decades.

In Europe, a study using WHO 1999 criteria showed that the prevalence of diabetes in Germany in 2000 was 16.7% in men and 8.6% in women at 55-59 years of age and 23.1% in men and 17% in women at 70-74 years of age.

The prevalence of diabetes in US whites in 1988-1994 was 5.9% in men and 4.8% in women at 40-49 years of age and reached a peak of 19.2% in men and 16.6% in women at 75 years or older. The overall percentage of diabetes in US was 13.9 million in 1995; it was increased to 21.9 million in the year of 2025.

The glucose is a major energy source for all mammalian cells and it is the principal source of energy for the brain. A constant supply of glucose must be

provided in order to ensure against hypoglycemia and the potentially catastrophic effect in cell of the nervous system. An interacting mechanism for maintaining the blood glucose within a relatively narrow range has evolved to accomplish these purposes. This involves the production of glucose by the liver, from glycogenolysis, gluconeogenesis and the peripheral clearance of glucose by tissues such as the skeletal muscle, an adipose tissue and the splanchnic bed, including the liver.[50] In a fasting state glucose disposal is used mainly by insulin independent tissues, whereas little glucose is metabolized in insulin dependent tissues. Hepatic glucose production is responsible for maintaining the flux of glucose. This is a necessary steps since the brain and other neuronal tissues use glucose at a constant rate.[51] This balance becomes disrupted in a post absorptive state, resulting in an increased glucose, plasma concentration that stimulates pancreatic β-cells to synthesize and secrete insulin. Insulin released into the portal vein and carried to the liver, where its primary function is to suppress hepatic glucose production.

Insulin mediates its physiologic function by binding to the insulin receptor, which is located at the plasma membrane in the liver, skeletal muscle and adipose tissue. The insulin stimulates glucose uptake into muscle and fat cells requires the movement of GLUT 4 containing vesicles from intracellular compartments to the plasma membrane. The translocation of GLUT 4 is containing vesicles from their intracellular pool to the plasma membrane. The fusion of the vesicles with the plasma membrane leads to the extracellular exposure of GLUT 4 proteins, which allows the uptake of glucose into the cells.[52]

Diabetic hyperglycemia causes a variety of pathological changes in small vessels, arteries and peripheral nerves leading to micro and macro vascular

complications such as diabetic nephropathy, neuropathy, retinopathy and coronary artery disease. Hyperglycemia increases the production of reactive oxygen species (ROS) inside the aortic endothelial cells.[53] Free radicals have been implicated in the causation of several diseases such as liver cirrhosis, atherosclerosis, cancer, diabetes etc., and compounds that can scavenge have great potential in ameliorating these disease process.

Hence, the maintenance of normal glucose homeostasis will prevent pathological relate to diabetes. The dramatic increase in these pathologies underlines the researcher's interest in finding bioactive phytochemical that has multiple therapeutic benefits in the treatment of diabetes and its complications.

The prevalence of type 2 diabetes mellitus has increased rapidly over the past decade. Oral medications are initiated when 2-3 months of diet and exercise alone are unable to achieve or maintain optional plasma glucose levels. Cardiovascular disease is the major cause of mortality among patients with NIDDM, accounting for 60-80% of deaths in these patients.[54] The oral agents used for treatment of type 2 diabetes are sulphonyl ureas, metformin, α-glucosidase inhibitor and peroxisome proliferator activated receptor agonists thiazolidinediones.[55]

Hypoglycemia is the most worrying side effect of sulphonyl ureas glipizide and glimepride are associated with a lower incidence of hypoglycemia. Sulphonyl ureas may not be the optimal first choice for obese patients.[56] Most of the related side effects of metformin are metallic taste, gastro - intestinal discomfort and nausea.[57] Thiazolidinediones has many side effects including lowering the number of red blood cells and plasma protein levels and severe liver toxicity.[58] The α-glucosidase inhibitor are gastrointestinal, including abdominal comfort, bloating

flatulence and diarrhea but are reversible with discontinuation.[59] Insulin, with all its recent advancements in availability and efficacy, has its own definite disadvantages, like wild swings in blood sugar levels, insulin allergy, insulin resistance and insulin neuropathy.[60] Therefore finding other antidiabetic agents, especially those made from natural sources an important goal for researchers in diabetes.

Diabetes can be induced by pharmacological, surgical or genetic manipulations in several animal species. Most experiments in diabetes are carried out on rodents.[61] In the majority of the studies, natural products mainly derived from plants have been tested in diabetes models induced by chemical agents such as streptozotocin and alloxan.[62] Based on the dose was administered by these agents syndromes similar to either type 1 or type 2 diabetes mellitus.[63] The cytotoxic action of these diabetogenic agents is mediated by reactive oxygen species, but both differ in their mechanism of action. Alloxan establish a redox cycle with the formation of superoxide radicals. These radicals undergo dismutation to hydrogen peroxide with a simultaneous massive increase in cytosolic calcium concentration, which causes rapid destruction of pancreas β-cells.[64] As streptozotocin induced animals were used in all the experiment works. It is used to induce both insulin dependent and non insulin dependent diabetes mellitus. Streptozotocin actions on β-cells are accompanied by characteristic alterations in blood insulin and glucose concentrations. Streptozotocin (STZ) impairs glucose oxidation and decreases insulin biosynthesis and secretion.[65] It was observed that STZ at first abolished the β-cells response to glucose. The temporary return of responsiveness then appears which is followed by its permanent loss and cells are damaged.[66] STZ enters the pancreas β-cells via a glucose transporter and causes alkylation of DNA.[67]

Despite the recent drugs to treat and prevent diabetic condition, its prevalence continues to increase at an alarming rate. Although several drugs targeted for carbohydrate hydrolyzing enzymes, release of insulin from pancreatic β-cells (sulphonyl urea), glucose utilization (biguanides), insulin sensitizers, PPAR – agonists (glitazones) are in clinical practice, some of the drugs are linked to liver toxicity, including a number of death from hepatic failure[68] and raising the symptoms and risk factors of heart disease leading to heart failure (rosiglitazone).[69] On the other hand, traditional medicinal plants with various principles and beneficial multiple activities like manipulating carbohydrate metabolism by various mechanisms, preventing β-cells damage, restoring integrity and function of β-cells, augmenting insulin releasing activity, improving glucose uptake and utilization and the antioxidant properties. This section reviews the multiple therapeutic approaches of phytochemical in combating diabetes mellitus and its complications.

The review shows the importance and the interest placed on medicinal plants in the drive to demonstrate their antidiabetic effects and to isolate the bioactive agents. Many ethnobotanical surveys of medicinal plants used as folk medicine by the local population have been performed in different parts of the world, including India, several plant species have been described as hypoglycemic. The studies are interesting to focus on experimental studies performed on hypoglycemic plants and their bioactive components. Several medicinal plants have been used as dietary adjunct and in the treatment of numerous diseases without proper knowledge of their function. Although phototherapy continues to be used in several countries, few plants have received scientific or medical scrutiny.

Herbal medicines are becoming popular in developed countries as there is a wide belief that herbal preparations being "natural" and are intrinsically harmless and produces desired pharmacological activities. So, there is a rapid demand for formulations of herbal origin. Many governments are strictly implementing laws to control different aspects of these herbal drugs. So, it is mandatory now all the plant drugs and its formulations should pass analytical tests prescribed for it.

REPORTED BIOLOGICAL ACTIVITIES FOR ITS RELATED SPECIES

- **Sangilimuthu Alagar Yadav *et al.,*** The methanolic extract was evaluated in different method like DPPH assay, Fe^{2+} chelating activity, nitric oxide radical scavenging activity, $ABTS^{+}$ assay, superoxide anion and Hydrogen peroxide radical assay was studied. The methanolic extract of *Barleria noctiflora* leaf and root showed significant *in vitro* antioxidant activity have been reported.[70]

- **Jaya Preethi P.,** A review of herbal medicine for diabetes mellitus was exhibited some medicinal plants an important role in the management of diabetes in developing countries. In this review *Barleria noctiflora* belonging to family Acantheceae in whole plant was used in the treatment of diabetes.[71]

- **Ranjit Singh *et al.,*** The ethanol extract of the *Barleria cristata* seeds 200mg/kg showed significantly reduced blood glucose level by alloxan induced diabetes in Wistar rats at 150 and 200g/kg body weight.[72]

- **Gambhire MN *et al.,*** The anti inflammatory activity of aqueous extract of *Barleria cristata* leaves showed significant inhibitory activity of carrageenan induced edema, prostaglandin activity and vascular permeability dose dependent in mice.[73]

- **Shyam T *et al.,*** The antidiabetic activity of methanol extracts of *Barleria montana* showed reduction of blood glucose level in STZ induced diabetic rats was dependent dose and duration of action.[74]

- **Geetha M *et al.,*** The alcoholic and aqueous extracts of *Barleria prionitis* leaf and root showed a significant fall in blood glucose level in diabetic rats have been reported.[75]

- **Shanaz Banu *et al.,***The methonalic extract of *Barleria montana leaves and Barleria cristata* roots showed the significant reduction of blood glucose level in STZ induced diabetic rats.[76]

- **Ajay Mandal *et al.,*** The wound healing activity of *Barleria lupulina* leaf showed treatment with ointment containing methanolic and aqueous extract significant ($P<0.001$) activity in excision and incision wound model.[77]

- **Sos skovso.**, The high fat diet summarized metabolic and pathology involved in the different stages of the type 2 diabetes progresses in humans. It affects the functional β cell mass that is insufficient insulin secretion. The animal model of High Fat Diet fed, Streptozotocin (HFD/STZ) induced rat model might be a suitable animal model of type 2 diabetes.[78]

At the time of starting research work there has been no work was published, so we have started doing work in this plant, meanwhy the author Sangilimuthu Alagar Yadav carried out antioxidant activities “DPPH, ABTS, Hydrogen peroxide, and NBT” methods and published in Augest 2012. This also proved that the plants its having very good antioxidant property. So we carried out the same antioxidant activity in the plant with different extracts.

4. PLAN OF THE WORK

Phase-I: Collection and authentication of the plant materials

Phase II: Pharmacognostical studies

1. Microscopical studies
    A. T.S of Leaf and Stem
    B. Powder Analysis
2. Quantitative Microscopy of Leaf constants
    A. Stomatal Number and Stomatal Index
    B. Vein-Islet and Vein Termination Number
    C. Palisade Ratio
3. Physicochemical Parameters
    A. Ash Value
        i. Determination of Total Ash Value
        ii. Determination of Water Soluble Ash Value
        iii. Determination of Acid Insoluble Ash Value
    B. Extractive Values
        i. Determination of Alcohol Soluble Extractive Value
        ii. Determination of Water Soluble Extractive Value
    C. Loss on Drying
    D. Fluorescence Analysis

Phase-III: Phytochemical studies of the extracts

1. Preparation of extracts and fractions
2. Qualitative phytochemical analysis
3. Estimation of total phenol content
4. Estimation of total flavonoids

Phase-IV: *In vitro* antioxidant and antidiabetic studies

A. *In vitro* antioxidant screening
   i. DPPH radical scavenging activity
   ii. ABTS radical Scavenging method
   iii. Scavenging of hydrogen peroxide
   iv. Lipid peroxidation inhibitory activity
   v. Hydroxyl radical scavenging by p-NDA method
   vi. Superoxide radical scavenging by alkaline DMSO method.

B. *In vitro* antidiabetic studies
   i. *In vitro* inhibition of α-amylase
   ii. *In vitro* inhibition of α-glucosidase.

Phase-V: Pharmacological screening of ethyl acetate fraction of *Barleria noctiflora*

A. Acute toxicity studies
B. Subacute toxicity study
   i. Average body weight

ii. Biochemical Parameter

iii. Haematological Parameter

iv. Histopathology of Liver and Kidney

C. *In vivo* antidiabetic studies.

i. Oral glucose tolerance test (OGTT)

ii. Antidiabetic activity

a. Average of Body weight

b. Blood glucose level

c. Blood Parameters

- Triglycerides
- Total Cholesterol
- Cholesterol content in lipoprotein fractions
- Alanine Transaminase
- Aspartate Transaminase
- Alkaline Phosphatase
- Estimation of glycosylated haemoglobin
- Estimation of serum insulin level
- Assay of creatinine
- Histopathological studies of pancreatic tissues

D. Wound healing activity

   i. Excision wound model

   ii. Incision wound model

Phase-VI: Isolation and characterization of phytoconstituents from *Barleria noctiflora* extract.

1. Column chromatography.

2. Spectral data study: IR, ^{1}H NMR, ^{13}C NMR spectroscopy.

5. PLANT PROFILE

5.1. PLANT INTRODUCTION

Figure 1: *Barleria noctiflora* plant photo

Botanical Name	**:**	*Barleria noctiflora L.f.*
Family	**:**	Acanthaceae.
Kingdom	**:**	Plantae
Phylum	**:**	Magnoliophyta
Class	**:**	Magnoliopsida
Order	**:**	Lamiales
Genus	**:**	*Barleria*

Species	**:**	*noctiflora*
Other Varieties	**:**	*B.cristata, B.lupulina, B.prionitis, B.montana*
Distribution	**:**	It occurs throughout the hotter parts of India, Africa, Sri Lanka and other parts of Asia.
Parts Used	**:**	Whole part
Organoleptic characters	**:**	The leaf is green and stems gray in color. The leaf is in oval like shape and stem cylindrical in shape. The plants are pungent odour and slightly sweet and sour taste.

Morphological Characters

Habit	**:**	A common under shrub occasionally found wild, but generally cultivated as a hedge plant or for its ornamental flowers.[79]
Height	**:**	0.6 – 1.5 m height.
Leaves	**:**	1.5 – 2 cm long, 1- 1.5cm wide, simple, elliptic, acuminate, entire, acute reticulate, glabrous above, glabrous or pubescent beneath; petiole short.[80]
Stem	**:**	1- 6 mm thick, terete, hard, glabrous, nodes swollen, branching at nodes, young stem gray, slightly four angled, usually with 2-3 divaricate spines at axil of leaf mature stem cylindrical with longitudinally arranged or scattered, a few mature stem slightly hollow.[81]

Flowers	**:**	Sessile, often solitary in the axils, becoming spicate above, laciculate, acute bristle tipped early glabrous bracteoles, bristle tipped, calyx divided almost to the base.[82]
Chemical Constituents	**:**	Barlerinoside, barlerin, acetylbarlerin, 7-methoxy diderroside and lupulinoside.

5.2. ETHNOMEDICAL INFORMATION

This is being widely used as folk and Ayurvedic medicine. It is widely distributed throughout tropical region of India, Africa, Sri Lanka and other parts of Asia.[83] *Barleria noctiflora* is a shrub and it grows up to 90 cm height. Many of the members of the Acanthaceae family are used as medication for asthma.[84] The genus *Barleria* includes 28 taxa and 26 species. It has 3 unique characters calyx 4-partile with 2 large outer segments and 2 smaller inner ones, spheroidal, honey - combed pollen grains and the predominant with double cystoliths.[85] Most of the *Barleria* species are potent anti- inflammatory, analgesic, anti leukemic, antitumor, anti-hyperglycemic, anti-amoebic, virucidal & antibiotic etc.

6. MATERIALS AND METHODS

6.1. INSTRUMENTS AND CHEMICALS

6.1.1. Instruments

Table 1: List of Instruments

Instrument	Company
Autoanalyser	Maxlyzer ultra auto analyzer**,** India.
Centrifuge	Remi (R-4c) laboratory centrifuge, Remi motors Ltd, Mumbai.
Haematology analyzer	Cosmic technology hematology analyzer, India.
pH meter	Systronics MKVI digital pH meter.
Glucometer	SD check glucometer, India.
^{13}C NMR	Bruker India Pvt Ltd., India.
FT-IR	Shimadzu FTIR 8400S, Japan.
Grinding Mill	Junior grindwell, chowdhry, J.U.C, Mumbai.
Homogenizer	Elvenjan homogenizer, Remi motors Ltd., Mumbai.
GC-MS spectroscopy	Perkinelmer India Pvt Ltd., India.
Melting point apparatus	Lab india melting point apparatus, India.
Proton NMR	Bruker India Pvt Ltd., India.
Rotary evaporator	Rotavapor R-205, Buchi laboratory equipments, Flawill, Switzerland.
Spectrophotometer	Shimadzu 1700 UV-Vis spectrophotometer, Shimadzu, Tokyo, Japan.

6.1.2. Chemicals and Drugs used

Table 2: List of Chemicals and Drugs (Analytical grade)

Chemicals	**Company**
Petroleum ether	S.D. Fine Chem, Ltd., India.
Chloroform	S.D. Fine Chem, Ltd., India.
Ethyl acetate	S.D. Fine Chem, Ltd., India.
n-butanol	S.D. Fine Chem, Ltd., India.
Ethanol	S.D. Fine Chem, Ltd., India.
Dimethyl sulfoxide	S.D. Fine Chem, Ltd., India.
2,2-Diphenyl-1-Picryl Hydrazyl (DPPH)	HiMedia, Mumbai, India.
2,2'-Azino-Bis(3-ethylbenz-Thiazoline-6 Sulfonic acid) diammonium salt (ABTS),	HiMedia, Mumbai, India.
Rutin	HiMedia, Mumbai, India.
p-Nitroso Dimethyl Aniline (p-NDA)	HiMedia, Mumbai, India.
Folin-Ciocalteu reagent	S.D. Fine Chem, Ltd., Biosar, India.
Ascorbic acid	S.D. Fine Chem, Ltd., Biosar, India.
Nitro Blue Tetrazolium (NBT)	S.D. Fine Chem, Ltd., Biosar, India.
Butylated Hydroxy Anisole (BHA)	S.D. Fine Chem, Ltd., Biosar, India.
Tannic acid	Loba Chemie Indo Co., Mumbai.
Thiobarbituric acid	Loba Chemie Indo Co., Mumbai.
Trichloroacetic acid	Loba Chemie Indo Co., Mumbai.
Ethylene Diamine Tetra Acetic acid (EDTA)	Loba Chemie Indo Co., Mumbai.
Glucose assay kit	Agappe Diagnostics, Kerala, India.
Cholesterol kit	Agappe Diagnostics, Kerala, India.
Low density lipoprotein cholesterol (LDL) kit	Agappe Diagnostics, Kerala, India.
High density lipoprotein cholesterol (HDL) kit	Agappe Diagnostics, Kerala, India.
Triglyceride assay kits	Agappe Diagnostics, Kerala, India.
Alkaline phosphatase (ALP)	Agappe Diagnostics, Kerala, India.
Aspartate transaminase (AST)	Agappe Diagnostics, Kerala, India.
Alanine transaminase (ALT)	Agappe Diagnostics, Kerala, India.
Streptozotocin	HiMedia, Mumbai, India.
α-amylase	HiMedia, Mumbai, India.
Potato starch,	Merck Ltd, Mumbai, India.
Maltose	Merck Ltd, Mumbai, India.
α- glucosidase	Sisco lab Ltd, Mumbai, India.
Tween-80 (were purchased for the study)	SD fine chemicals, Mumbai, India.
Glibenclamide (were obtained as gift sample)	Aventis Pharma, Mumbai, India.
Acarbose (were obtained as gift sample)	Orchid Pharma Ltd, Chennai, India.
Commercial animal feed	Sai Durga Feeds and Food, Bangalore.

6.1.3. Plant Materials

Barleria noctiflora was collected during the winter season in and around Erode District, Tamilnadu, India. It was identified and authenticated by Prof. P. Jayaraman, Director, National Institute of Herbal Science, Chennai-45, Tamilnadu, India (Ref no: PARC/2011/1015), and the voucher specimen was put down at the same institute for future reference.

6.2. PHARMACOGNOSTICAL EVALUATION

6.2.1. Microscopical Studies

Microscopical techniques provide detailed information about the crude drugs by virtue of its two main analytical uses. Its property to magnify permits the fine structures of minute objects to be visualized and thereby confirm the structural details of the plant drugs under evaluations. The crude drug microscopical inspection of plant origin is necessary for the identification of the powdered and grounded materials. To establish the microscopical characters of any herbal drug, considerable skill and experience on microscopy of crude drugs is important.

Crude drugs can be identified by morphological characteristics; in case of doubt the same can be investigated for histological characteristics to confirm the identity of crude drugs. However, in spite of these facts microscopy is a great necessity in the identification of powders. The powder drugs can be recognized based on the presence or absence of different cell types criteria on their cytomorphological characters, e.g. parenchyma, collenchymas, fibers, stone cells, vessels, trichomes, secretory cells, epidermal cells etc.

A. Transverse section (TS) of Leaf and Stem

Select healthy plant of *Barleria noctiflora* and normal organ. The necessary samples of different organs were cut and removed from the plant and fixed in FAA (Formalin 5 ml + Acetic acid 5 ml + 70% of Ethanol 90 ml) after 24 hrs of fixing the specimen were dehydrated with a graded series of Tertiary Butyl Alcohol (TBA) as per the schedule.[86] Infiltration of the specimen was carried by gradual addition of paraffin wax (melting point 58-60ºC) until TBA solution attained super saturation. The specimens were cast into paraffin blocks.

B. Sectioning

The paraffin embedded specimens were sectioned with the help of rotary microtome. The thickness of the section wouldbe 10-12 µm, de-waxing of the sections was by customary procedure. The sections were stained with toluidine blue as per the methods.[87] The staining results were remarkably good and some cytochemical reactions were also obtained. The dye rendered pink color to the cellulose walls, blue to the lignified cells, dark green to suberin, violet to the mucilage, blue to the protein bodies, etc. wherever necessary sections were also stained with safranin and fast green and potassium iodide (for starch). Glycerin mounted temporary preparation were made from macerated/cleared materials. Powdered materials of different parts were cleared with sodium chloride and mounted in glycerin medium after staining. Different cell component was studied and measured.[88,89]

C. Powder Analysis

The dried powder of whole plant of *Barleria noctiflora* was passed through sieve no 60 and examined for its microscopic characters. The powder of drugs was boiled with chloral hydrate to remove the coloring matter, mounted on the glass slides using glycerin and covered with a cover slip and viewed under microscope.[90]

D. Photomicrograph

Microscopic descriptions of tissue are supplemented with micrographs wherever necessary. Photographs of different magnifications were taken with Nikon lab photo 2 microscopic units. For the study of crystals, starch grains and lignified cells, polarized light was employed, since these structures have birefringent property, under polarized light they appear bright against a dark background. Magnifications of the figures are indicated by the scale-bars. Descriptive terms of the anatomical features are as given in the standard anatomy book.[91]

6.2.2. Quantitative Microscopy of Leaf Constants

Quantitative analytical microscopy is used for the measurement of cell contents of the crude drugs and thus helps in their identification, characterization and standardization. This method is based upon a comparison between the characteristics observed in the sample and those of standard materials of known origin. Such comparison can be applied to the positive identification of the botanical, geographical and other sources of a drug, to evaluate the quality of drug. While evaluating an herbal drug, the quantitative microscopical methods can be two types. One involves the measurement of the size of the individual features and the other involves estimation of the occurrence of a particular feature in a specific quantity of materials.

A. Stomatal Number and Stomatal Index

The clear piece of the middle part of leaf by boiled with chloral hydrate solution. Peel out of upper and lower epidermis separately by forceps. Keep it on slide and mount in glycerin water then draw a square of 1 mm by means of a stage micrometer and camera lucida on a drawing paper. The stage micrometer was replaced by the cleared leaf preparation, focus under the same magnification and trace the epidermal cells and stomata, count the number of stomata present in the area of 1 sq.mm, include the cell if at least half of its area lies within the square. Record the result for each of the ten fields and the average number stomata was calculated per sq.mm. The stomatal index is the percentage which the number of stomata forms to total number of epidermal cells. Stomatal index can be calculated by using the formula

$$I = \frac{S}{E+S} X\ 100$$

I = stomatal index

S = no of stomata per unit area

E = no of epidermal cells in the same unit area

B. Vein-Islet and Vein Termination Number

Clear pieces of the leaf by boiling in chloral hydrate solution for about 30 min and mount the preparation in glycerin water and arrange camera lucida and drawing paper. Place stage micrometer on the microscope and using 16 mm objective and then draw a square of 1 mm by means of area, placed the slide with clear leaf and trace of the veins which are included within the square. Count the number of vein islet and vein termination in the sq.mm, where the islets are

intersected by the sides of the square, include those on two adjacent sides and exclude those islets on the other sides. Find the average number of vein islets and vein termination from the four adjoining squares, to get the value for 1 sq.mm.

C. Palisade Ratio

A clear piece of the leaf by boiling in chloral hydrate solution for about 30min and mount the preparation in glycerin water and arrange camera lucida and trace the outlines of 4 continuous epidermal cells, then focus down to palisade layer and trace off sufficient cells to cover the tracing of the epidermal cells and palisade cells were counted under the four epidermal cells. Calculate the average number of cells beneath single epidermal cells. Repeat the determination for five groups of four epidermal cells from different parts of the leaf. Calculate the average from the results five groups.[92]

6.2.3. Physicochemical Parameters

A. Ash Values

Ash values are useful in determining the purity and quality of crude drugs, especially in the powdered form.

The crude drug of ash content is generally taken to be the remaining residue after the incineration. Ash standards are recognized for a number of official drugs. The standards are usually getting a higher limit on the total ash or acid insoluble ash permitted.

The residue of total ash is the remaining after incineration. The acid insoluble ash is the division of the total ash, which are insoluble in diluted hydrochloric acid.

The residue or ash yielded by organic chemical compounds as a rule, the amount of inorganic matters is measure present as an impurity. In common cases, the inorganic matters are present in small amounts. The purification process, which are difficult to remove and not objectionable if only traces are present. The determining the quality and purity of ash values are helpful in the crude drugs in powder form.

The procedures given in Indian pharmacopoeia were used to determine the crude drug ash values such as total ash and acid insoluble ash.

i. Determination of Total Ash Value

Accurately weighed about 3 gm of dried powdered drug of the *Barleria noctiflora* was taken in a silica crucible and incinerated in a muffle furnace at a temperature not exceeding 450°C until free from carbon, cool and weigh, the charred mass exhaust with hot water, residue was collect on ashless filter paper, and incinerate the residue and filter paper until the white ash or nearly so. Calculate the total ash percentage with reference to the air dried drug.

ii. Determination of Water Soluble Ash Value

The total ash was obtained and boiled with 25 ml of water for 5 min. Then the insoluble matter was collected on an ash less filter paper, washed with hot water for 15 min at a temperature not exceeding 450˚C. The insoluble matter was subtracted from the weight of total ash. The variation in weight represents the water soluble ash. The water soluble ash percentage was calculated with reference to the air dried drug.

iii. Determination of Acid Insoluble Ash Value

The obtained ash directed under total ash was boiled with 25 ml of 2M Hcl for 5 min. To collect insoluble matters on ash less filter paper and washed with hot water and weighed, then acid insoluble ash percentage was calculated with reference to the air dried drug.

B. Determination of Extractive Values

Extractive values of coarse powdered crude drugs are useful for their evaluation, the extraction of the drug with an exacting solvent yield a solution containing different phytoconstituents. The phytoconstituents composition in that exacting solvent depends upon the drug nature and used solvent. Further, this value indicates the present crude drug in nature constituents.

i. Determination of Alcohol Soluble Extractive Value

Weigh accurately 5 gm of air-dried coarse powder of the *Barleria noctiflora* was soaked with 100 ml of ethanol (90%) in a closed flask for 24 hrs, frequently shaking during the first 6 hrs and stand to allow for 18 hrs. Then it was filtered rapidly against the loss of the solvent. The 25 ml of the filtrate was evaporated to dryness in a tarred flat bottomed shallow dish, dried at 105°C and weighed. The percentage of ethanol soluble extractive value was calculated with reference to the air-dried drug.

ii. Determination of Water Soluble Extractive Value

Weigh accurately 5 gm of coarsely powdered drug of the *Barleria noctiflora* and macerate with 100 ml of water in closed flak for 24 hrs, shaking frequently during the first 6 hrs and allow to stand for 18 hrs. Thereafter, it was filtered rapidly taking precautions against loss of the solvent. Then filtrate was evaporated to

dryness in a tarred flat bottomed shallow dish, dried at 105°C and weighed. The water soluble extractive percentage was calculated with reference to air dried drug.

C. Loss on Drying

The percentage loss on weight determines the amount of volatile matter of any type (including water) that can be driven off under the condition specified (Hot air oven or Desiccators). If the sample is large crystal form, then reduce the particle size by quick crushing to a powder.

Procedure

The powdered drug of 1.5 gm was weighed accurately in a porcelain dish, which was formerly dried at 105˚C in hot air oven to constant and then weighed. From the weight difference, then calculate the percentage loss of drying with reference to the air dried substance.

D. Fluorescence Analysis

The organic molecules absorb light usually over a range of specific wavelength and many of molecules re-emit such radiations. This phenomenon is called as luminescence. The substance is receiving the exciting rays; the phenomenon is defined as fluorescence. The quantitative evaluation is possible in fluorescence analysis using the fluorescence produced by a compound in UV light. The dried Powder was treated with various chemical reagents and exposed to visible, daylight and ultraviolet light to study their fluorescence behavior.[93]

6.2.4. Extraction

(A) Successive Extraction

Five hundred grams (500 gm) of shade dried powdered aerial part of *Barleria noctiflora* were extracted using Soxhlet apparatus, successively with

petroleum ether, chloroform and ethanol for 72 hrs each. The extracts were concentrated to dryness in a rotavapor. The yield of the extract was determined. All the extracts were stored in a refrigerator at 4°C until further use.

(B) Crude Extraction

Five hundred grams (500 gm) of shade dried powdered aerial part of *Barleria noctiflora* were extracted with ethanol in a Soxhlet apparatus for 72 hrs. The yield of greenish brown solid was 55.3 gm (11.06% w/w). The dried powder (50 gm) was extracted with water (250 ml) by maturation for seven days and concentrated to yield a greenish brown solid (5.7 gm, 11.4% w/w). All the extracts were concentrated to dryness in a rotavapor under reduced pressure and controlled temperature (40-50°C). All the extracts were stored in a refrigerator at 4°C until further use.

(C) Fraction

Twenty grams (20 gm) of the dried crude ethanol extract were taken in a stoppered flask, containing 200 ml of water and shaken mechanically for 1-2 hrs in a flask shaker. The ethanol extract was not completely soluble in water. The water insoluble portion of ethanol extract was separated using filtration and further fractioned with ethyl acetate and n-butanol using the same procedure. The supernatants obtained from the above fraction were concentrated and evaporated to dryness and their percent yield was determined.

6.2.5. Qualitative Phytochemical Analysis

A systematic and complete study of crude drugs should include a complete investigation of both primary and secondary metabolites derived from plant

metabolism. The different qualitative chemical tests are to be performed for establishing profiles of extracts for their nature of chemical composition.[94]

A) Test for carbohydrates

1. Molisch Test: It extracts treated with α-naphthol and concentrated sulphuric acid along the sides of the test tube. Purple colour or reddish violet colour was produced at the junction between two liquids.

2. Fehling's Test: To the extracts equal quantity of Fehling's solution A and B is added. Heat gently; the brick red precipitate is obtained.

3. Benedict's test: To the 5 ml of Benedict's reagent, add 8 drops of solution under examination. Mix well, boiling the mixture vigorously for 2 min and then cool. Red precipitate is obtained.

4. Barfoed's test: To the 5 ml of the Barfoed's solution add 0.5 ml of solution under examination, heat to boiling, a red precipitate is formed.

B) Test for Alkaloids

1. **Dragendroff's Test:** To the extract, add 1 ml of Dragendroff's reagent Orange red precipitate is produced.

2. **Wagner's test:** To the extract add Wagner's reagent. The reddish brown precipitate is produced.

3. **Mayer's Test:** To the extract add 1 ml or 2 ml of Mayer's reagent. The dull white precipitate is produced.

4. **Hager's Test:** To the extract add 3 ml of Hager's reagent yellow Precipitate is produced.

C) Test for Steroids and Sterols

1. **Liebermann Burchard test:** Dissolve the test sample in 2 ml of chloroform in a dry test tube then add 10 drops of acetic anhydride and 2 drops of concentrated sulphuric acid. The solution becomes red, then blue and finally bluish green in colour.

2. **Salkowski test:** Dissolve the test solution in chloroform and add equal volume of conc. sulphuric acid. Bluish cherry red and purple color is noted in chloroform layer, whereas acid assumes marked green fluorescence.

D) Test for Glycosides

1. **Legal's test:** Sample is dissolved in pyridine; sodium nitropruside solution is added to it and made alkaline. Pink red colour is produced.

2. **Baljet test:** To the drug sample, sodium picrate solution is added. Yellow to orange colour is produced.

3. **Borntrager test:** Add a few ml of dilute sulphuric acid to the test solution. Boil, filter and extract the filtrate with ether or chloroform. Then organic layer is separated to which ammonia is added, pink, red or violet colour is produced in organic layer.

4. **Killer Killani test:** Sample is dissolved in acetic acid containing traces of ferric chloride and transferred to the surface of concentrated sulphuric acid. At the junction of liquid reddish brown color is produced which gradually becomes blue.

E) Test for Saponins

Foam test: About 1 ml of alcoholic sample is taken in a graduated cylinder and it is diluted to 20 ml with distilled water and shaken for 15 min. The formation of 1 cm layer of foam indicates the presence of saponins.

F) Test for Flavonoids

Shinoda test: To the sample, magnesium turnings and then concentrated hydrochloric acid is added. Red colour is produced.

H) Tests for Tannins and Phenolic Compounds

The Phenol content in the raw material of *Barleria noctiflora* extract was estimated by using following reagents.

To 2-3 ml of extract, add few drops of following reagents:

a). 5% $FeCl_3$ solution: Deep blue-black color.

b). Lead acetate solution: White precipitate.

c). Gelatin solution: White precipitate.

d). Acetic acid solution: Red color solution.

e). Dilute iodine solution: Transient red color.

f). Dilute HNO_3: Reddish to yellow color.

I) Test for Fixed Oils and Fatty acids

1. Spot test:

A small quantity of the extract is placed between two filter papers. Oil stain produced with any extract shows the presence of fixed oils and fats in the extracts.

2. Saponification test

Few drops of 0.5N alcoholic potassium hydroxide are added to the extract with a few drops of phenolphthalein solution. Later the mixture is heated on a water bath for 1-2 hr soap formation indicates the presence of fixed oils and fats in the extracts.

K) Test for Proteins and Amino acids

1. Biuret test: Add 1 ml of 40% sodium hydroxide and 2 drops of 1% copper sulphate to the extract, a violet colour indicates the presence of proteins.

2. Ninhydrin test: Add 2 drops of freshly prepared 0.2% Ninhydrin reagent to the extract and heat. A blue colour develops indicating the presence of proteins, peptides or amino acids.

3. Xanthoprotein test: To the extract, add 20% of sodium hydroxide or ammonia. Orange colour indicates presence of aromatic amino acid.

6.2.6. Estimation of Total Phenol Content

Recent studies have shown that many dietary polyphenolic constituents derived from plants are more effective antioxidants than vitamins E or C, and thus might contribute to the protective effects. It is possible to assess the extent to which the total antioxidant potential of plant extracts can be accounted by the activities of individual polyphenols. Hence, the total phenol content of the extracts was determined by using the Folin-Ciocalteu method.[95] This method is based on the phenolic oxidation groups with phosphotungstic and phosphomolybdic acids. After oxidation a green blue complex formed was measured at 760 nm.

Reagents

1. **Folin-Ciocalteu reagent:** Commercially available Folin-Ciocalteu reagent was diluted (1:10) with distilled water and used.
2. **Sodium bicarbonate:** 7.5 g of sodium bicarbonate was dissolved in 100 ml of distilled water and used.

Preparation of Test and Standard Solutions

The ethanolic extract, aqueous extract and the standard tannic acid (50 mg each) were dissolved separately in 50 ml of methanol. These solutions were serially diluted with methanol to obtain the lower dilutions.

Procedure

Methanolic solution of the extracts in the concentration of 1 mg/ml was used in the analysis. The reaction mixture was prepared by mixing 0.5 ml of methanolic solution of extract, 2.5 ml of 10% Folin-Ciocalteu reagent and 2.5 ml 7.5% $NaHCO_3$.[95] Blank was concomitantly prepared**,** containing 0.5 ml methanol, 2.5 ml 10% Folin-Ciocalteu reagent and 2.5 ml of 7.5% of NaHCO3. Thereafter the samples were incubated in a thermostat at 45°C for 45 min. The absorbance was determined using spectrophotometer at 760 nm. The samples were prepared in triplicate for each analysis and the mean value of absorbance was obtained. The same procedure was repeated for the standard solution of tannic acid and the calibration line was constructed. Based on the measured absorbance, the concentration of phenolic was read (µg/ml) from the calibration line; then the content of phenolic in the extracts was expressed in terms of tannic acid equivalent (TAE) (µg of TAE/g of extract).

6.2.7. Estimation of Total Flavonoid Content

Flavonoids are a class of plant secondary metabolites. It can be classified in to bioflavonoids, isoflavonoids and neoflavonoids. Flavonoids have been a wide range of biological and pharmacological activities. The total flavonoids content was determined as the Aluminium chloride used in colorimetric method. The absorbance was determined using spectrophotometer at 415 nm.

Reagents

Aluminium chloride solution: 2 gm of Aluminium chloride was dissolved in 100 ml of methanol.

Preparation of Test and Standard Solutions

The extracts and the standard rutin (50 mg each) were dissolved separately in 50 ml of methanol. These solutions were serially diluted with methanol to obtain the lower dilutions.

Procedure

The sample contained 1ml of methanol solution of the extracts in the concentration of 1mg/ml and 1 ml of 2% $AlCl_3$ solution. The samples were incubated for an hour at room temperature. The absorbance was determined using spectrophotometer at 415 nm. The samples were prepared in triplicate for each analysis and the mean value of absorbance was obtained. The same procedure was repeated for the standard solution of rutin and the calibration line was constructed. Based on the measured absorbance, the concentration of flavonoid was read (µg/ml) on the calibration line; then, the content of flavonoid in the extracts was expressed in terms of rutin equivalent (RE) (µg of RE/g of extract).[96]

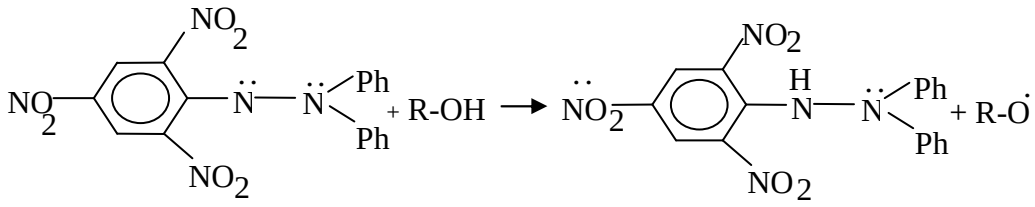
6.3. *IN VITRO* ANTIOXIDANT STUDIES OF EXTRACTS

To study the comparative antioxidant activity of the plant extracts, *in vitro* antioxidant studies were conducted. The extracts having better antioxidant property were chosen for *in vivo* studies. The scavenging activities of the different extracts of plant against different radicals were carried out according to the procedures described below. In all the experiments, the absorbance was measured against a blank solution. A control was performed without adding extract or standard. The final concentration of the extracts or standard was 1000, 500, 250, 100, 50, 25, 10 and 5 µg/ml. The results of all the *in vitro* antioxidant scavenging activities were expressed in terms of IC_{50}, which is the concentration required by the sample to scavenge 50% of free radicals.

The *in vitro* antioxidant activity was studied by using the different extractions such as Petroleum ether extract of *Barleria noctiflora* (PEBN), Chloroform extract of *Barleria noctiflora* (CEBN), Ethanolic extract of *Barleria noctiflora* (EEBN), Crude ethanolic extract of *Barleria noctiflora* (CEEBN) and Aqueous extract of *Barleria noctiflora* (AEBN).

A. Diphenyl Picryl Hydrazyl (DPPH) Radical Scavenging Activity

DPPH assay is supported on the measurement of antioxidant scavenging ability towards the stability of DPPH radicals.[97] The free radical DPPH is purple in color in methanol and is reduced to the corresponding hydrazine, which is yellow in color when it reacts with hydrogen donor.



Purple color DPPH (2,2-diphenyl-1-picryl hydrzyl)

Yellow stable phenoxy radical

It is a decoloration assay, with addition of the antioxidant is evaluated to DPPH solution in methanol and the decrease in the absorbance is measured at 517 nm.

Reagents

DPPH solution (0.1 mM): 22 mg of DPPH was accurately weighed and dissolved in 100 ml of methanol. From this stock solution, 18 ml was taken and diluted to 100 ml with methanol to obtain 0.1 mM DPPH solution.

Preparation of Test and Standard Solutions

The extracts and the standard ascorbic acid (20 mg each) were separately dissolved in 20 ml of methanol. These solutions were serially diluted with methanol to obtain the lower dilutions.

Procedure

To 2 ml methanolic solution of 0.1 mM DPPH was added separately into 200 µl of sample extracts and standard of ascorbic acid (5-1000µg/ml) with 0.8 ml methanol. The mixture was mixed thoroughly and kept in the dark for 1 hr. The control sample was prepared by mixing 2 ml of DPPH and 1 ml methanol.[98] The absorbance was measured at 517 nm using a spectrophotometer. Samples were

measured in three replicates reading. Percentage inhibition of DPPH scavenging activity was calculated by using following formula.

% inhibition = [Abs control –Abs sample / Abs control] x 100.

B. ABTS Radical Scavenging Method

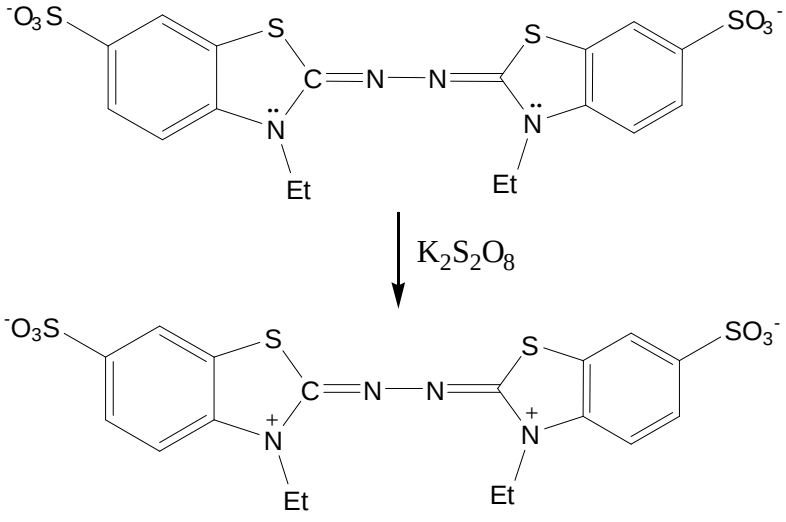
In ABTS assay, the absorbance of radical cation of $ABTS^{+}$, this has a wavelength characteristic at 734 nm. In this assay, the radical is generated in a stable form using potassium persulphate as ABTS radical cation, a blue green chromogen. Afterwards, the formed colored radical is mixed with antioxidant in the reaction medium and the colored radical is converted back to colorless ABTS, the percentage inhibition of absorbance at 734 nm is calculated.[99]

Reagents

ABTS (191.8 mg) was dissolved in 50 ml of methanol to give 7 mM concentration of ABTS reagent, and 2.4 mM potassium persulphate was prepared.

Preparation of Test and Standard Solutions

The extracts and the standard ascorbic acid (20 mg each) were separately dissolved in 20 ml of methanol. These solutions were serially diluted with methanol to obtain the lower dilutions.



ABTS radical cation

Procedure

The radicals of $ABTS^+$ cation were produced by the reaction in between of 7 mM ABTS solution and 2.4 mM potassium persulfate solution (1:1), in the dark room it was stored at room temperature for 12-16 hrs before use.[100] $ABTS^+$ solution was diluted with methanol to attain an absorbance of 0.7 at 734 nm. After the addition of 30 µl of plant extract / standard ascorbic acid (5-1000µg/ml) and 3 ml diluted $ABTS^+$ solution, the absorbance was measured after 30 min. A suitable blank was run with each assay. All the reading was carried out in triplicate. The absorbance of percentage inhibition at 734 nm was calculated using the formula is mentioned in earlier assay.

C. Scavenging of Hydrogen Peroxide

Hydrogen peroxide is generated *in vivo* by several oxidase enzymes. There is increasing evidence that hydrogen peroxide is scavenged, either directly or indirectly via its reduction product hydroxyl radical ($OH^{\bullet}$). In this method, a scavenger is incubated with hydrogen peroxide, the loss of hydrogen peroxide measured spectrophotometrically at 230 nm.[101]

Preparation of Test and Standard Solutions

The extracts and the standard ascorbic acid (20 mg each) were separately dissolved in 20 ml of methanol. These solutions were serially diluted with methanol to obtain series of aliquots.

Procedure

The hydrogen peroxide (40 mM) of 2 ml was prepared in phosphate buffer (pH 7.4) and 1 ml of methanol sample (5-1000 µg/ml of extract of plant / standard of ascorbic acid) was added to a hydrogen peroxide solution.[102] The absorbance was

determined at 230 nm after 10 min against a blank solution containing phosphate buffer without hydrogen peroxide. The experiment was repeated in triplicate. The percentage of hydrogen peroxide scavenging by the extract and standard compound was calculated using earlier formula.

D. Lipid Peroxidation Inhibitory Activity

Lipid peroxidation is a process in which molecular oxygen is incorporated into an unsaturated lipid (LH) to form lipid hydroperoxides (LOOH). Iron stimulates the lipid peroxidation by decomposing lipid hydroperoxides (LOOH) to form alkoxyl ($LO^{\bullet}$) and peroxyl ($LOO^{\bullet}$) radicals or by directly reacting with molecular oxygen to produce hydroxyl radicals ($OH^{\bullet}$). Alkoxyl, peroxyl, and hydroxyl radicals initiate lipid peroxidation by abstracting hydrogen atoms from unsaturated fatty acids (LH), a reaction followed by oxygen uptake.[103]

$$Fe^{2+} + LOOH \longrightarrow LO^{\bullet} + OH^{\bullet} + Fe^{3+}$$

Lipid hydroperoxide Peroxyl Radical Hydroxyl Radical

$$Fe^{3+} + LOOH \longrightarrow LO^{\bullet} + H^{+} + Fe^{2+}$$

$$LO^{\bullet} + LH \longrightarrow LOH + L^{\bullet}$$

$$LO_2^{\bullet} + LH \longrightarrow LOOH + L^{\bullet}$$

$$OH^{\bullet} + LH \longrightarrow H_2O + L^{\bullet}$$

$$L^{\bullet} + O_2 \longrightarrow LO_2^{\bullet}$$

Preparation of Test and Standard Solutions

The extracts and the standard (20 mg) were separately dissolved in 20 ml of freshly distilled Dimethyl sulfoxide (DMSO). These solutions were serially diluted with freshly distilled DMSO to obtain the lower dilutions.

Preparation of Rat Brain Homogenate

The rat brain homogenate was prepared according to the described method.[104] Albino rat brain was removed and washed with ice–cold saline. The brain was homogenized in 9 volumes of ice–cold phosphate buffer (pH 7.4) using a glass homogenizer and centrifuged at 1000 rpm for 10 min. The supernatant was stored at 70°C until the lipid peroxidation experiment.

Procedure

The lipid peroxidation was initiated by the 1 ml of tissue homogenate, the addition of 0.1 ml of $FeSo_4$ (25 μm), 0.1 ml of ascorbate (100 μm) and 0.1 ml of KH_2Po_4 (10 mm) and the volume was made up to 3 ml with distilled water and incubated at 37ºC for 1 hr. Then 1 ml of 5% Trichloroacetic acid (TCA) and 1 ml of Thiobarbituric acid (TBA) was added to this reaction mixture and the tubes were boiled for 30 min, in a boiling water bath.[105] This was centrifuged at 3500 rpm for 10 min. In the test system homogenate was incubated with various concentrations of extracts (5-1000 μg/ml). The extent of inhibition of lipid peroxidation was evaluated by the estimation of Thiobarbituric acid reactive substances (TBARS) level by measuring the absorbance at 532 nm was calculated using the formula is mentioned in earlier assay.

E. Scavenging of Hydroxyl Radical by p-NDA Method

The hydroxyl radical is measured by inhibition of p-Nitroso Dimethyl Aniline (p-NDA) bleaching by hydroxyl radical.[106] The radical is generated during Fenton reaction. In this reaction, complex of iron EDTA reacts with hydrogen peroxide in the presence of ascorbic acid to produce hydroxyl radical, which can bleach p-NDA specifically. Hydroxyl radical shows scavenging activity by

bleaching inhibition and scavenging percentage as absorbance is measured at 440nm.

$$Fe^{3+} + EDTA \longrightarrow Fe^{3+}\text{-EDTA complex}$$

$$Fe^{3+}\text{-EDTA complex} + \text{Ascorbate} \longrightarrow Fe^{2+}\text{-EDTA complex} + \text{Oxidized Ascorbate}$$

$$Fe^{2+}\text{-EDTA complex} + H_2O_2 \longrightarrow Fe^{3+}\text{-EDTA} + OH^{\bullet} + OH^{-}$$

$$(Fe^{3+} + O_2^{-} \longrightarrow Fe^{2+} + O_2)$$

Preparation of Test and Standard Solutions

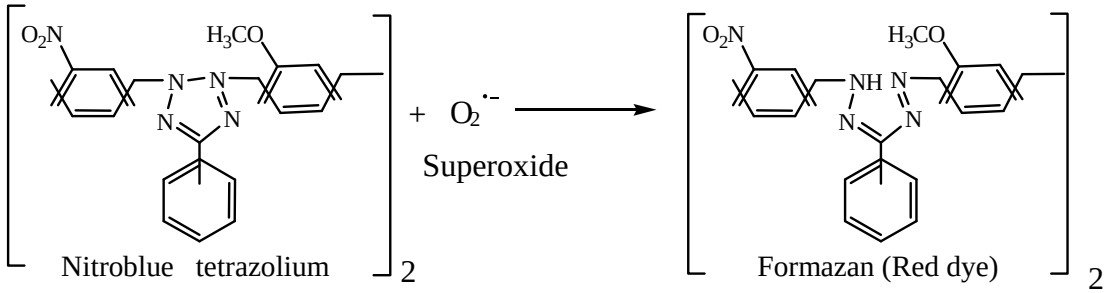
The extracts and the standard ascorbic acid (20 mg each) were dissolved separately in 20 ml of freshly distilled DMSO. These solutions were serially diluted with freshly distilled DMSO to obtain the lower dilutions.

Procedure

Various concentrations of the extracts / standard ascorbic acid (5-1000 µg/ml) in 0.5 ml of distilled DMSO solution were added each 0.5 ml of a mixture containing ferric chloride (0.1 mM), EDTA (0.1 mM), ascorbic acid (0.1 mM), hydrogen peroxide (2 mM) and p-NDA (0.01 mM) in phosphate buffer (pH 7.4, 20 mM) to produce a final volume of 3 ml. Absorbance was measured spectrophotometrically at 440 nm.[107] All the measurements were carried out triplicate. Percentage inhibition was calculated in earlier formula.

F. Scavenging of Superoxide Radical by Alkaline DMSO Method

In the alkaline DMSO method, generate the superoxide radical by addition of sodium hydroxide to the air saturated dimethyl sulfoxide (DMSO). The superoxide remains stable in solution, which decrease nitro blue tetrazolium to formazan at room temperature and can be measured at 560 nm. Superoxide scavenger capable of reacting inhibits the formation of a red dye formazan.[108]



Preparation of Test and Standard Solutions

The extracts and the standard ascorbic acid (20 mg each) were separately dissolved in 20 ml of freshly distilled DMSO. These solutions were serially diluted with freshly distilled DMSO to obtain the lower dilutions.

Procedure

The generated superoxide residue stable in solution and reduces Nitroblue Tetrazolium (NBT) into formazan dye at room temperature which can be measured at 560 nm.[109] Briefly, 0.1 ml of NBT (1 mg/ml) was added to the reaction mixture containing 1 ml of alkaline DMSO (1 ml DMSO containing 5 mM NaOH in 0.1 ml water) and 0.3 ml of the plant extracts / standard ascorbic acid (5-1000µg/ml) in distilled DMSO at various concentrations, to give a final volume of 1.4 ml. The absorbance was measured at 560 nm. Percentage inhibition was calculated in earlier formula.

6.4. *IN VITRO* ANTI DIABETIC ACTIVITY

Diabetes is a disease, which still eludes effective and satisfactory cure. It may be possible that the immensely rich plant resources of our country can definitely provide effective hypoglycemic agents and it has been estimated that more than 3000 species of plants have been used throughout the world to treat diabetes. A novel antidiabetic drug should possess hypoglycemic at low concentration against β

cells and should be safe against higher concentrations. Hence, the study of *in vitro* antioxidant activity shows potent in ethanol extract selected and fractioned with different solvents from the ethyl acetate and n-butanol were tested for *in vitro* antidiabetic activity by inhibition of α-amylase and inhibition of α-glucosidase. The results were expressed in IC_{50}, which is the concentration of the fraction to inhibit enzyme by 50%.

A. In vitro Inhibition of α-amylase

Preparation of Test and Standard Solutions

The Ethyl acetate fraction of *Barleria noctiflora* (EAFBN) and n-butanol fraction of *Barleria noctiflora* (NBFBN) and the standard Acarbose (20 mg each) were separately dissolved in 20 ml of freshly prepared 5% distilled DMSO. These solutions were serially diluted with freshly prepared distilled DMSO to obtain the lower dilutions.

Procedure

The different concentrations (5–1000 μg/ml) of EAFBN and NBFBN, standard Acarbose were prepared in 5% DMSO. 500 μl of test/standard was added to 500 μl of α-amylase (0.5 mg/ ml) and was incubated for 10 min at room temperature. Then added 500 μl of 1% starch solution and incubated for another 10 min. After that 1 ml of the 3, 5-dinitrosalicylicacid as a colouring reagent was added to the reaction mixture and heated in a boiling water bath for 5 min. After cooling, it was diluted with 10 ml of distilled water. The absorbance was then measured at 540 nm against the reagent blank. The α-amylase inhibition was expressed as percentage of inhibition and the IC_{50} values determined by linear regression plots with varying

concentration of fraction against percentage inhibition.[110] The percentage inhibition was calculated employing the following formula.

% inhibition = [(Abs of control – Abs of test)/ Abs of control] X 100

B. In vitro Inhibition of α-glucosidase

Preparation of Test and Standard solutions

The Ethyl acetate fraction of *Barleria noctiflora* (EAFBN) and n-butanol fraction of *Barleria noctiflora* (NBFBN) and the standard Acarbose (20 mg each) were separately dissolved in 20 ml of freshly prepared 5% distilled DMSO. These solutions were serially diluted with freshly distilled DMSO to obtain the lower dilutions

Procedure

The test concentration (5–1000 μg/ml) of EAFBN and NBFBN /standard Acarbose was prepared from the stock solution (1 mg/ml) in 5 % DMSO. 500 μl of the test / standard was added to 500 μl of α-glucosidase (1 U/ml) and was incubated for 5 min at room temperature. Then added 500 μl of maltose (37 mM) solutions and incubated for 30 min. After that 1 ml of the glucose kit reagent was added to the reaction mixture and kept aside for 15 min. 1 ml of Tris buffer was then added to the mixture. The absorbance was then measured at 505 nm against the reagent blank. The α-glucosidase inhibition was expressed as percentage of inhibition and the assays were carried out in triplicate and the IC_{50} value determined by linear regression plots with varying concentration of plant fraction against percentage of inhibition was calculated in earlier formula.[111]

6.5. PHARMACOLOGICAL SCREENING OF *BARLERIA NOCTIFLORA*

In the *in vitro* studies, the Ethyl acetate fraction of *Barleria noctiflora* (EAFBN) exhibited potent *in vitro* anti diabetic activity. Hence, we selected Ethyl acetate fraction of *Barleria noctiflora* (EAFBN) for *in vivo* evaluation of acute toxicity, sub acute toxicity, anti diabetic activity and wound healing activities.

6.5.1. Acute Toxicity studies

In the evaluation of the assessment and toxic characteristics of substances, resolution of acute oral toxicity is typically an initial step. It provides the information on health hazards, a short-term exposure by the oral route. Acute oral toxicity is the adverse effect occurring within a short time of oral administration, dose of a substance or various doses given within 24 hrs. Acute toxicity study data may serve as a basic source for classification and labeling. LD_{50} (median lethal dose), oral, is a statistically derived dose of substance can be anticipated to cause death in fifty percent (50%) of animals when administered by oral route. LD_{50} value expressed the terms of substance per unit weight of the animal (mg/kg). It is an initial step in establishing a dosage regimen in sub chronic and further studies may provide primary information on the mode of toxic action of a substance.

Animals

The experimental animals were processed in accordance with the instruction given by our institutional ethics committee for the purpose of control and supervision of experiments on animals (CPCSEA). Healthy female albino mice (20-25 gm) were used for the study. Animals were kept in standard polypropylene cage and maintained under standard laboratory conditions of temperature (24 ± 1ºC), 12 hrs dark light cycle, standard diet and water *ad libitum*. The study protocol was

approved by the institutional ethics committee (JKKMMRFCP/ IAEC/2013/013) and all the procedure was performed in accordance with the recommendations for the proper care and use of laboratory animals.

Procedure

Acute oral toxicity testing was carried out in accordance with the OECD guideline 423 Acute Toxic Class Method.[112] The test was conducted in 4 groups each containing 3 animals for ethyl acetate fraction of *Barleria noctiflora* in each step. Healthy adult female albino mice weighing 20–25 gm was procured and kept in cages under ambient temperature (22 ± 3°C) with 12 hr light/dark cycle. The animals were randomly selected, marked and kept in their cages for 5 days prior to dosing for acclimatization to laboratory conditions. The animals were fasted over-night, but water provided ad libitum and received a single dose of (5, 50, 300, 2000 mg/kg, b.w., p.o.) each group of ethyl acetate fraction of *Barleria noctiflora*. After the administration, food was withheld for a further 3–4 hrs. Animals were observed after the dosing individually once during the first 30 min, periodically during the first 24 hrs (with special attention during the first 4 hrs) and the day after that for a period of 14 days. Once daily side of the cage observations included changes in the mucous membrane (nasal), eyes, skin and respiratory rate, circulatory (blood pressure and heart rate), central nervous system (gait, tremors, drowsiness, and convulsions) and autonomic (urinary incontinence, defecation salivation, lacrimation, perspiration and piloerection) changes.

6.5.2. Sub acute toxicity

In the evaluation of the toxic characteristics of a substance, determination of oral toxicity using repeated doses carried out by subacute toxicity testing. The tested

substance is administered orally and daily in graduate doses to several groups. The experimental animals were dosed with daily for a period of 28 days. Once a week detailed clinical observation should be made in all animals. Clinical biochemistry determination to investigate major toxic effects in tissues, specifically the effects on kidney and liver, performed on blood samples. Full histology carried out on preserving organs and tissues. It provides information on the effect of repeated oral exposure and can indicate the need for further long term studies. It is an initial step in establishing a dosage regimen in sub chronic and chronic studies may provide the initial information on mode of toxic action of the substance.

Animals

The experimental animals were processed in accordance with the instruction given by our institutional ethics committee for the purpose of control and supervision of experiments on animals (CPCSEA). Healthy Wister rats (150-200gm) were used for the study. Animals were kept in standard polypropylene cage and maintained under standard laboratory conditions of temperature (24 ± 1ºC), 12 hrs dark light cycle, standard diet and water *ad libitum*. The study protocol was approved by the institutional ethics committee (JKKMMRFCP/ IAEC/2013/013) and all the procedure was performed in accordance with the recommendations for the proper care and use of laboratory animals.

Preparation of test drug

The ethyl acetate fraction of *Barleria noctiflora* (4 gm) was suspended in 50 ml of saline of 1% of Tween 80. This suspension was administered orally to the animals with the help of an intragastric catheter, at the doses of 100, 250 and 500 mg/kg body weight, respectively.

Procedure

Subacute toxicity study was conducted according to OECD test guideline 407.[113] Wister albino rats weighing 160 ± 10 gm were used. They were allowed to acclimatize to the laboratory conditions for 7 days and were maintained on standard animal feeds and provided with water *ad libitum*. The animals were weighed and divided into four groups of six animals each. After fasting the rats overnight, the first group served as a control group received a dose of 1 ml of 1% Tween 80 solution orally, while the rest of the group were given ethyl acetate fraction of *Barleria noctiflora* at doses of 100, 250 and 500 mg/kg, b.w., p.o. the dispersed with 1% Tween 80 solutions once a day for 28 days. The body weight mortality and clinical observation were recorded daily. At the end of the experiment, they were anaesthetized with ether and blood collected via retro orbital puncture in two tubes: one with EDTA for immediate analysis of haematological parameters and another in centrifuge tube was centrifuged within 5 min of collection at 4000rpm for 15 min to separate the serum, which was analyzed for glucose, total cholesterol, total triglyceride, HDL-cholesterol, LDL-cholesterol, Asparate Aminotransferase (AST), Alanine Aminotransferase (ALT) using an auto analyzer (Maxlyzer Ultra). Blood was collected in EDTA tubes for measurement of Red Blood Cell (RBC), White Blood Cell (WBC), Haemoglobin concentration (Hb), Packed cell volume (PCV %) using Haematology analyzer (Cosmic technology Ltd). Finally rats were sacrificed; the heart, liver and kidney were taken out and weighed. Liver and kidney were fixed in neutral buffer formalin for 72 hrs and stained with hemotoxylin and eosin stain for histopathology examination. The slides were examined by qualified pathologist.

6.5.3. *In vivo* Antidiabetic studies

The Pancreas is an exocrine and endocrine tissue. The pancreas exocrine part consists of grape like a bunch of secretory cells that form sacs known as acne, which connect to ducts that ultimately empty into duodenum. The endocrine part consists of island of endocrine tissue (islet of Langerhans), which is dispersed throughout the pancreas. The important hormones secreted by islet cells are insulin and glucagon. Herbs are known to play a vital role in the management of diabetes. Ayurveda, the ancient system of Indian medicine, identified liver diseases and recommended a number of herbal remedies.

Streptozotocin (STZ) is synthesized from Streptomycetes achromogenes and is used to induce both type 1 and type 2 diabetes mellitus (IDDM and NIDDM). Streptozotocin actions on β-cells are characteristic alterations in blood insulin and concentration of glucose. After the injection of STZ in 2 hrs the hyperglycemia is observed with an associated drop in blood insulin. About 6 hrs later, hypoglycemia occurs to increase the levels of insulin. Finally, it develops hyperglycemia and decreases the blood insulin levels. The changes in blood glucose and concentration of insulin reflect abnormalities in β-cell function. STZ damage glucose oxidation and decreases insulin biosynthesis and secretion. It observed in STZ at first abolished the response of β-cell to glucose, which is followed by its permanent loss and cells damaged. STZ is taken up with pancreatic β- cells via glucose transporter GLUT 2. A GLUT 2 reduced expression was found to prevent the diabetogenic action of STZ. The STZ action on intracellular results is DNA changes in pancreatic β-cells fragmentation. The reason for the β- cell death of STZ-induced is due to DNA alkylation. The frequently used single administration intravenous dose in adult rats to induce type 1 is between 40 and 60 mg/kg body weight, but higher doses are

also used. The intraperitoneal administration of STZ in single dose below 40 mg/kg body weight may be ineffective. Type 2 can easily be induced in rats by intravenous or intraperitoneal treatment with 100 mg/kg b.w. but the high fat diet induced rats are requiring the low dose of STZ effective in type 2 diabetes.

Several plants have been reported for their hypoglycemic property by reducing the sugar and maintain the level. The Glibenclamide is approved for use as an oral hypoglycemic drug in diabetes. The ethyl acetate fraction of *Barleria noctiflora* tested for hypoglycemic activity using the development of HFD and low dose of Streptozotocin (STZ) treated type 2 diabetic rats. Hence, the present study was undertaken to evaluate its *in vivo* antidiabetic activity.

Animals

The experimental animals were processed in accordance with the instruction given by our institutional ethics committee for the purpose of Control and Supervision of Experiments on Animals (CPCSEA). Wister rats (150-200gm) were used for the study. Animals were kept in standard polypropylene cages and maintained under standard laboratory conditions of temperature (24±1ºC), 12 hrs dark light cycle, standard diet and water *ad libitum*. The study protocol was approved by the institutional ethics committee (JKKMMRFCP/ IAEC/2013/013) and all the procedure was performed in accordance with the recommendations for the proper care and use of laboratory animals.

Preparation of test drug

The ethyl acetate fraction of *Barleria noctiflora* (4 g) was suspended in 50 ml of saline of 1% of Tween 80. This suspension was administered orally to the

animals with the help of an intragastric catheter, at the doses of 100, 200 and 400 mg/kg body weight, respectively.

Preparation of standard

Glibenclamide (0.1 gm) was suspended in 50 ml of saline of 1% of Tween 80. This suspension was administered orally to the animals with the help of an intragastric catheter, at the dose of 10 mg/kg body weight.

A. Oral Glucose Tolerance Test (OGTT)

Rats were divided into five groups of six animals each. The 1st group received normal saline of 1% of Tween 80, 2nd group was administered Glibenclamide (10 mg/kg, b.w., p.o.) and remaining groups received EAFBN at 100 mg/kg, 200 mg/kg and 400 mg/kg, b.w.,p.o. All groups received a glucose solution (2 g/kg) 30 min after the administration of the test drug. The blood sample was withdrawn from tip of tail and blood glucose levels were estimated at 30,60,90 and 120 min using one touch glucometer (SD check, India).

B. Antidiabetic activity

i. Preparation of streptozotocin (STZ)

Streptozotocin (350 mg) was diluted in 50 ml of 0.1 Mol/L of cold citrate buffer (pH 4.5). This solution was administered intraperitoneally to the animals, at the dose of 40 mg/kg body weight.

ii. Preparation of high fat diet

High fat diet (HFD) was prepared[114] and consisted of 73% of a normal diet, 25% of coconut oil and 2% of dietary cholesterol, all of commercial grade.

iii. Development of HFD and low dose of STZ treated type 2 diabetic rats

The animals were fed with high fat diet twice a day for two weeks except normal control followed by type 2 diabetes mellitus was induced in overnight fasted rats administering a single dose of freshly prepared solution of streptozotocin (40 mg/kg. b.w. i.p) in 0.1 Mol/L of cold citrate buffer (pH 4.5). The STZ treated animals were allowed to drink 5% glucose solution over night to drug induced hypoglycemia. After 7 days of injection of STZ rats with moderate diabetes having persistent glycosuria and hyperglycemia (blood glucose >250 mg/dl) were used for further experimentation.[115]

iv. Experimental design

The rats were divided into seven groups of six rats (n=6) each. Group 1 and Group 2 served as normal control rats and diabetic control rats were given 1 ml of 1% Tween 80. Group 3 served as high fat diet (HFD) normal control were given 1 ml of 1% Tween 80. Group 4 served as standard and was treated with 1% Tween 80 containing glibenclamide (10 mg/kg, b.w., p.o.). Group 5, Group 6 and Group 7 were treated with doses of (100 mg/kg, 200 mg/kg and 400 mg/kg, b.w., p.o.) EAFBN in 1% of Tween 80. Except the normal control and HFD control remaining all the animals was induced with HFD and STZ. All the suspension were prepared with 0.9% of normal saline. The treatments were continued daily for 28 days. Blood samples were collected from the tip of rat tail and blood glucose levels were estimated at 0, 7^{th}, 14^{th},21^{st} and 28^{th} days of treatment using one touch glucometer (SD check, India). Body weight was measured initially and during the treatment period. On the 29^{th} day blood was collected by retro-orbital puncture from the inner canthus of the eye under mild ether anesthesia using capillary tubes in fresh vials and the serum separated. Serum parameters like triglycerides, total cholesterol, low-

density lipoproteins (LDL), high density lipoproteins (HDL), alkaline phosphatase (ALP), aspartate transaminase (AST), alanine transaminase (ALT), urea, creatinine, glycated haemoglobin and insulin were determined using standard kits obtained from Agappe diagnostics, Cochin, India and using a semi auto analyzer (Maxlyzer ultra). Immediately after the blood collection, the animals were sacrificed; hemidiaphragm and liver tissues were dissected out for estimation of glycogen, pancreas for histopathological studies.

v. Estimation of glycogen and glucose uptake by Hemidiaphragm and liver glycogenolysis

The liver tissue was separated into two portions, one for the glycogen estimation and another for liver glycogenolysis. The liver tissue was homogenized in 5 % w/v trichloroacetic acid and its glycogen content were determined.[116] The hemidiaphragms and liver tissues were carefully excised and placed immediately in ice cooled perfusion solution with the following composition: NaCl (0.687%), KCl (0.04%), $MgSO_4$ (0.014%), $CaCl_2$ (0.028%), $NaHPO_4$ (0.014%) and $NaHCO_3$ (0.21%). Glucose was added to another batch of the perfusate at a concentration of 400 mg%. This perfusate was used to study the glucose uptake/ transfer processes. The hemidiaphragms were incubated at 37°C for 1.5 hr with appropriate aeration to enable stirring and also to provide oxygen to the tissue. At the end of the incubation period glucose concentration in the perfusate was assayed. The hemidiaphragm were removed, rinsed in water and dried in an oven at 55–60°C for 4–5 hrs or till a constant weight was obtained. The glucose uptake during the incubation period was calculated in terms of mg per100 mg dry weight of the hemidiaphragm. Similarly, liver slices were incubated in the glucose enriched perfusate. The glucose

concentration in the perfusate over the incubation period was determined in terms of mg per gm of the dry weight of liver.[117]

Evaluation of parameters

i. Estimation of changes in body weight of the animals

The body weight of all rats was measured on starting day (0 day) of the experiment and 28th day of the experiment. Both initial and final body weights were noted and reported.

ii. Triglycerides (TGL)

Triglycerides catalyze the following reactions:

$$\text{Triglycerides} \xrightarrow{\text{Lipase}} \text{Glycerol + Fatty Acid}$$

$$\text{Glycerol + ATP} \xrightarrow{\text{GK}} \text{Glycerol-3-Phosphate + ADP}$$

$$\text{Glycerol-3-Phosphate + } O_2 \xrightarrow{\text{GPO}} \text{Dihydroxy acetone phosphate + } H_2O_2$$

$$2H_2O_2 + \text{Aminoantipyrine + 4-Chlorophenol} \xrightarrow{\text{POD}} \text{Chinonimine + } 4H_2O_2$$

Where, GK represents Glycerokinase, GPO represents Glycerol-3-Phosphate Oxidase and POD represents Peroxidase. The absorbance of the sample and standard were measured against the blank at 546 nm. The triglyceride levels are expressed as mg/dl.

Procedure

To 1 ml of the reaction solution from an Agappe Diagnostics kit for triglycerides, 100 µl of the sample or standard was added, mixed well and incubated at 37ºC for 10 min. The absorbance of sample and standard were measured against reagent blank within 60 min at 546 nm. The concentration of triglyceride was calculated using the formula,

Triglycerides [mg/dL] = ($A_{sample}/A_{standard}$) x Concentration of standard

iii. Total Cholesterol (TC)

Cholesterol and its esters are released from lipoproteins by detergents. Cholesterol esterase hydrolyzes the esters. In the subsequent enzymatic oxidation by cholesterol oxidase, H_2O_2 is liberated. This is converted into a colored quinoneimine in a reaction with 4-aminoantipyrine and phenol catalyzed by peroxidase.

$$\text{Cholesterol ester} + H_2O \xrightarrow{CHE} \text{Cholesterol} + \text{Fatty Acid}$$

$$\text{Cholesterol} + O_2 \xrightarrow{CHO} \text{Cholesterol-3-one} + H_2O_2$$

$$2\,H_2O_2 + \text{Amino antipyrine} + \text{Phenol} \xrightarrow{POD} \text{Quinoneimine} + 4\,H_2O$$

Where, CHE is Cholesterol Esterase, CHO is Cholesterol Oxidase and POD is Peroxidase. The absorbance of the sample and the standard was measured against the reagent blank at 546 nm. Cholesterol level is expressed as mg/dl.

Procedure

To 1 ml of the reaction solution from an Agappe Diagnostics kit for cholesterol, 100 µl of the sample or standard was added, mixed well and incubated at 37 ºC for 5 min. The absorbance of sample and standard were measured at 546 nm within 60 min. The concentration of cholesterol was calculated by using the formula,

Cholesterol [mg/dL] = (A sample/A standard) x Concentration of standard

iv. Cholesterol content in lipoprotein fractions

Cholesterol in the lipoprotein fractions was also determined by following method as described earlier. HDL cholesterol was analyzed in the supernatant obtained after precipitation of serum with phosphotungstic acid/Mg^{2+}. LDL cholesterol was calculated as follows:

$$\text{LDL - C} = \text{Total cholesterol - HDL – (Triglycerides/5)}$$

The levels of HDL and LDL-cholesterol are expressed as mg/dl.

v. Alanine Transaminase (ALT)

ALT catalyzes the following reactions:

$$\text{2-Oxoglutarate + L-Alanine} \xrightleftharpoons{\text{ALT}} \text{Glutamate + pyruvate}$$

$$\text{Pyruvate + NADH + H}^{+} \xrightleftharpoons{\text{LDH}} \text{Lactate + NAD}^{+}$$

Where, LDH is Lactate Dehydrogenase. The rate of NADH consumption was measured spectrophotometrically at 340 nm and is directly proportional to the ALT activity in the sample. ALT level was expressed as U/L.

Procedure

Reagents 1 and 2 of Agappe diagnostics kit for ALT were mixed at the ratio of 4:1 and the temperature was maintained at 30ºC. To 50 µl of the sample, 0.5 ml of the reagent solution was added and mixed. After 1 min, the decrease in absorbance was measured every min for 3 min at 340 nm.

$$\text{Enzyme activity [U/I]} = (\Delta \text{ A/ min}) \times 2143$$

Where, Δ A is the decrease in absorbance per min.

vi. Aspartate Transaminase (AST)

AST catalyzes the following reactions:

$$\text{2-Oxoglutarate + L-Alanine} \xrightleftharpoons{\text{AST}} \text{glutamate + oxaloacetate}$$

$$\text{oxaloactetate + NADH + H}^{+} \xrightleftharpoons{\text{MDH}} \text{Malate + NAD}^{+}$$

Where, MDH is Malate Dehydrogenase. The rate of NADH consumption was measured spectrophotometrically at 340 nm and is directly proportional to the AST activity in the sample. AST level was expressed as U/L.

Procedure

Reagents 1 and 2 of Agappe diagnostics kit for AST were mixed at the ratio of 4:1 and the temperature was maintained at 30 ºC. To 50 µl of the sample, 0.5 ml of the reagent solution was added and mixed. After 1 min, the decrease in absorbance was measured every min for 3 min at 340 nm.

$$\text{Enzyme activity [U/I]} = (\Delta A/\text{ min}) \times 2143$$

Where, Δ A is the decrease in absorbance per min.

vii. Alkaline Phosphatase (ALP)

ALP catalyzes by the following reaction:

$$\text{4-Nitrophenyl phosphate} + H_2O \xrightleftharpoons{\text{ALP}} \text{Phosphate} + \text{4-Nitrophenolate}$$

The rate of increase in 4-nitrophenolate was determined spectrophotometrically at 405 nm and is directly proportional to the ALP activity in the sample. Alkaline phosphatase level in serum, liver and kidney tissue homogenates was expressed as U/L.

Procedure

Reagents 1 and 2 of Agappe diagnostics kit for ALP were mixed at the ratio of 4:1 and the temperature was always maintained at 30 ºC. To 20 µl of the sample, 1 ml of the reagent solution was added and mixed. After 1 min, the increase in absorbance was measured every min for 3 min at 405 nm. The activity was calculated by using the formula,

Enzyme activity [U/I] = (Δ A/ min) x 2754

Where, Δ A is the increase in absorbance per min.

2754 is Assay factor

viii. Estimation of Glycated haemoglobin (HbA_{1c})

Haemoglobin is that portion of blood which carries the oxygen. One gram of haemoglobin is capable of combining with 1.36 ml of oxygen under optimal physiological condition. The normal value of haemoglobin as reported in human blood is 14.5g %. Glycated haemoglobin was estimated by the Bannon's method using an Agappe Diagnostics kit.

Reagents

1. Lysing reagent
2. Ion exchange resin tube
3. Distilled water

Procedure

Step A: Haemolysate Preparation

250 µl of lysing reagent was taken in a test tube, to this 50 µl of well mixed whole blood was added and mixed well. The mixture was incubated for five minutes at room temperature to allow complete lysis of R.B.C.

Step B: Glyco Haemoglobin (GHb) Separation

0.1 ml of Haemolysate from step A was added in to ion exchange resin tubes. The resin tube was agitated by a vortex mixer for 5 min. The resin was allowed to settle. Then the supernatant was separated. The absorbance's of the supernatant was measured at 420 nm in a colorimeter.

Step C: Total Haemoglobin (THb) Estimation

Distilled water 5 ml was taken in a glass tube. To this 0.02 ml of haemoglysate from Step A was added, mixed well and the absorbance was measured at 420 nm in a colorimeter.

The GHb in % was calculated by the following formula

$$\text{GHb \%} = \frac{\text{Abs. of GHb}}{\text{Abs. of THb}} \times 4.61$$

Where, 4.61= Assay factor

From this GHb %, HbA1c and Mean blood glucose (MBG) was calculated by the standard chart provided with kit. The values were expressed as % of haemoglobin.

ix. Estimation of serum insulin level

Insulin was assayed in serum using an Agappe Diagnostics by Enzyme Linked Immunosorbant Assay (ELISA) technique.

Reagents

1. Monoclonal anti-insulin antibody
2. **Enzyme conjugate :** Anti-insulin antibodies conjugated to horseradish peroxidase
3. **Standard :** Human insulin
4. **Solution A :** Buffer solution containing hydrogen peroxide
5. **Solution B :** Tetramethylbenzidine
6. Concentrated wash buffer
7. **Stop solution :** 2 N HCL

Procedure

25 µl of the serum was dispensed in micro wells coated with anti-insulin antibody. To this, 100 µl of the enzyme conjugate was dispensed into each well, mixed for 5 sec and incubated at 25°C for 30 min. The wells were rinsed five times with washing buffer. Then, 100 µl of solution A and then 100 µl of solution B were dispensed into each well. This was incubated for 15 min at room temperature. The reaction was stopped by adding 50 µl of 2 N HCl to each well and read at 450 nm.

The values are expressed as µU/ml serum.

x. Assay of Creatinine

Creatinine forms a yellow-orange compound in alkaline solution with picric acid. At the low picric acid concentration used in this method, a precipitation of protein does not take place. The concentration of the dyestuff formed over a certain reaction time is a measure of the creatinine concentration. As a result of the rapid reaction between creatinine and picric acid, later secondary reaction does not cause interference. This method, thus distinguishes itself by its high specificity.

Procedure

Buffer solution and the picric acid solution froman Agappe diagnostics kitt forcreatinge were mixed in the ratio of 1:1 and incubated for about 10 min before use. To 1 ml of this reagent solution, 0.20 ml of sample/standard was added, and mixed well. The absorbance was measured exactly after 1 min and 5 min at 492 nm. The concentration of creatinine was calculated by using the following formula

$$\text{Creatinine concentration (mg/dl)} = \frac{A_2 - A_1}{ASt_1 - ASt_2}$$

A_1 and A_2 = absorbance of sample after 1 and 5 min

ASt_1 and ASt_2 = Absorbance of standard after 1 and 5 min

xi. Assay of Urea

The ammonia formed from urease action reacted with phenol in the presence of hypochlorite to form indophenols, which with alkali gave a blue coloured compound. The nitro prusside acted as a catalyst in increasing the rate of reaction. The intensity of the colour obtained was directly proportional to the amount of urea present in the sample.

Procedure

Each 20 µl of serum and working standard with 200 µl of urease buffer solution at 37°C for 15 min, 5 ml of the phenol nitroprusside solution was added to it, followed by 5 ml of the hypochlorite reagent. It was placed in a water bath at 37°C for 15 min and the absorbance was measured against reagent blank at 630 nm. For reagent blank, 20 µl of distilled water was added to 200 µl of urease buffer. Urea content in the sample was calculated as

Urea concentration (mg/dl) = Abs of sample / Abs of standard X 100

B. Histopathological studies of Pancreatic tissues

Pancreatic tissues isolated from rats were used for histopathological studies. The tissue in each group was cut into small portions measuring 1 cm, fixed with 10% formaldehyde solution, dehydrated in gradually increasing concentrations of ethanol (50–100 %), cleared in xylene and embedded in paraffin. Sections of 5 µm thickness were prepared. Haemotoxylin and eosin were used for staining and later the microscopic slides of pancreatic tissue were photographed under 100X magnifications.

6.5.4. Wound Healing Activity

Wounds are physical injuries that result in an opening or breaking of the skin. Healing is a dynamic process that may take place in two ways viz., 'regeneration', the replacement of lost tissue by similar tissue, and 'repair', by contrast is the replacement of lost tissue by a new structure known as 'granulation tissue', that mature to form the scar tissue. Wound healing by either of the two ways is to restore the lost continuity. It is, thus considered incomplete until there is firm knitting or disrupted surfaces by collagen, obliteration of dead space, surface covering and restoration of normal function.

Wound healing is a complex process that results in the contraction and closure of the wound and restoration of a functional barrier. Cutaneous wound repair is accompanied by an ordered and definable sequence of biological events starting with wound closure and progressing to the repair and remodeling of damaged tissue. Repair of injured tissues includes inflammation, proliferation, and migration of different cell types. Inflammation, which constitutes a part of the acute response, results in a coordinated influx of neutrophils at the wound site.[118] In spite of tremendous advances in the pharmaceutical drug industry, the availability of drugs capable of stimulating the process of wound repair is still limited.[119]

There is general consent that reactive oxygen species (ROS) are injurious to the wound healing process due to their harmful effects on cell and tissues. Topical applications of compounds with free radical scavenging properties in patients have been shown to improve wound healing and to protect tissues from oxidative damage.[120] Based on *in vitro* antioxidant activity and its ethnomedical information, it

was of interest to study the wound healing activity of the ethyl acetate fraction of *Barleria noctiflora*.

Streptozotocin (STZ) treated type 2 diabetic rats

The animals were fed with high fat diet twice a day for two weeks except normal control followed by type 2 diabetes mellitus was induced in overnight fasted rats administering a single dose of freshly prepared solution of streptozotocin (40 mg/kg. b.w. i.p) in 0.1 Mol/L of cold citrate buffer (pH 4.5). The STZ treated animals were allowed to drink 5% glucose solution over night to drug induced hypoglycemia. After 7 days of injection of STZ rats with moderate diabetes having persistent glycosuria and hyperglycemia (blood glucose >250 mg/dl) were used for further experimentation.

Procedure

For the assessment of the wound healing activity excision and incision wound models were used. Animals were divided into five groups of 6 animals used for each of the excision and the incision wound models. The normal controls (Group 1) were applied with simple ointment B.P. Diabetic controls (Group 2) were applied with simple ointment B.P. Diabetic positive controls. (Group 3) were applied with Nitrofurazone 0.2% w/w, (Group 4 & Group 5) were applied with ethyl acetate fraction of *Barleria noctiflora* (EAFBN) 5% w/w and 10%w/w with simple ointment B.P. Except the normal control remaining all the animals was induced by STZ.

A. Excision wound model

Rats were inflicted with excision wounds, according to the method of Morton and Melone.[121] Under light ether anesthesia, the hair was shaved from the

dorsal thoracic central region. One excision wound was inflicted by cutting away a 200 mm^2 full thickness of skin from a predetermined area. The animals were closely observed without any infection. Excision wound was induced in diabetic as well as normal rats. Each animal was maintained in a separate gauge till the end of the study. The treatment was done topically in all the cases. Animals are kept in separate cages. The day on which wound was made to consider as day '0'. Wound contraction rate was monitored by planimetric measurement of the wound area on 4^{th}, 8^{th}, 12^{th} and 16^{th} days. This was achieved by tracing the wound on a graph paper. Reduction in the wound area expressed as percentage of the original size. Epithelialization period was noted as the number of days after wounding required for the eschar to fall off leaving no raw wound behind.

% of contraction = Initial wound size - specific day wound size / Initial wound size X 100

B. Incision model

The rats were anesthetized with anesthetic ether and two longitudinal parvertebral incision of 6 cm length were made through the skin and cutaneous muscle at a distance of about 1.5 cm from the midline on each side of the depilated back. After the incision the parted skin was sutured 1cm apart using a surgical thread and curved needle.[122] The wound was left undressed. The drugs were topically applied to the wound once a day, till complete healing. The sutures were removed on the eighth post wound day. The skin breaking strength of the 10 day old wounds was measured.[123]

6.6. STATISTICAL ANALYSIS

All the values were expressed as mean ± Standard Error of Mean (SEM) and were analyzed for significance by ANOVA and groups were compared with Dunnett

test, using In Stat v.2.02 software (GraphPad Software Inc.). Differences between groups (p Value) were considered significant at $P<0.05$ level.

6.7. ISOLATION AND CHARACTERIZATION OF PHYTOCONSTITUENTS

Isolation is a part of natural product chemistry, through which, it is possible to separate components and the biologically active one can be incorporated as ingredients in the modern system of medicine. The column chromatography technique is widely used for the separation, isolation and purification of the natural products. The principle involved in this is the adsorption/partition towards the adsorbent/support packed into the column. By changing the polarity of the mobile phase, the separation can be achieved by column chromatography. Characterization of the isolated compounds can be carried out by different analytical techniques like, Ultra Violet (UV), Infrared (IR), Nuclear Magnetic Resonance (NMR) and Mass Spectroscopy (MS). The ethyl acetate fraction was subjected to column chromatography separation to isolate their phytoconstituents.

6.7.1. Separation of the Ethyl acetate fraction of *Barleria noctiflora*

The ethyl acetate fraction of *Barleria noctiflora* (5 gm) was subjected to chromatography over silica gel 60-120 mesh (250 gm) of column length 100 cm and a diameter of 3 cm. Elution was carried out with solvents and solvent mixtures of increasing polarities. The fractions were collected in 100 ml portions and concentrated to 10 ml monitored on TLC (silica gel G as adsorbent, with suitable solvent system). The fractions that showed similar spots were mixed. The chromatographic details are given in Table-3.

Table 3: Column chromatography of Ethyl acetate fraction of *Barleria noctiflora*

Fraction No.	Eluent	Residue on evaporation
1-4	Petroleum ether	No residue
5-7	Petroleum ether – chloroform (50:50)	No residue
8-10	chloroform	No residue
11-13	chloroform – ethyl acetate (90:10)	No residue
14-16	chloroform – ethyl acetate (80:20)	Pale Yellowish residue
17-19	chloroform – ethyl acetate (70:30)	Pale white residue
20-22	chloroform – ethyl acetate (60:40)	Yellowish residue
23-25	chloroform – ethyl acetate (50:50)	Yellowish residue
26-28	chloroform – ethyl acetate (40:60)	Yellowish residue
29-32	chloroform – ethyl acetate (20:80)	Yellowish brown residue
33-35	chloroform – ethyl acetate (10:90)	Yellowish white residue
36-38	ethyl acetate	Yellowish white residue
39-42	ethyl acetate – methanol(90:10)	Brown residue
43-45	ethyl acetate – methanol (80:20)	Brown residue
46-48	ethyl acetate – methanol (50:50)	Brown residue

The column chromatography fractions 17-19 yielded a pale white amorphous solid and the TLC profile showed single spot. It was designated as compound-I. All the remaining fractions were of low yields and exhibited several spots on TLC. Hence, these were not processed further, compound-I was subjected to purification and pure compounds obtained were subjected to TLC, physical, chemical and spectroscopic studies for their characterization.

Compound-I

The pale white amorphous solid was recrystalised in chloroform. Upon recrystalization, white solid were separated out. The white solid showed a single spot on TLC. The compound was designated as C1 (yield 4.9 mg, 0.0025%).

7. RESULTS AND ANALYSIS

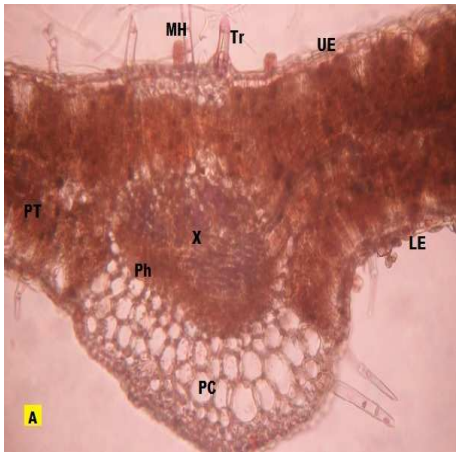
7.1. PHARMACOGNOSTICAL EVALUATION

7.1.1. Microscopical studies

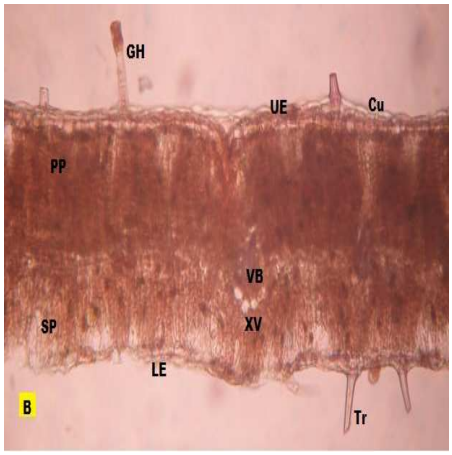
A. T.S of the leaf

The T.S. Of leaf through the midrib shows the presences of unicellular hair are present in the epidermis. The upper epidermis and lower epidermis were along the midrib is narrow comprising of small, slightly appellate thick walled cells with thick cuticle cells. The ground tissue of the midrib is homogenous and Parenchyma cells. The xylem part of the strand has several long parallel compact lines of xylem elements. The elements are narrow, elliptical and thin walled. Phloem occurs in broad and the continuous arc along the abaxial side of the xylem. The midrib was shown the trichome, and palisade tissue (Figure- 2A).

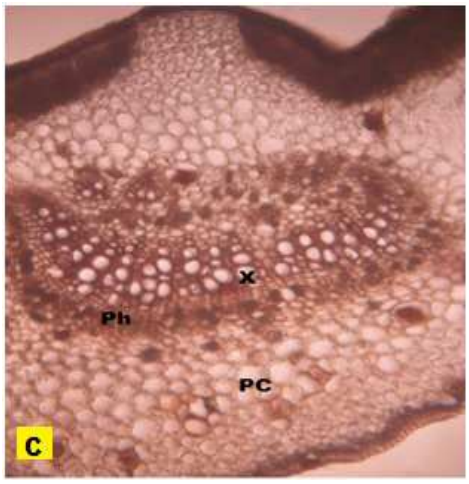
The lamina part shows the palisade spongy parenchyma tissues. The palisade cells are wide, cylindrical and compact. The epidermis is thin and has narrow rectangular thick cuticularised cells. The upper epidermis shows glandular hair, cuticle and trichome. It has a spongy parenchymatous ground tissue of vascular strand with dark bundle sheath. The center of lamina part shows xylem vessels and vascular bundle (Figure-2B). A sector enlarged section shows the presence of phloem, xylem, and parenchyma glandular cells (Figure-2C).



2A: T.S. of Midrib



2B: T.S. of Lamina



2C: T.S. of Enlarge section

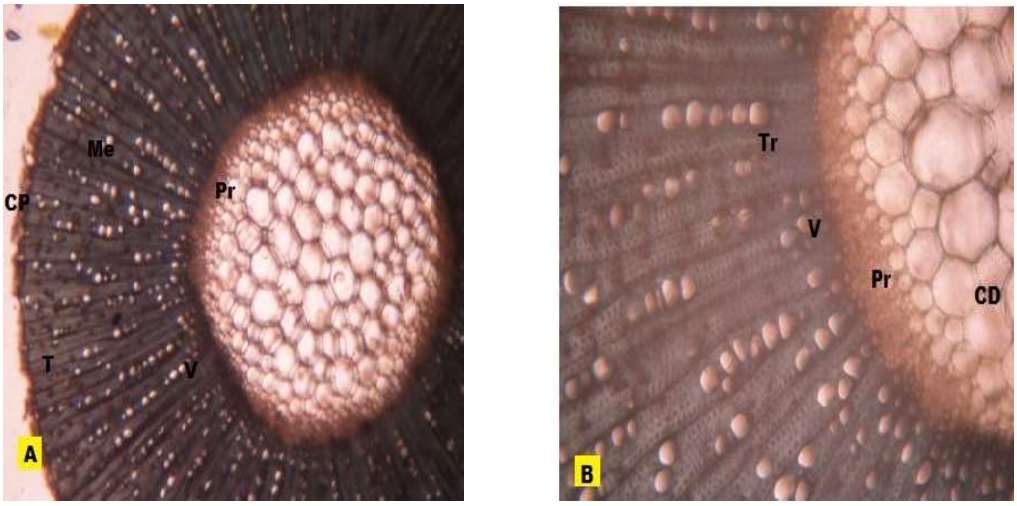
Figure 2: T.S. of *Barleria noctiflora L.* leaf

[Abbreviations: PT: Palisade tissue, Ph: Phloem, PC: Parenchyma cells, X: Xylem, MR: Midrib, LE: Lower epidermis, UE: Upper epidermis, Tr: Trichome, MH: Multicellular hair, PP: Palisade parenchyma, SP: Spongy parenchyma, VB: Vascular bundle, GH: Glandular hair, Cu: Cuticle.**]**

B. T.S. of Stem

The T.S. of stem shows the vascular cylinder consists of a thin, discontinuous layer of crushed phloem and a wider zone of meta xylem elements. Phloem has dilated rays and parallel lines of elements. Pith is wide and parenchymatous cells are circular to angular, compact and no intercellular spaces the

presence of protoxylem and vessels (Figure-3A). A sector enlarged stem section was clearly shown the presence of trachied, vessels, protoxylem, xylem and depositions cells (Figure-3B).



3A: T.S. of stem 3B: T.S. of Enlarge section

Figure 3: T.S. of *Barleria noctiflora L.* Stem

[Abbreviations: CB: Crushed phloem, T: Trachieds, Me: Metaxylem, Pr: protoxylem, V: Vessels, CD: Cell depositions.**]**

7.1.2. Powder microscopy

The powder is pale green in color with a pungent odour and slightly sweet and sour taste. The microscopic study of powder revealed the presence of a bunch of vessels, bundle of fiber, transgenital parenchyma cells. The lengths of fiber are nearly 2 mm. The unicellular covering trichome which were slightly curved and diacytic stomata. The seen in piece of epidermal tissue with trichome, parenchymatous tissue, trachied stone cells and vessels elements (Figure-4).

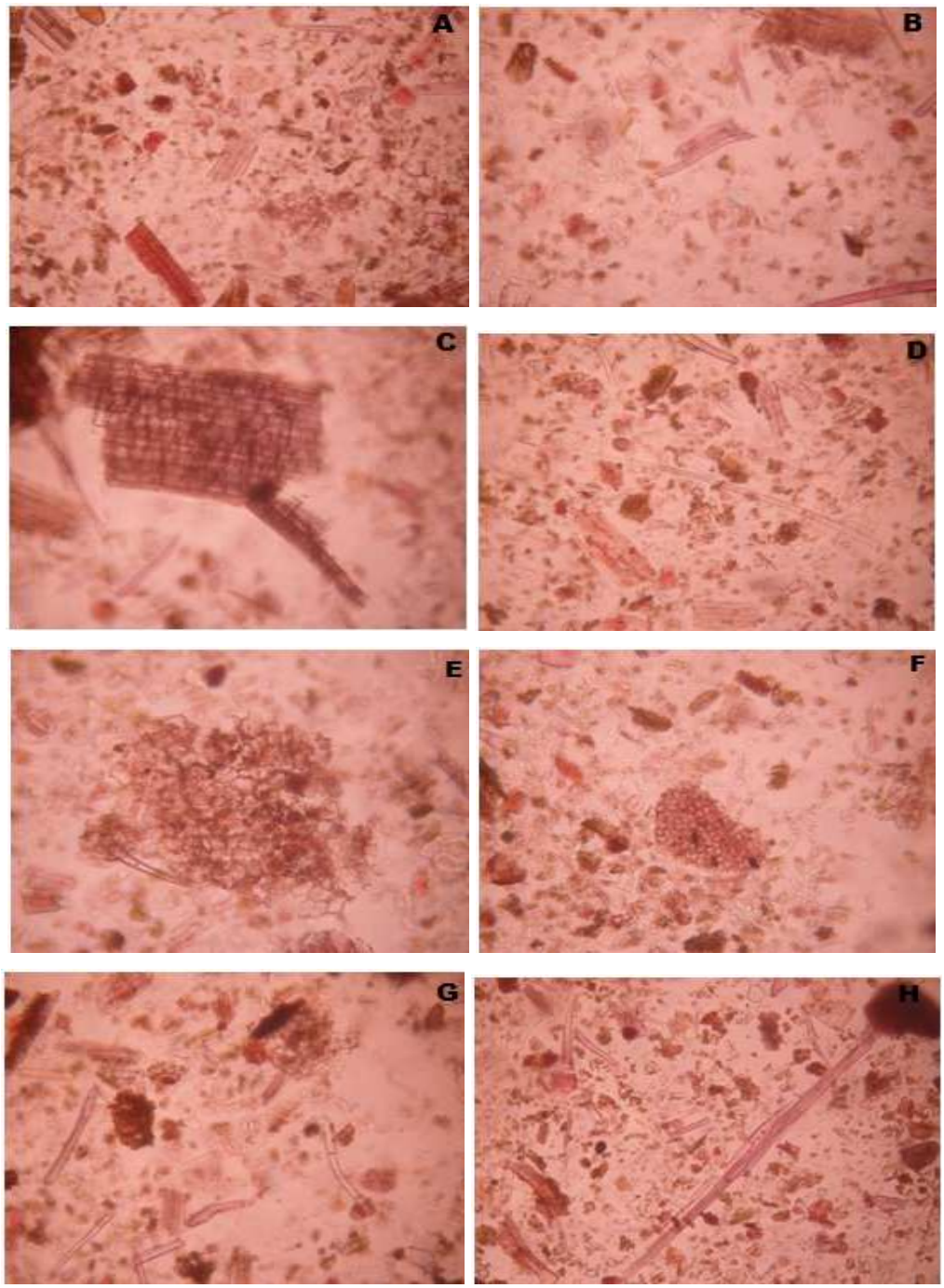


Figure 4: Powder microscopy of *Barleria noctiflora L.*

A: Bunch of vessels, B: Bundle of fiber, C: Transgenital parenchyma cells, D: length of fiber, E: Epidermal tissue with trichome, F: Parenchymatous tissue, G: Stomata, H: Trachied stone cells.

7.1.3. Quantitative Microscopy

The observed values for stomatal number, stomatal index, vein islets, vein termination number and palisade ratio (Table-4).

Table 4: Quantitative Microscopic data

S. No.	Parameters	Value in 1 sq.mm (average of 10 fields)
1	Stomatal number	21.9
2	Stomatal index	9.9
3	Vein-islet number	4.3
4	Vein termination number	4.2
5	Palisade ratio	8.1

7.1.4. Physicochemical Parameters

The physicochemical evaluation of Total ash, water soluble ash, acid insoluble ash, water soluble extractive value, alcohol soluble extractive value and loss on drying were calculated and recorded. The total ash value, water soluble ash and acid insoluble ash were 13.5, 9 and 2% w/w respectively (Table-5). Extractive values of alcohol soluble and water soluble extractive value were 7.36 and 8.24% w/w. The moisture content of the drug is not too high as the general requirement for moisture content in crude is not more than 14% w/w. The moisture content (loss on drying) was 2.67% w/w (Table-6).

A. Ash values

Table 5: Data for ash values for powdered of *Barleria noctiflora*

Ashes	Ash values (%w/w)
Total ash	13.5
Water soluble ash	9
Acid insoluble ash	2

B. Extractive values and Loss on drying

Table 6: Data for extractive values and loss on drying for powdered of *Barleria noctiflora*

Analytical parameters	Percentage (%w/w)
Alcohol soluble extractive	7.36
Water soluble extractive	8.24
Loss on drying	2.67

7.1.5. Fluorescence analysis

Powdered drug under an ultra violet and ordinary light when treated with different reagents emitted various color radiation, which help in identifying the drug in powder form (Table-7).

Table 7: Florescence analysis of *Barleria noctiflora* powder

Reagents	Daylight	Short UV	Long UV (365nm)
Powdered drug	Yellowish green	Yellowish green	Pale green
Powder + 1 N NaOH	Pale yellow	Pale green	Dark green
Powder + 1 N HCl	Pale yellow	Pale green	Pale green
Powder +50% H2SO4	Dark brown	Brownish green	Brownish green
Powder + H_2O	Pale green	Pale greenish yellow	Greenish yellow
Powder + Ammonia	Pale brown	Pale brown	Pale brown
Powder +50%HNO3	Dark yellow	Pale yellow	Dark greenish yellow
Powder + picric acid	Pale yellow	Greenish yellow	Dark green
Powder + $Fecl_3$	Brownish Green	Brownish green	Dark green
Powder + Methanol	Pale green	Pale green	Light green

7.1.6. Preparation of extracts and phytochemical studies

The nature of the extracts and their yields are given in Table-8 The percentage yield of crude ethanolic extract and water extract of *Barleria noctiflora* was found to be 11.06% w/w & 11.4% w/w, respectively.

Table-8: The percentage Yields of *Barleria noctiflora* of different extraction.

Extraction	Quantity used for extraction		Nature of the extract	Yield %
	Powder (g)	Solvent (ml)		
Petroleum ether[S]	500	2500	Greenish semi solid	1.56
Chloroform[S]	500	2500	Greenish semi solid	2.354
Ethanol[S]	500	2500	Brownish green semi solid	6.7
Ethanol[C]	500	2500	Greenish Brown semi solid	11.06
Water[C]	50	250	Greenish brown solid	11.4

S – Successive, C –Crude

7.1.7. Preliminary phytochemical analysis

Preliminary phytochemical analysis revealed the presence of flavonoides, alkaloids, saponins, glycosides, tannins, phenols, protein and carbohydrates in water and ethanol extract. The steroids are present in chloroform and petroleum ether extract (Table-9). The crude drug of powder was the presence of vitamin C, vitamin D and inorganic elements like sodium, iodine, phosphate, and chloride.

Table 9: Preliminary phytochemical screening of *Barleria noctiflora*.

Chemical constituents	Water extract	Alcohol extract	Chloroform extract	Petroleum ether extract
Flavonides	+	+	-	-
Alkaloids	+	+	-	-
Phenols	+	+	-	-
Saponins	+	+	-	-
Steroids	-	-	+	+
Glycosides	+	+	-	-
Tannins	+	+	-	-
Protein	+	+	-	-
Carbohydrate	+	+	-	-

+ Denotes the presence of the respective class of compounds.

- Denotes the absence of the respective class of compounds.

7.1.8. Estimation of Total Phenol Content

The total phenolic content of *Barleria noctiflora* of crude ethanolic and aqueous extract was obtained using the regression calibration curve Y=0.0058X-0.0425, R^2=0.998 and is expressed as tannic acid equivalents. The total phenol content of the Ethanolic extract of *Barleria noctiflora* (EEBN) was found to be 282 µg/ml and aqueous extract of *Barleria noctiflora* (AEBN) was found to be 305 µg/ml respectively (Figure-5). Total phenolic content was high in the ethanolic extract compared to the aqueous extract. It shows the ethanolic extract of *Barleria noctiflora* posse's high antioxidant ability.

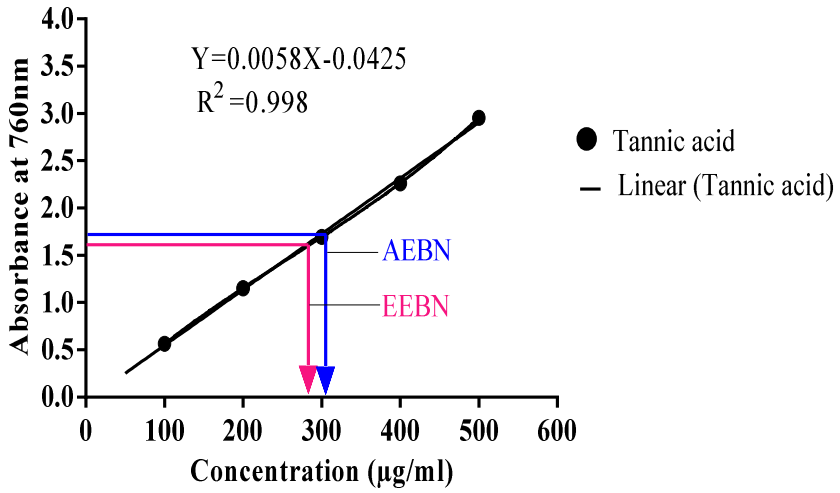


Figure 5: Total Phenolic content

7.1.9. Estimation of Total Flavonoid Content

The total flavonoides content was obtained using the regression calibration curve Y=0.0012X-0.042, R^2=0.997 with Rutin equivalent. The Total flavonoid content of the ethanolic extract was found to be 226 µg/ml and aqueous extract was found to be 311 µg/ml respectively (Figure-6). Total flavonoid content was high in the ethanolic extract compared to the aqueous extract.

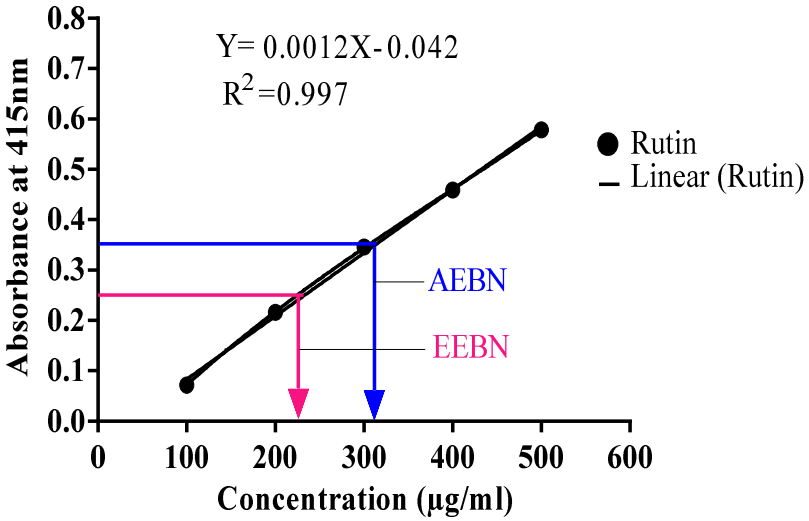


Figure 6: Total Flavonoid Content

7.2. *IN VITRO* ANTIOXIDANT STUDIES OF EXTRACTS

The *in vitro* antioxidant activity were used different extraction of Petroleum ether extract of *Barleria noctiflora* (PEBN), Chloroform extract of *Barleria noctiflora* (CEBN), Ethanolic extract of *Barleria noctiflora* (EEBN), Crude ethanolic extract of *Barleria noctiflora* (CEEBN) and Aqueous extract of *Barleria noctiflora* (AEBN).

A. Diphenyl Picryl Hydrazyl (DPPH) Radical Scavenging Activity

The antioxidant activity of DPPH method, the ethanolic extract of *Barleria noctiflora* showed good antioxidant activity. The IC_{50} values were found in PEBN 367.16±1.93, CEBN 304.2±3.75, EEBN 214.5±1.15, CEEBN 202.36±2.67, AEBN 256.73±1.82 and standard ascorbic acid is 200.46±2.79 respectively (Table-10 and Figure-7). The DPPH is a stable free radical, which has been widely accepted as a tool for estimating free radical scavenging activities of antioxidants. DPPH is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule. The reduction capability of DPPH radical is determined by the decrease in absorbance at 517 nm induced by antioxidants. The experimental data of the extracts revealed that the extracts are likely to have the effects of scavenging free radicals. Among all the extracts the crude ethanol extract showed more potent DPPH radical scavenging activity like the standard used. From the result we observe that a dose dependent relationship in the DPPH radical scavenging activity.

Table 10: *In vitro* antioxidant activity of *Barleria noctiflora*

Extract	IC_{50} values ± SE (µg/ml) by methods*					
	DPPH	**ABTS**	**H_2O_2**	**Lipid peroxidation**	***p*-NDA**	**Alkaline DMSO**
Pet Ether	367.16±1.93	271.4±0.51	268.03±0.05	336.16±1.34	330.43±3.43	349.03±0.68
Chloroform	304.2±3.75	254.03±0.33	256.9±0.90	204.63±0.08	186.06±0.66	264.73±0.23
Successive Ethanol	214.5±1.15	166.73±2.05	196.9±0.10	179.23±0.12	147.4±0.25	166.36±0.12
Crude Ethanol	202.36±2.67	128.33±0.44	174.76±1.08	152.23±0.74	146.76±0.44	151.11±0.05
Crude Aqueous	256.73±1.82	210.86±0.27	236.26±0.20	195.06±0.14	176.2±0.30	181.03±0.51
Standard	200.46±2.79	106.6±0.17	151.83±0.12	132.03±0.06	136.73±0.27	139.76±0.66

***Average of three determinations; Data are expressed as mean ± SEM**

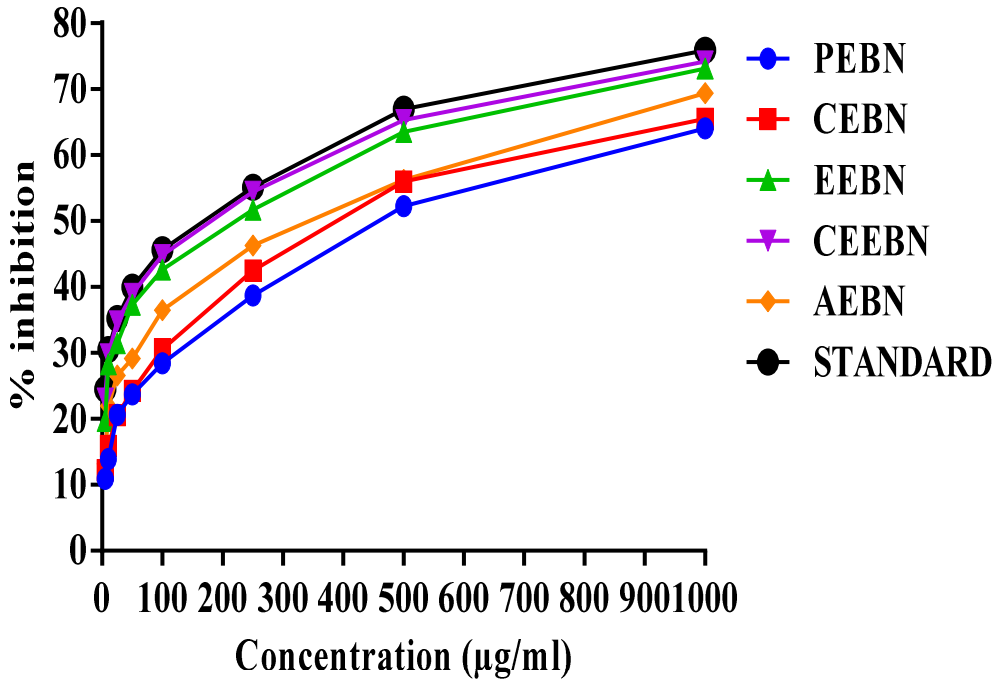


Figure 7: DPPH radical scavenging activity

B. ABTS Radical Scavenging Method

In the ABTS method, the extracts showed the potent radical scavenging activity in a concentration dependent manner in all the extracts. The IC_{50} values were found in PEBN 271.4±0.51, CEBN 254.03±0.33, EEBN 166.73±2.05, CEEBN 128.33±0.44, AEBN 210.86±0.27 and standard ascorbic acid is 106.6±0.17, respectively (Table-10 and Figure-8). ABTS radical scavenging activity is relative, which involves a more drastic, radical, chemically produced and is often used for screening complex antioxidant mixtures such as plant extracts, beverages and biological fluids. The ability in both the organic and aqueous media and the stability in a wide pH range raised the interest in the use of ABTS for the estimation of antioxidant activity. Hence, the extracts radical scavenging activity showed a direct role of its phenolic compounds in free radical scavenging.

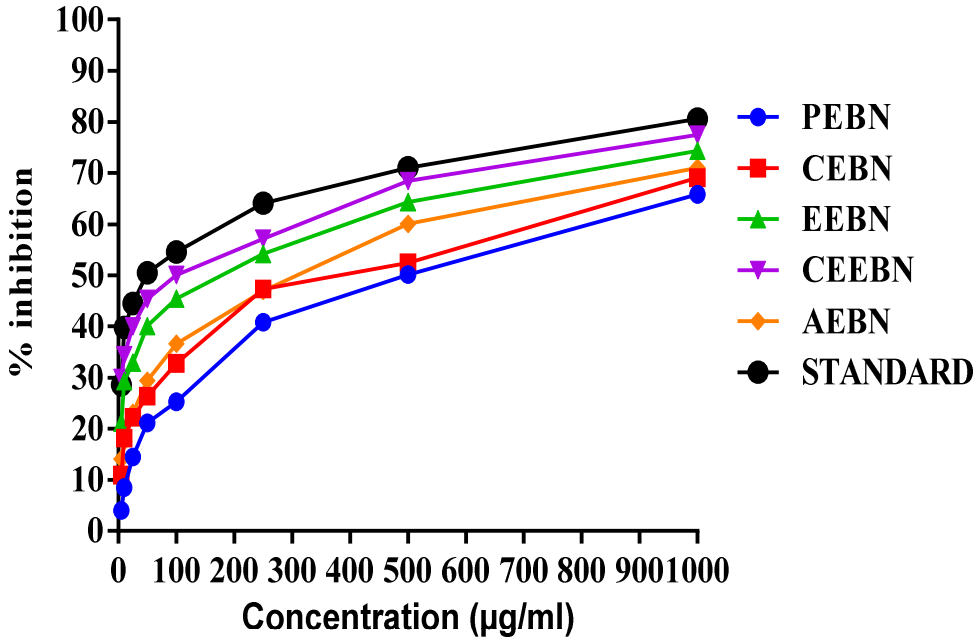


Figure 8: ABTS radical scavenging activity

C. Scavenging of Hydrogen Peroxide

Superoxide radical is known to be a very harmful species to cellular components as a precursor of more reactive species. The superoxide radical is known to be produced *in vivo* and can result in the formation of hydrogen peroxide via dismutation reaction. Moreover, the conversion of superoxide and hydrogen peroxide into more reactive species. Hydrogen peroxide itself is not very reactive, but sometimes is toxic to cell because it may give rise to hydroxyl radical in the cells. Therefore, removing of hydrogen peroxide is very important for antioxidant defense in the cell system. The ascorbic acid used as a standard with an IC_{50} value of 151.83±0.12 and the extracts IC_{50} values were found in PEBN 268.03±0.05, CEBN 256.9±0.90, EEBN 196.9±0.1, CEEBN 174.76±1.08 and AEBN 236.26±0.20, respectively (Table-10 and Figure-9).

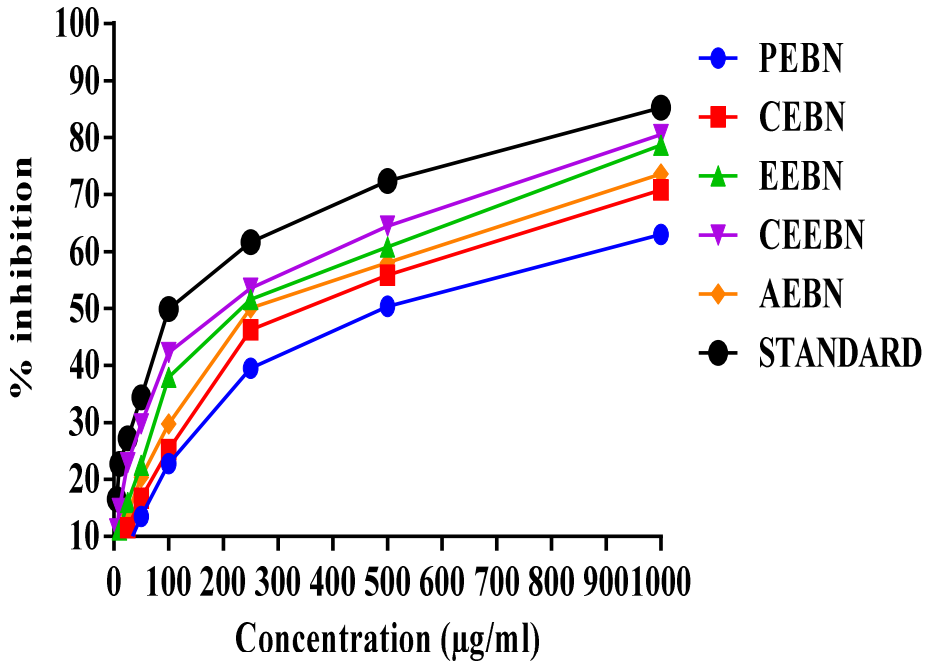


Figure 9: Scavenging of hydrogen peroxide

D. Lipid Peroxidation Inhibitory Activity

In the lipid peroxidation method, the extracts of successive and crude ethanolic extracts exhibit potent activity compare to other extracts. The IC_{50} values were found in PEBN 336.16±1.34, CEBN 204.63±0.08, EEBN 179.23±0.12, CEEBN 152.23±0.74, AEBN 195.06±0.14 and standard ascorbic acid is 132.03±0.06, respectively (Table-10 and Figure- 10).

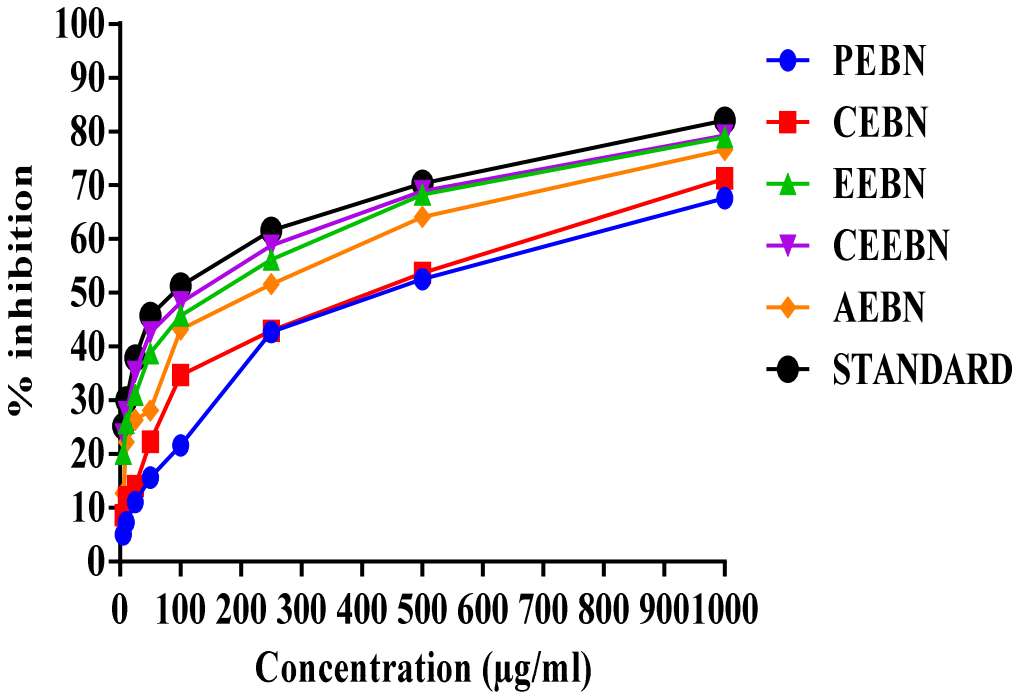


Figure 10: Lipid Peroxidation Inhibitory Activity

E. Scavenging of Hydroxyl Radical by p-NDA Method

In the p-NDA method, among the oxygen radicals, the hydroxyl radical is the most reactive and induces severe damage to adjacent biomolecules. In p-NDA method, the hydroxyl radical is generated through Fenton reaction. In this reaction, iron-EDTA complex reacts with hydrogen peroxide in the presence of ascorbic acid to generate hydroxyl radical which can bleach p-NDA specifically. The extracts show potent scavenging activity by inhibition of bleaching of p-NDA. The EEBN and CEEBN extracts showed similar IC_{50} value 147.4±0.25 and 146.76±0.44 compared to standard ascorbic acid IC_{50} value of 136.73±0.27. The extracts showed PEBN 330.43±3.43, CEBN 186.06±0.66 and AEBN 176.2±0.30, respectively (Table-10 and Figure-11). The scavenging activity may be due to the presence of various phytochemical including polyphenols and flavonoids in the extracts.

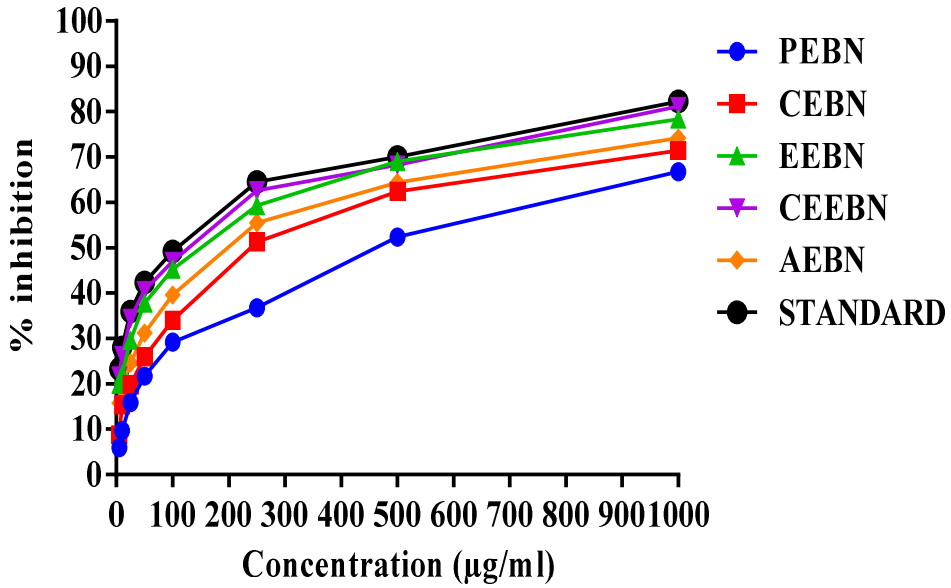


Figure 11: Hydroxyl radical scavenging activity *p*-NDA method

F. Scavenging of Superoxide Radical by Alkaline DMSO Method

The alkaline DMSO method, the extracts was moderately inhibited the superoxide radical generation. The IC_{50} values were found in PEBN 349.03±0.68, CEBN 264.73±0.23, EEBN 166.36±0.12, CEEBN 151.11±0.05, AEBN 181.03±0.51and standard ascorbic acid is 139.76±0.66, respectively (Table-10 and Figure-12). Therefore, the phenolic compounds of extracts may be involved in scavenging hydrogen peroxide. The extracts are found to be an efficient scavenger of superoxide radical generated in the alkaline DMSO system. The result clearly indicates that the plant extracts have a noticeable effect as scavenging superoxide radical.

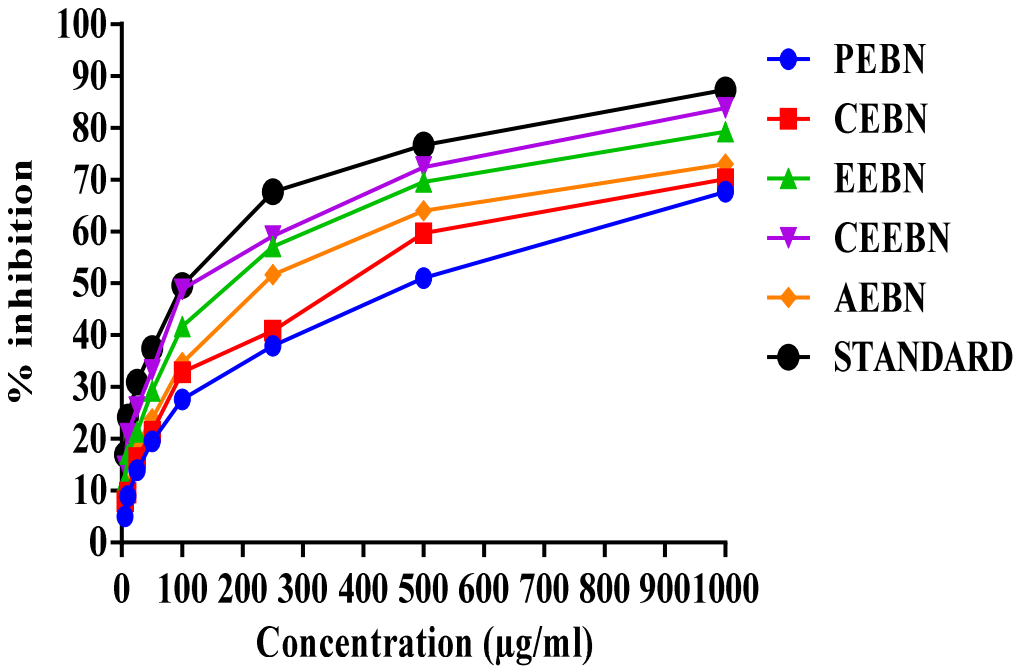


Figure 12: Superoxide radical scavenging activity by alkaline DMSO method

7.3. *IN VITRO* ANTI DIABETIC ACTIVITY

7.3.1. In vitro inhibition of α-amylase

The Ethyl acetate fraction of *Barleria noctiflora* (EAFBN) and n-butanol fraction of *Barleria noctiflora* (NBFBN), elicited a dose dependent inhibition of α-amylase enzyme activity. The α-amylase inhibitory effect of the EAFBN was found to be ranging from 14.62 % to 84.63% when studied at concentrations 5–1000 µg/ml. At the same concentration range the inhibitory effect of NBFBN was found to be ranging from 12.58% to 82.25%, whereas the effect of standard drug acarbose ranged from 19.60% to 88.89%. The IC_{50} of EAFBN was found to be 114.7± 0.15 µg/ml, whereas NBFBN showed at 147.8±0.40 µg/ml. The IC_{50} of acarbose was found to be 88.92±0.25µg/ml (Table-11 and Figure-13).

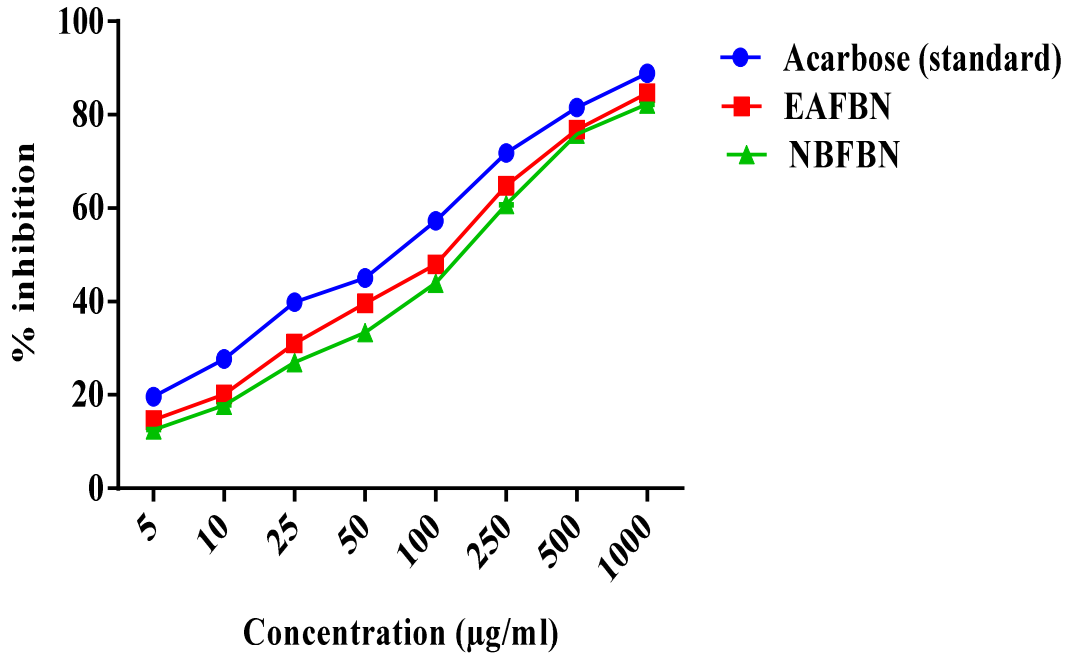


Figure 13: *In vitro* α-amylase inhibitory activity of *Barleria noctiflora* fraction

Table 11: *In vitro* α-amylase inhibitory activity of *Barleria noctiflora* fractions

Test substance	Concentration (µg/ml) % inhibition								IC_{50} (µg/ml)
	5	10	25	50	100	250	500	1000	
Acarbose (Standard)	19.60±0.02	27.71±0.02	39.88±0.03	45±0.01	57.25±0.03	71.79±0.02	81.52±0.02	88.89±0.03	88.92±0.25
EAFBN	14.62±0.01	20.14±0.02	31.04±0.02	39.65±0.02	47.95±0.05	64.75±0.03	76.79±0.02	84.63±0.01	114.7±0.15
NBFBN	12.5±0.03	17.83±0.02	26.95±0.02	33.37±0.03	43.93±0.01	60.76±0.03	75.88±0.01	82.25±0.05	147.8±0.40

Values are expressed as mean± SEM of triplicate measurement.

7.3.2. In vitro inhibition of α-glycosidase

The fractions of EAFBN and NBFBN, elicited a dose dependent inhibition of α-glycosidase enzyme activity. The α-glycosidase inhibitory effect of the EAFBN was found to be ranging from 12.47 % to 83.83% when studied at concentrations 5–1000 µg/ml. At the same concentration range the inhibitory effect of NBFBN was found to be ranging from 11.55% to 81.41%, whereas the effect of standard drug acarbose ranged from 18.44% to 88.20%. The IC_{50} of EAFBN was found to be 104.93± 0.28 µg/ml, whereas NBFBN showed at 138.96±0.17 µg/ml. The IC_{50} of acarbose was found to be 97.96±0.51 µg/ml (Table-12 and Figure-14).

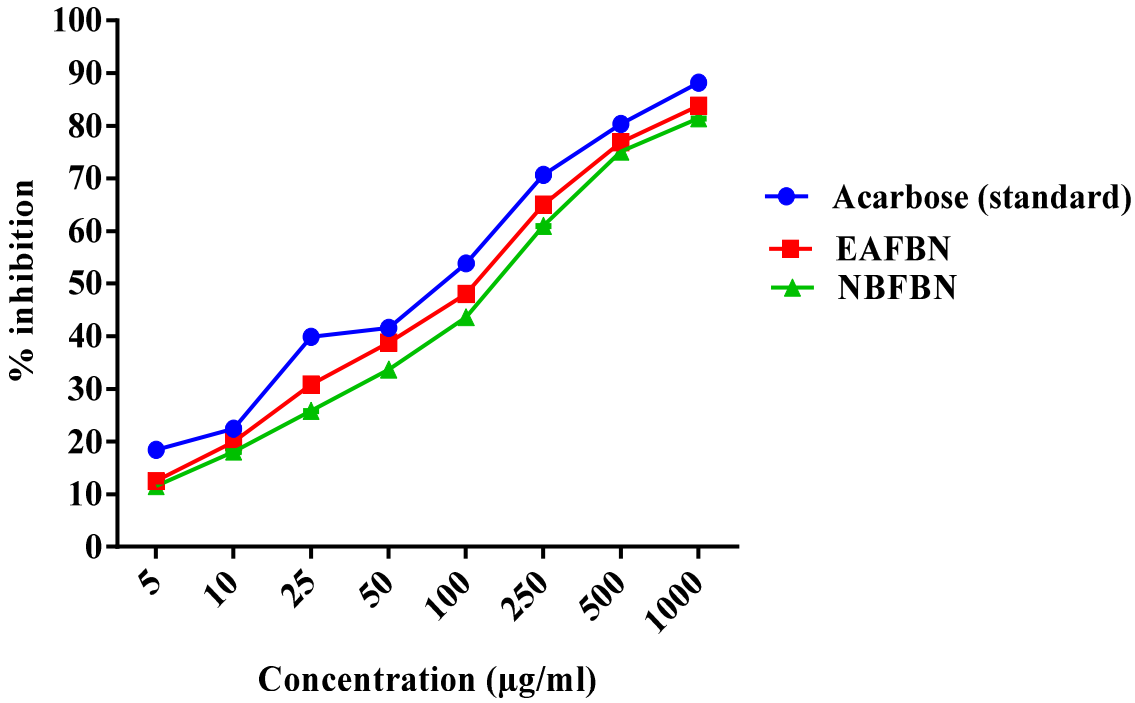


Figure 14: *In vitro* α-glycosidase inhibitory activity of *Barleria noctiflora* fractions.

Table 12: *In vitro* α-glycosidase inhibitory activity of *Barleria noctiflora* fractions

Test substance	Concentration (µg/ml) % inhibition								IC_{50} (µg/ml)
	5	10	25	50	100	250	500	1000	
Acarbose (Standard)	18.44±0.07	22.46±0.13	39.90±0.03	41.56±0.05	53.85±0.04	70.68±0.05	80.32±0.07	88.20±0.03	97.96±0.51
EAFBN	12.47±0.02	19.90±0.03	30.83±0.01	38.81±0.02	48.03±0.02	65±0.01	76.87±0.04	83.83±0.04	104.93±0.28
NBFBN	11.55±0.03	18.08±0.04	25.82±0.06	33.69±0.02	43.63±0.02	60.97±0.02	75.08±0.01	81.41±0.03	138.96±0.17

Values are expressed as mean± SEM of triplicate measurement.

7.4. PHARMACOLOGICAL SCREENING OF *BARLERIA NOCTIFLORA*

7.4.1. Acute Toxicity Studies

In the acute toxicity study, there was no lethality was observed in any groups of animals after the treatment of ethyl acetate fraction of *Barleria noctiflora* (EAFBN). During the course of study, no difference in the body weight, the behavior, sensory nervous system responses and abnormal changes were detected. The EAFBN was more than 2000 mg/kg and cannot be categorized under the GHS (Globally Harmonized System) category.

7.4.2. Sub acute toxicity

In the subacute toxicity study, there were no significant differences in the body for all the rats (Table-13 and Figure-15). Toxicity signs such as salivation, lacrimation and piloerection were not observed. The effects of the fraction on the organs are summarized (Table-14 and Figure-16). The macroscopic examinations of the organs of the animals treated with various doses of the fraction did not show any changes in color compared with the control. Also, there were significant reduction changes in the organ weight of the treated animals in high dose compared with the control.

Table 13: The effects of the EAFBN on body weight changes in sub acute toxicity studies.

Treatment groups	Average bodyweight in gm ± SEM				
	Day 1	Day 7	Day 14	Day 21	Day 28
Control	169.50±3.35	174±3.42	176.66±3.56	178.67±3.16	180.16±3.11
EAFBN (100mg/kg)	164.67±2.90	167±2.98	169.33±3.04	172.33±2.98	174.67±3.07
EAFBN (250mg/kg)	164±1.88	165.5±2.07	167±2.11	168±2.11	169.16±2.08
EAFBN (500mg/kg)	166.83±1.40	167.67±1.38	168.33±1.43	169.17±1.47	170±1.36

Values are given in average body weight (g) ±SEM for groups of six animals (n=6) each. The level of significance was taken at $p < 0.05$ are compared to control on the corresponding day (One way ANOVA followed by Dunnett test).

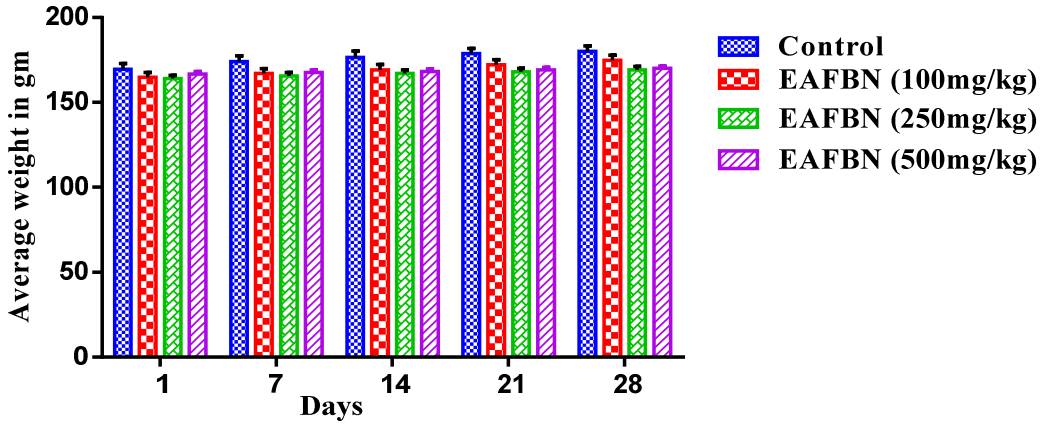


Figure 15: The effects of the EAFBN on body weight in sub acute toxicity studies.

Table 14: The effects of EAFBN organ weight changes in sub acute toxicity studies

Treatment groups	Average organ weight in gm ± SEM		
	Kidney	**Heart**	**Liver**
Control	0.94±0.017	0.47±0.007	4.93±0.023
EAFBN (100mg/kg)	0.97±0.007	0.48±0.007	5.12±0.026**
EAFBN (250mg/kg)	0.92±0.012	0.47±0.009	5.10±0.018**
EAFBN (500mg/kg)	0.87±0.010*	0.46±0.009	4.77±0.024**

Values are given in average organ weight (g) ± SEM for groups of six animals (n=6) each. **p < 0.01, * p < 0.05 denotes when compared to control (One way ANOVA followed by Dunnett test).

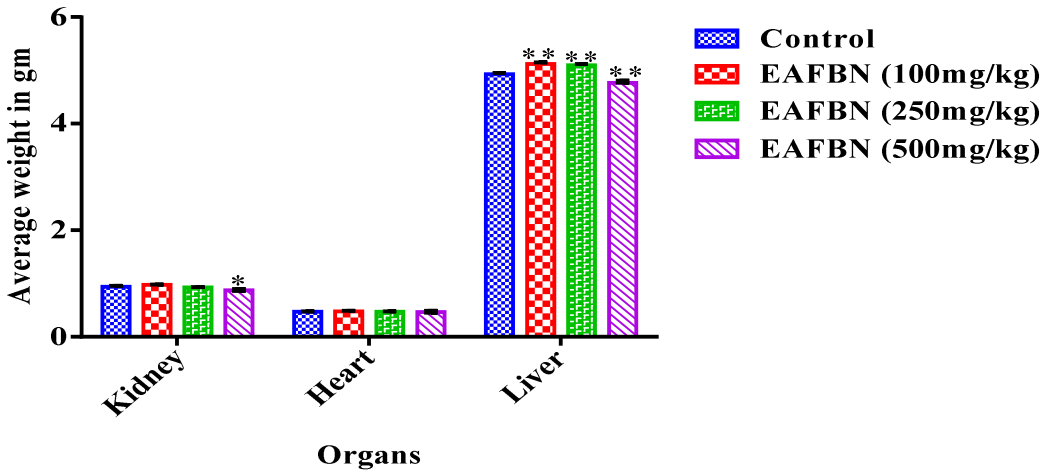


Figure 16: The effects of EAFBN organ weight in sub acute toxicity studies.

Effect of EAFBN on sub acute toxicity on biochemical parameter

The effect of biochemical parameters are summarized in Table-15 and Figure-17. The glucose level was exhibited significant (p<0.01) decrease in ethyl acetate fraction of *Barleria noctiflora* (EAFBN), when compared to control group. The treatment with the fraction at the doses of (100 mg/kg, 250 mg/kg & 500

mg/kg) was found to be 83.83±0.94, 65.66±1.68 & 58.50±1.68 mg/dl, showed a significant decrease in glucose level compared to control (102.33±1.49 mg/dl).

In the normal control, the total cholesterol level was found to be 87.5±2.20 mg/dl. EAFBN at a dose of 100mg/kg, 250mg/kg & 500mg/kg treatment showed 94±1.46, 86.83±1.07 and 73.33±1.22 mg/dl respectively. The values are not significant at the dose of 100 and 250 mg/kg. However, the dose of 500 mg/kg showed significantly ($p<0.01$) reduce cholesterol level when compared to normal control.

The triglyceride level of EAFBN significantly ($p<0.01$) increase at 100 mg/kg and 250mg/kg body weight doses (73.5±1.33 and 89.66±1.05 mg/dl, respectively) and significantly ($p<0.01$) decrease at 500 mg/kg body weight dose 78.66±2.06 mg/dl, when compared to a normal control group (62.16±2.08 mg/dl).

The lipid profile of HDL - Cholesterol level of EAFBN significant ($p<0.01$) increase the treatment at 100,250 and 500 mg/kg body weight doses showed 144.16±1.22, 159.01±2.51 and 182.16±3.32 mg/dl, when compared to control group (60.66±1.05 mg/dl). The LDL – Cholesterol level of EAFBN was found 96.33±1.40, 93±1.15and 83.66±2.06 mg/dl at the doses of 100,250,500 mg/kg showed significant ($p<0.01$), respectively. Hence, in the doses were increased simultaneously LDL-Cholesterol level was decreased.

The heart and liver release AST and ALT, and an elevation in serum concentration are an indicator of liver and heart damage. The EAFBN showed the AST level was 62.50±1.89, 59.16±1.07 and 59±0.96 IU/L at the doses of 100,250 and 500 mg/kg. However, there was no significant increase in AST level, when compared to normal control. The ALT level was a significant decrease ($p < 0.01$) at

100, 250 and 500 mg/kg bodyweight doses (41.5±1.33, 39.16±1.13 and 37.16±0.79 IU/L, respectively), when compared to control group.

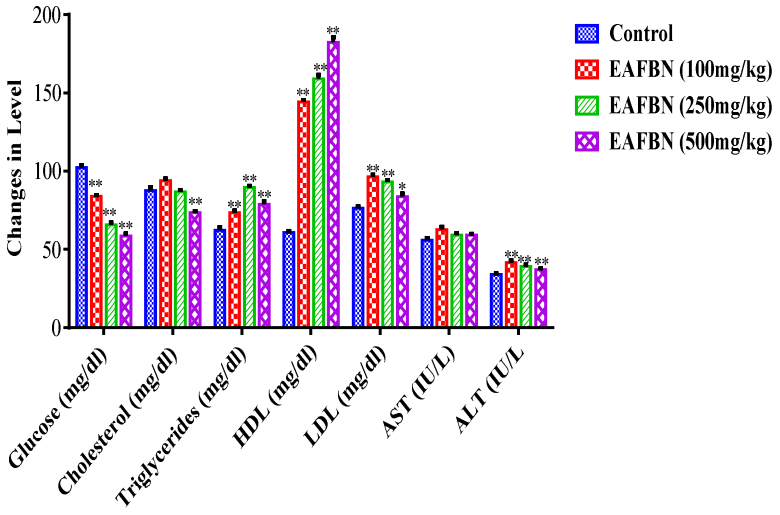


Figure 17: The effects of EAFBN biochemical profile of sub acute toxicity studies.

Table 15: The effects of EAFBN biochemical profile of sub acute toxicity studies.

Parameter	Treatment groups			
	Control	**EAFBN (100mg/kg)**	**EAFBN (250mg/kg)**	**EAFBN (500mg/kg)**
Glucose (mg/dl)	102.33±1.49	83.83±0.94**	65.66±1.68**	58.50±1.68**
Cholesterol (mg/dl)	87.5±2.20	94±1.46	86.83±1.07	73.33±1.22**
Triglycerides (mg/dl)	62.16±2.08	73.5±1.33**	89.66±1.05**	78.66±2.06**
HDL (mg/dl)	60.66±1.05	144.16±1.22**	159.01±2.51**	182.16±3.32**
LDL (mg/dl)	76.33±1.22	96.33±1.40**	93±1.15**	83.66±2.06*
AST (IU/L)	55.83±1.53	62.50±1.89	59.16±1.07	59±0.96
ALT (IU/L	33.83±1.01	41.5±1.33**	39.16±1.13**	37.16±0.79**

Values are given in average mean ± SEM for groups of six animals (n=6) each. **p < 0.01, * p < 0.05 denotes when compared to control (One way ANOVA followed by Dunnett test).

The haematological parameter shown in (Table-16 and Figure-18), RBC, WBC, Hb and Packed cell volume effect were not significant of EAFBN at the dose of 100,250 and 500 mg/kg bodyweight. The results showed, there was no changes in haematological level when compared to normal control may be due to the non toxic of these doses.

Table 16: The effects of (EAFBN) haematological parameters of sub acute toxicity studies.

Parameter	Treatment groups			
	Control	EAFBN (100 mg/kg)	EAFBN (250 mg/kg)	EAFBN (500 mg/kg)
RBC ($x10^6/\mu l$)	8.83±0.17	9.03±0.06	9.20±0.09	9.71±0.10
WBC ($x10^3/\mu l$)	11.86±0.14	12.45±0.20	12.36±0.20	12.83±0.12
Haemoglobin (g/dl)	14.20±0.22	14.50±0.11	14.70±0.11	14.90±0.15
PCV %	47.16±0.47	47.66±0.49	45.83±0.60	43.66±1.05

Values are given in average mean ± SEM for groups of six animals (n=6) each. $p < 0.05$ are compared to control (One way ANOVA followed by Dunnett test).

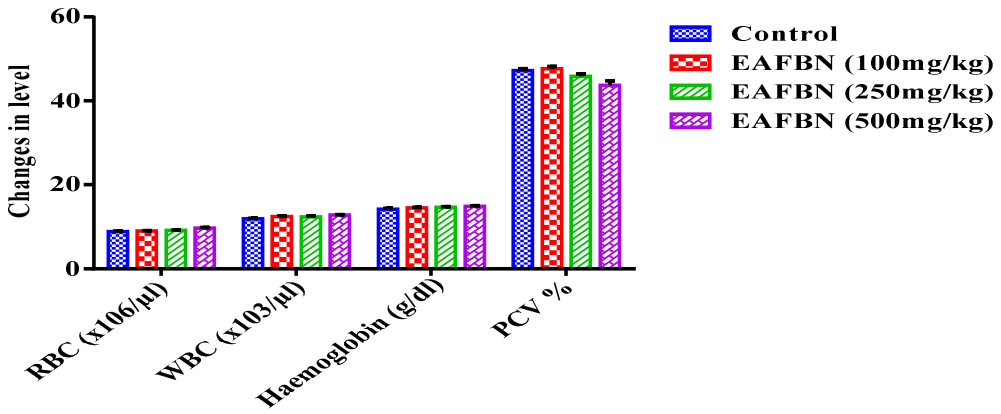


Figure 18: The effects of (EAFBN) haematological parameters of sub acute toxicity studies.

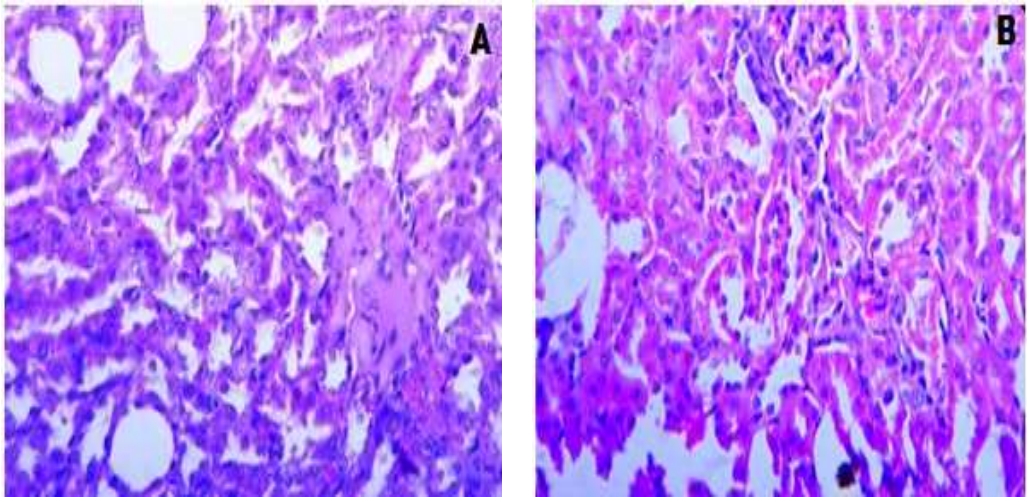
Histopathology of Kidney

The histological picture of the kidney section of normal rats exhibited normal architecture with glomerular and tubular structures. Mild lymphocytic infiltrate with few glomeruli showing increased bowman's space. Tubules show mild edematous changes (Figure-19A). The kidney section of EAFBN 100 mg/kg treated rats showed normal architecture with glomerular and tubular structures. No mesangial hyperplasia, no degenerative changes and cast in the tubules noted, Mild edema and no degenerative changes (Figure-19B). The kidney section of EAFBN 250 mg/kg treated rats showed normal architecture with glomerular and tubular structures and Mild lymphocytic infiltrate with increased bowmans space (Figure-19C). The kidney section of EAFBN 500 mg/kg treated rats exhibited normal architecture with glomerular and tubular structures. Mild lymphocytic infiltrate with increased bowman's space along with mild distortion of tubular epithelial cells (Figure-19D).

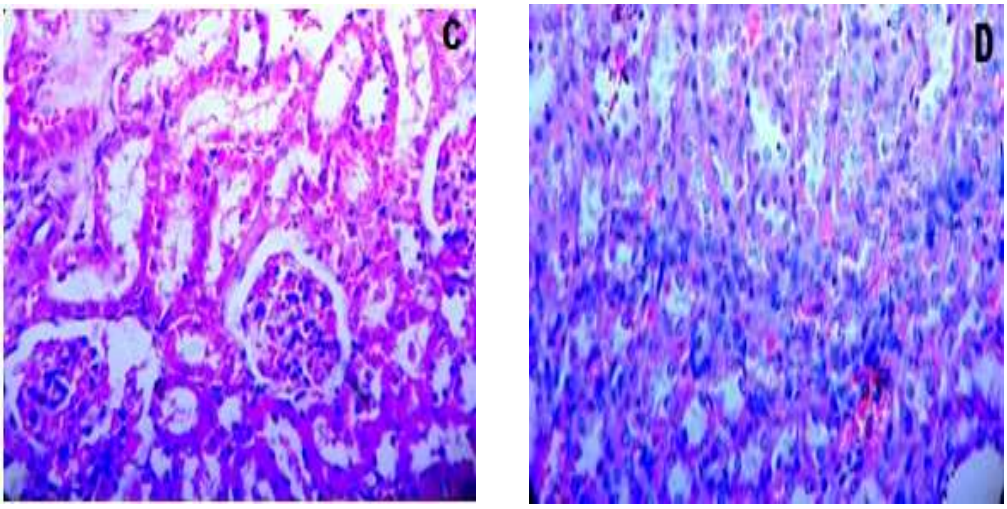
Histopathology of Liver

The histological picture of the liver section of normal rats exhibited normal liver parenchyma with no distortion of architecture, with normal lobules and acini. No portal central bridging necrosis and sinusoids showing no specific pathology. Hepatocytes were showing focal areas with features of mild ballooning with the pale granular cytoplasm. There were no cell shrinkage, intra acidophilic bodies, bile stasis and coffer cell prominence were noted (Figure-20A). The liver section of EAFBN 100 mg/kg treated rats showed normal liver parenchyma with no distortion of architecture, with normal lobules and acini. No portal central bridging necrosis and sinusoids showing no specific pathology. Hepatocytes showing no features of ballooning of cells, cell shrinkage, intra acidophilic bodies, bile stasis and kuffer cell prominence (Figure-20B). The liver section of EAFBN 250 mg/kg treated rats

showed normal liver parenchyma with no distortion of architecture, with normal lobules and acini. No portal central bridging necrosis and sinusoids showing no specific pathology. Hepatocytes were showing no features of ballooning and showing lylmphocytic infiltrate surrounding the portal tract. There was no cell shrinkage, intra acidophilic bodies, bile stasis and kuffer cell prominence (Figure-20C). The liver section of EAFBN 500 mg/kg treated rats exhibited normal liver parenchyma with no distortion of architecture, with normal lobules and acini. No portal central bridging necrosis and sinusoids showing no specific pathology. Hepatocytes showing no ballooning of cytoplasm, focal bile stasis noted, cell shrinkage, intra acidophilic bodies, bile stasis and kuffer cell prominence (Figure-20D).

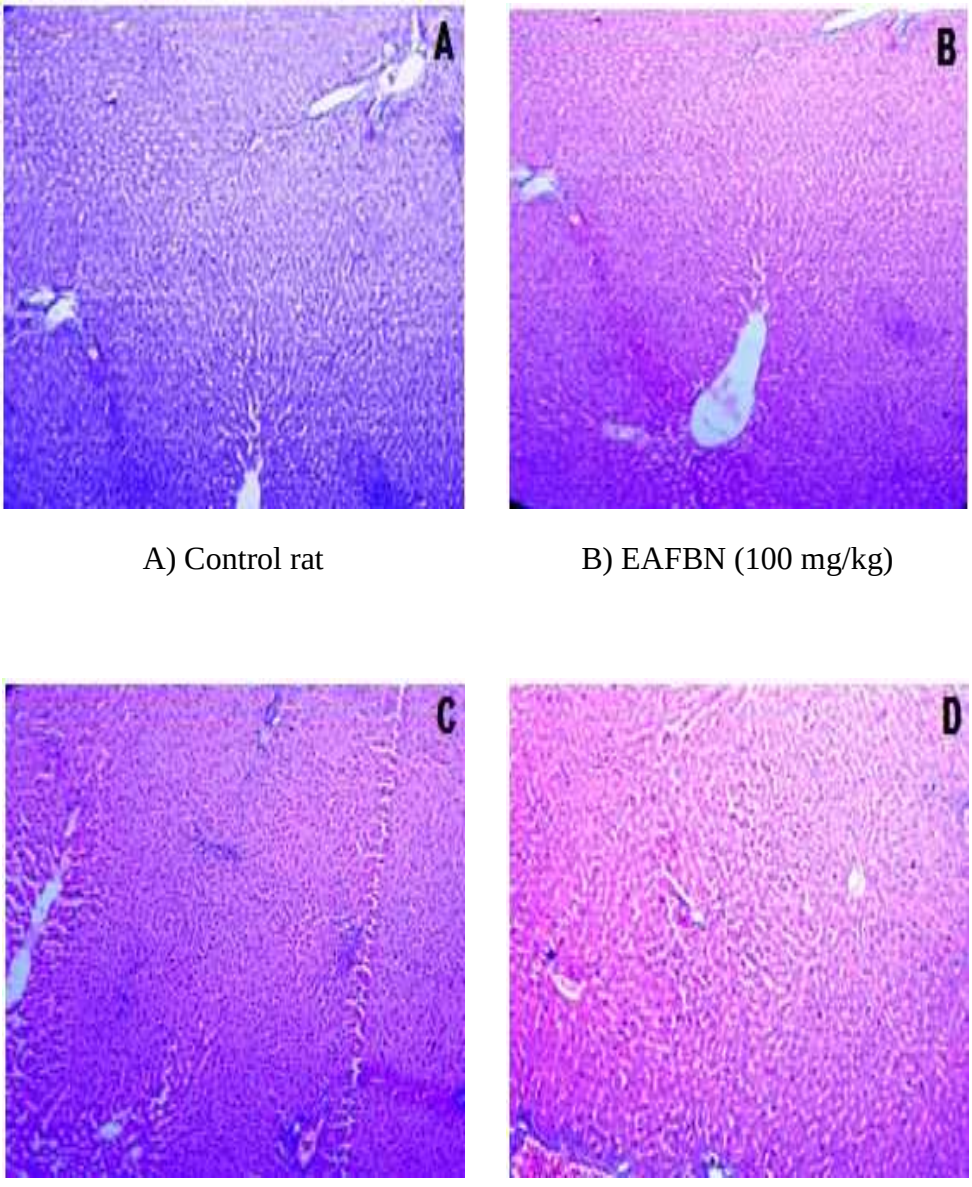


A) Control rat B) EAFBN (100 mg/kg)



C) EAFBN (250 mg/kg) D) EAFBN (500 mg/kg)

Figure 19: Histology of kidney in sub acute toxicity studies.



A) Control rat B) EAFBN (100 mg/kg)

C) EAFBN (250 mg/kg) D) EAFBN (500 mg/kg)

Figure 20: Histology of Liver in sub acute toxicity studies.

7.5. *IN VIVO* ANTIDIABETIC STUDIES

A. Oral glucose tolerance test (OGTT)

In an OGTT, A significant ($p<0.01$) decrease in the fasting glucose level was observed for glucose loaded animal when compared to normal control. The EAFBN doses of 100, 200 &400 mg/kg have shown increase the tolerance for glucose and blood glucose levels were significantly reduced in the dose dependent after oral administration (2 g/kg). The tested drug of EAFBN 400 mg/kg and standard drug showed significant ($p < 0.01$) activity at 30 min onwards and at the time of 120 min all the doses of drug shows significant ($p < 0.01$) activity (Table-17 and Figure-21).

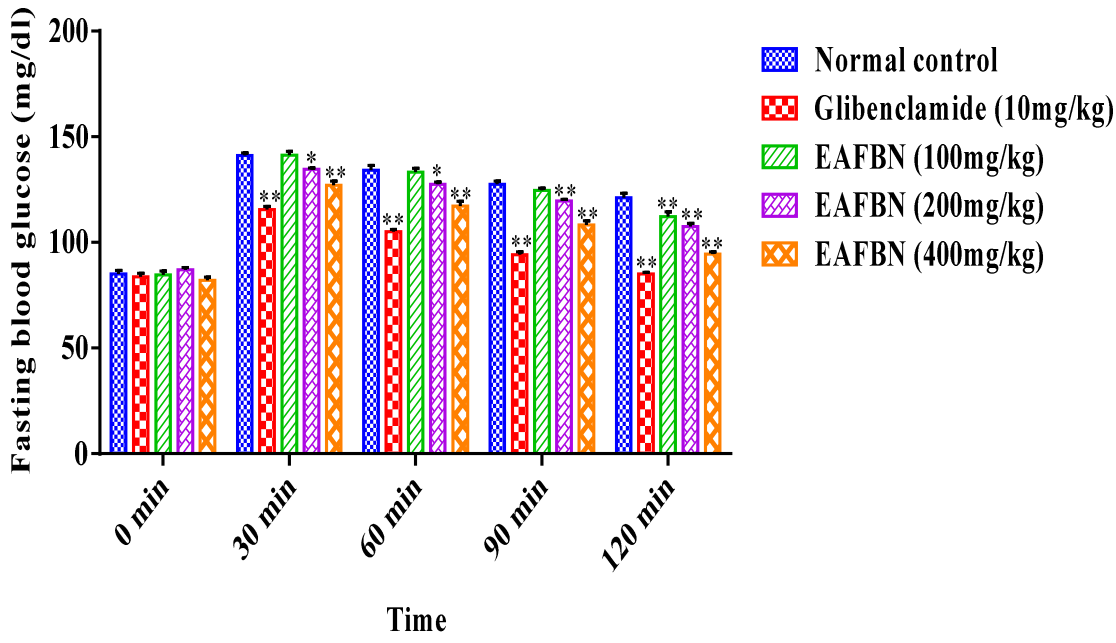


Figure 21: The effects of EAFBN on oral glucose tolerance test (OGTT) in rats.

TABLE 17: The effects of EAFBN on oral glucose tolerance test (OGTT) in rats.

Groups	Fasting blood glucose (mg/dl) at different time (min) after the treatment				
	0	**30**	**60**	**90**	**120**
Normal control	85±1.71	141.16±1.24	134.33±2.02	127.66±1.43	121.16±2.01
Glibenclamide (10mg/kg)	83.83±1.66	115.66±1.45**	105.16±0.90**	94.33±1.20**	85.16±0.79**
EAFBN (100mg/kg)	84.66±1.83	141.33±1.68	133.33±1.68	124.5±1.33	112.33±2.02**
EAFBN (200mg/kg)	87.16±0.94	134.5±0.76*	127.66±0.84*	119.5±0.95**	107.5±1.52**
EAFBN (400mg/kg)	82.16±1.35	127.16±1.86**	117.33±2.10**	108.33±1.85**	94.34±1.28**

Values are given in mean ±SEM for groups of six animals.**p < 0.01,* p < 0.05 denotes when compared to control.

B. Anti Diabetic activity

Changes in body weight

The body weight was slightly increased in the normal control, but High Fat Diet (HFD) control significantly (P<0.01) increased compared to initial body weight during the 28 days. Whereas STZ induced diabetic control rats were significant by (P<0.01) decreased body weight. Standard as well as the EAFBN treatment significantly (P<0.01) prevented this reduction in body weight. While, there was a marginal reduction of animal weight in these groups, when compared to initial body weights (Table-18 and Figure-22).

TABLE 18: The effects of EAFBN on body weight in HFD-STZ induced diabetic rats.

Groups	Body weight (g)	
	Initial	**Final (% Change)**
Normal control	165.5±3.53	180.66±3.80 (+15.16)
Diabetic control	191.66±1.20	141.16±1.85 (-50.50)**
HFD control	184.16±2.98	204±2.93 (+19.84)**
Diabetic + Glibenclamide (10mg/kg)	190±2.22	177.5±2.23 (-12.5)**
Diabetic + EAFBN (100mg/kg)	192.33±1.97	157±1.63 (-35.33)**
Diabetic + EAFBN (200mg/kg)	193±1.69	165.83±0.90 (-27.17)**
Diabetic + EAFBN (400mg/kg)	192.33±2.09	172.5±1.64 (-19.83)**

Values are given in mean ±SEM for groups of six animals each. **p < 0.01 denotes when all the group was compared with normal control.

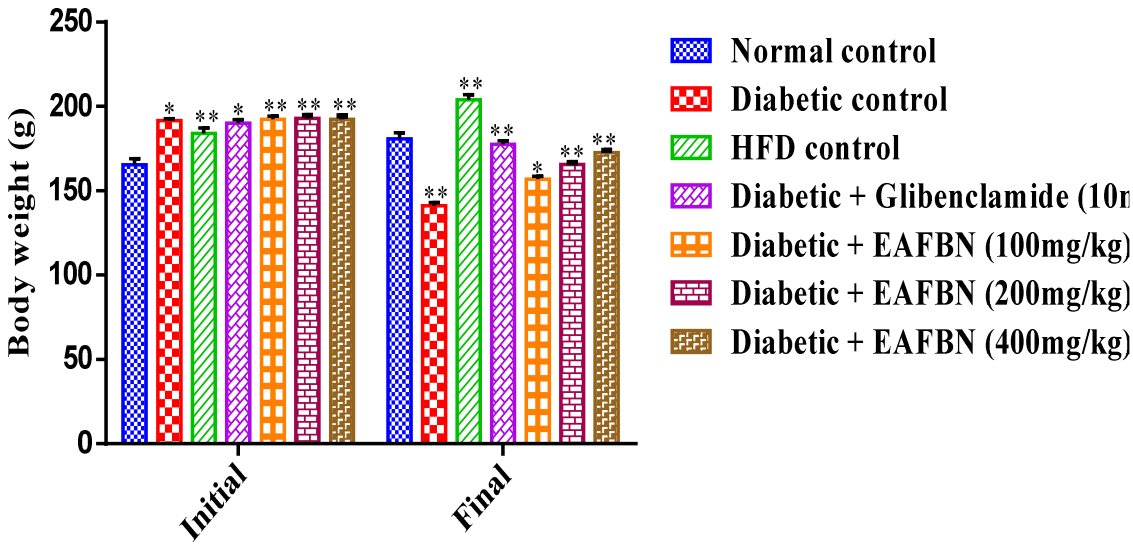


Figure 22: The effects of EAFBN on body weight in HFD-STZ induced diabetic rats.

Changes in blood glucose level

The diabetic rats showed a significant increase in the fasting blood glucose levels and the HFD control rats showed a slightly significant increase when compared to normal control. The treatment of HFD-STZ induced diabetic rats with the EAFBN (100,200 and 400 mg/kg) and standard (10 mg/kg) showed significant ($P<0.01$) decrease in the fasting blood glucose levels were compared to diabetic control (Table -19 and Figure-23).

TABLE 19: The effects of EAFBN on fasting blood glucose levels in HFD-STZ induced diabetic rats.

Groups	Fasting blood glucose (mg/dl)				
	0 day	**7 day**	**14 day**	**21 day**	**28 day**
Normal control	87.83±1.07	89±1.36	88±0.94	86.16±0.94	88.17±0.70
Diabetic control	285.16±3.32**	292.16±3.38**	300.83±4.67**	303.33±4.67**	310.33±2.57**
HFD control	107.83±2.76**	101.67±1.96	102.5±1.66*	102.83±1.10*	105.67±1.20**
Diabetic + Glibenclamide (10mg/kg)	282.67±3.95	207.66±6.52**	181.33±4.54**	150.5±4.00**	125.83±2.04**
Diabetic + EAFBN (100mg/kg)	282.5±4.61	246.67±3.62**	226.33±4.19**	195.5±3.95**	179.17±3.53**
Diabetic + EAFBN (200mg/kg)	275.5±4.75	240.83±2.49**	203.5±3.95**	172.16±3.51**	142±3.76**
Diabetic + EAFBN (400mg/kg)	278.33±6.50	216.66±4.29**	192.33±4.52**	157.5±5.16**	131±3.58**

Values are given in mean ±SEM for groups of six animals each. **$p < 0.01$, * $p < 0.05$ denotes when diabetic control and HFD control was compared with the normal control and treated groups were compared with the diabetic control on the corresponding day.

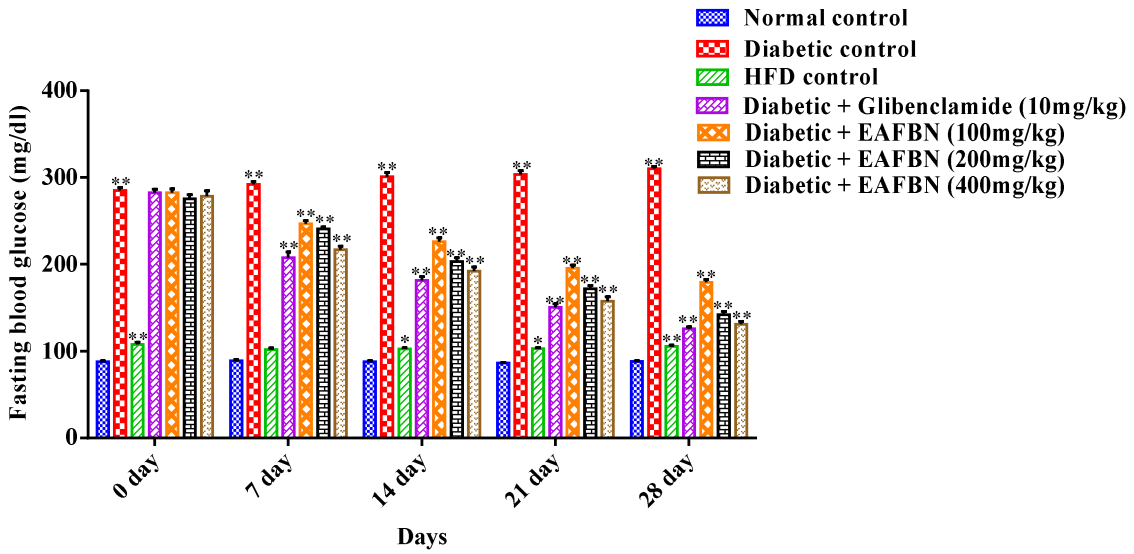


Figure 23: The effects of EAFBN on fasting blood glucose levels in HFD-STZ induced diabetic rats.

Changes in Lipid profile

The serum lipid values of Cholesterol, Triglycerides, HDL-Cholesterol and LDL-Cholesterol were shown in Table -20 and Figure-24. The cholesterol level of diabetic control and HFD control rats showed significant ($P<0.01$) increase the level when compared to normal control. The HFD-STZ induced diabetic rats treated with EAFBN (100, 200 and 400 mg/kg) and standard (10 mg/kg) showed significant ($P<0.01$) decrease level when compared to diabetic control.

The triglyceride level were showed significant ($P<0.01$) decrease for treated with EAFBN (100, 200 and 400 mg/kg) and standard (10 mg/kg), when compared to diabetic control. Hence the triglyceride level of diabetic control and HFD control rats showed significant ($P<0.01$) increase the level, when compared to normal control.

The LDL-cholesterol level of diabetic control rats showed significant ($P<0.01$) and HFD control rats were significant ($p<0.05$) increases, when compared to normal control. The HFD induced diabetic rats treated with EAFBN (100, 200 and 400 mg/kg)

and standard (10 mg/kg) showed significant (P<0.01) decrease level, when compared to diabetic control.

The HDL-Cholesterol level were showed significant (P<0.01) increase for treated with EAFBN (100, 200 and 400 mg/kg) and standard (10 mg/kg), when compared to diabetic control. Hence the triglyceride level of diabetic control rats showed significant (P<0.01) decrease the level, when compared to normal control.

However the treatment of standard and EAFBN showed a marked reversal of changes in the serum lipid parameters as compared to diabetic rats.

TABLE 20: The effects of EAFBN on serum lipid profile in HFD-STZ induced diabetic rats.

Groups	Cholesterol (mg/dl)	Triglycerides (mg/dl)	LDL (mg/dl)	HDL (mg/dl)
Normal control	110.67±1.45	86.66±2.20	89.66±1.60	37.33±1.02
Diabetic control	291.66±3.72**	244.33±4.02**	195.5±3.29**	24.33±1.25**
HFD control	149.5±6.00**	146.5±2.40**	99±1.41*	34.66±1.02
Diabetic + Glibenclamide (10mg/kg)	154.33±1.83**	109.33±2.14**	79.83±1.35**	46.16±1.47**
Diabetic + EAFBN (100mg/kg)	188.66±2.78**	189.16±2.04**	140.83±1.81**	33.16±1.42*
Diabetic + EAFBN (200mg/kg)	174.5±2.07**	156.16±2.38**	104.66±1.76**	40.33±1.22**
Diabetic + EAFBN (400mg/kg)	159.83±2.19**	130±2.65**	91.5±2.54**	45±1.73**

Values are given in mean ±SEM for groups of six animals each. **p < 0.01, * p < 0.05 denotes when diabetic control and HFD control was compared with the normal control and treated groups were compared with the diabetic control.

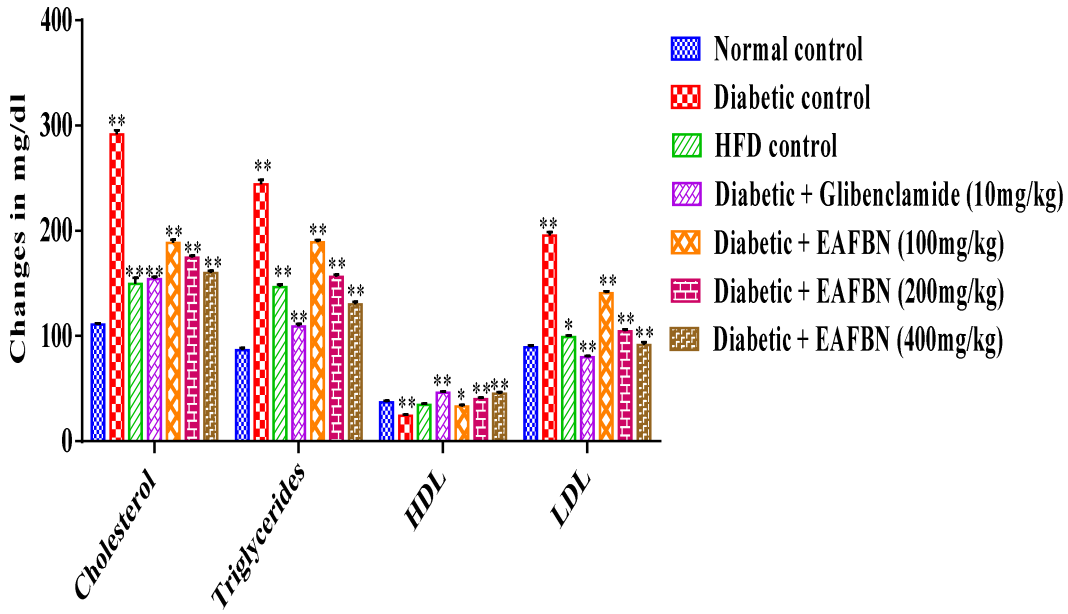


Figure 24: The effects of EAFBN on serum lipid profile in HFD-STZ induced diabetic rats.

Changes of Alkaline Phosphatase (ALP)

The significant (P<0.01) increase the level of ALP was observed for HFD-STZ induced diabetic rats when compared to normal control. A significant (P<0.01) reversal change was observed for EAFBN (100, 200 and 400 mg/kg) and standard (10 mg/kg) body weight dose when compared to diabetic control.(Table-21 and Figure-25).

Changes of Asperate Amino Transaminase (AST)

The significant (P<0.01) increase the level of AST was observed for HFD-STZ induced diabetic rats when compared to normal control. A significant (P<0.01) decreases were observed for EAFBN (100, 200 and 400 mg/kg) and standard (10 mg/kg) body weight dose when compared to diabetic control. The HFD control rats were produced no significant change when compared to normal (Table-21 and Figure-25).

Changes of Alanine Amino Transaminase (ALT)

The significant (P<0.01) increase the level of ALT was observed for STZ induced diabetic rats when compared to normal control. A significant (P<0.01) reversal to decreases was observed for EAFBN (100,200 and 400 mg/kg) and standard (10 mg/kg) body weight dose when compared to diabetic control. The HFD control rats were showed no significant change when compared to normal (Table-21 and Figure-25).

Changes in Urea level

A significant (P<0.01) decrease the level of urea was observed for EAFBN (100, 200 and 400 mg/kg) and standard (10 mg/kg) body weight dose when compared to diabetic control. The HFD control rats were showed no significant change when compared to normal. The significant (P<0.01) increase the level was observed for STZ induced diabetic rats when compared to normal control (Table-22).

Changes in Creatinine level

A significant (P<0.01) increase in the creatinine level was observed for STZ induced diabetic rats and HFD control when compared to normal control. A significant (P<0.01) decreased level was observed for EAFBN (100,200 and 400 mg/kg) and standard (10 mg/kg) dose when compared to diabetic control (Table-22).

TABLE 21: The effects of EAFBN of serum marker enzymes in HFD-STZ induced diabetic rats.

Groups	ALP (IU/L)	AST (IU/L)	ALT (IU/L)
Normal control	62±1.80	77.5±1.76	40±2.08
Diabetic control	156±3.89**	183.5±3.00**	126.5±1.56**
HFD control	68.33±1.60	79.16±1.30	42.33±1.66
Diabetic + Glibenclamide (10mg/kg)	66.66±1.28**	79.66±1.58**	44.5±1.38**
Diabetic + EAFBN (100mg/kg)	117.16±2.30**	150±3.16**	92.5±1.54**
Diabetic + EAFBN (200mg/kg)	90±2.06**	101.66±2.20**	68.66±1.52**
Diabetic + EAFBN (400mg/kg)	68.66±2.71**	82.16±1.81**	52.5±1.66**

Values are given in mean ±SEM for groups of six animals each. **$p < 0.01$, * $p < 0.05$ denotes when diabetic control and HFD control was compared with the normal control and treated groups were compared with the diabetic control.

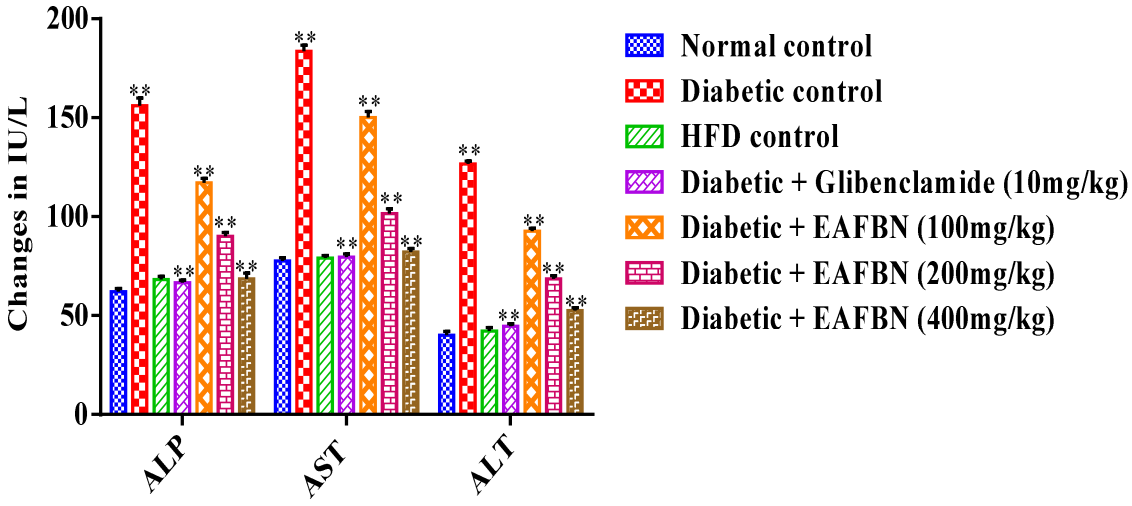


Figure 25: The effects of EAFBN of serum marker enzymes in HFD-STZ induced diabetic rats.

Changes in insulin level

Increase the level of insulin (P<0.01) was observed for EAFBN (100, 200 and 400 mg/kg) and standard (10 mg/kg) body weight dose when compared to diabetic control. The HFD control rats were found to be not significant change and significant (P<0.01) decrease the level was observed for STZ induced diabetic rats when compared to normal control (Table-22 and Figure- 26).

Changes in Glycolated haemoglobin level

A significant (P<0.01) increased level of the glycolated hemoglobin level was observed for STZ induced diabetic rats and HFD control when compared to normal control. A significant (P<0.01) decrease the level of was observed for EAFBN (100, 200 and 400 mg/kg) and standard (10 mg/kg) body weight dose when compared to diabetic control (Table-20 Figure- 26).

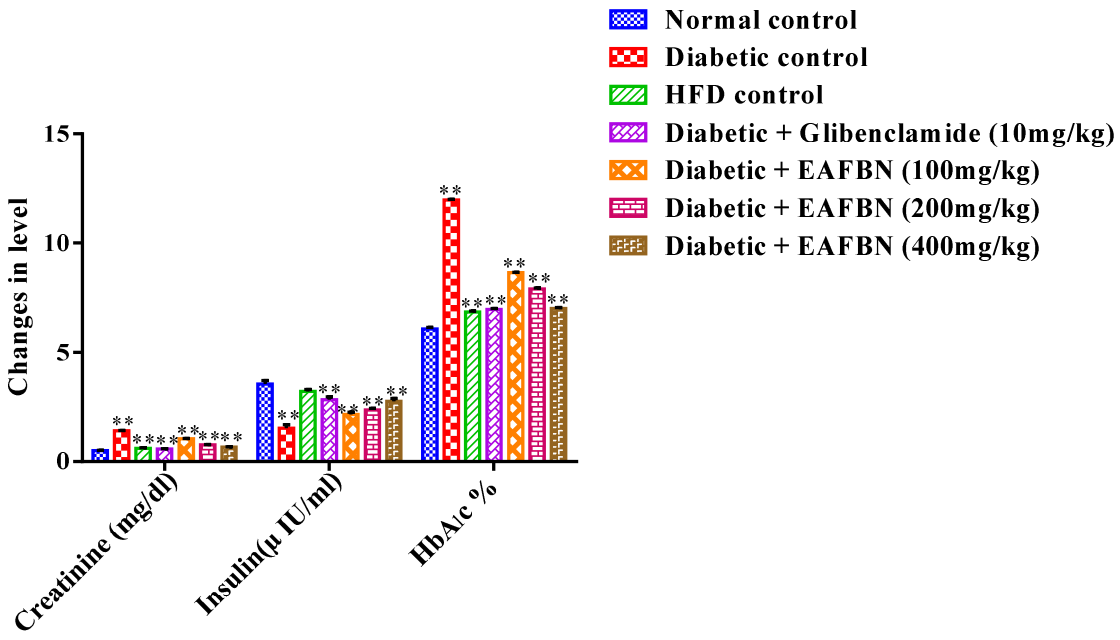


Figure 26: The effects of EAFBN on serum profile in HFD-STZ induced diabetic rats.

TABLE 22: The effects of EAFBN on serum profile in HFD-STZ induced diabetic rats.

Groups	Urea (mg/dl)	Creatinine (mg/dl)	Insulin(µ IU/ml)	HbA_1c (%)
Normal control	26.83±1.22	0.51±0.01	3.55±0.16	6.07±0.07
Diabetic control	63.66±1.62**	1.41±0.02**	1.53±0.15**	11.97±0.03**
HFD control	30.83±0.87	0.61±0.02**	3.21±0.10	6.85±0.04**
Diabetic + Glibenclamide (10mg/kg)	35.67±1.33**	0.58±0.01**	2.83±0.13**	6.97±0.03**
Diabetic + EAFBN (100mg/kg)	39.50±1.11**	1.05±0.01**	2.15±0.11**	8.64±0.03**
Diabetic + EAFBN (200mg/kg)	37±1.36**	0.77±0.01**	2.36±0.07**	7.91±0.03**
Diabetic + EAFBN (400mg/kg)	34.50±1.31**	0.67±0.01**	2.75±0.14**	7±0.06**

Values are given in mean ±SEM for groups of six animals each. **p < 0.01 denotes when diabetic control and HFD control was compared with the normal control and treated groups were compared with the diabetic control.

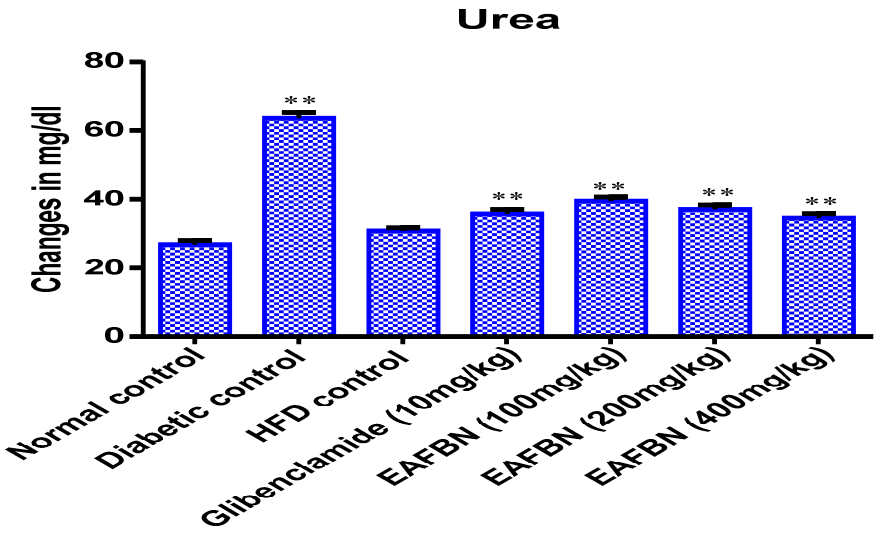


Figure 27: The effects of EAFBN on serum urea profile in HFD-STZ induced diabetic rats.

Effect of Liver glycogen, Glucose uptake and Glucose transport

There was a marked significant (P<0.01) reduction in the liver glycogen levels of diabetic rats from 4.02 mg/gm tissue (in normal control rats) to 2.27 mg/gm tissue was observed. Glibenclamide treatment elicited 3.77 mg/gm significant (P<0.01) increase in liver glycogen levels, while EAFBN (100, 200 and 400 mg/kg) treatment showed 2.83 mg/gm, 3.17 mg/gm and 3.72 mg/gm significant (P<0.01) increase in liver glycogen levels when compared with the untreated diabetic rats (Table -23 and Figure-28).

The glucose uptake by hemidiaphragms showed a significant (P<0.01) reduction of diabetic rats when compared to control group. The glucose uptake were significant (P<0.01) enhancement of rats treated with EAFBN and glibenclamide were seen when compared to diabetic control (Table -23 and Figure-28).

The glucose transport showed a significant (P<0.01) enhancement in diabetic rats as compared to normal rats. Also, both EAFBN and Glibenclamide treated rats showed a significant (P<0.01) inhibitory effect on glucose transport in liver tissue (Table -23 and Figure-28).

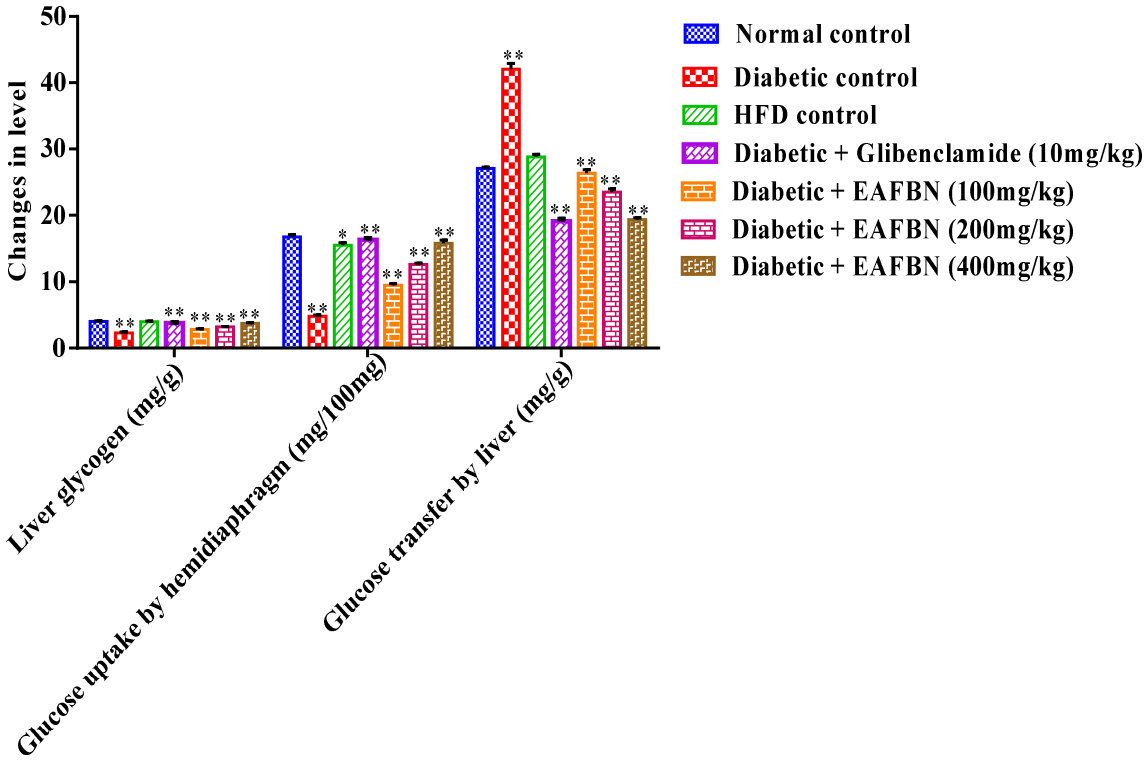


Figure 28: The effects EAFBN on Liver glycogen, Glucose uptake by hemidiaphragm the liver Glucose transport by liver in HFD-STZ induced diabetic rats.

TABLE 23: The effects EAFBN on Liver glycogen, Glucose uptake by hemidiaphragm and Glucose transport by the liver in HFD-STZ induced diabetic rats

Groups	Liver glycogen (mg/gm of wet tissue)	Glucose uptake by hemidiaphragm (mg/100mg)	Glucose transport by liver (mg/gm)
Normal control	4.02±0.10	16.78±0.30	27.05±0.22
Diabetic control	2.27±0.15**	4.76±0.20**	42.03±0.85**
HFD control	3.98±0.06	15.51±0.40*	28.83±0.35
Diabetic+ Glibenclamide (10mg/kg)	3.77±.073**	16.31±0.24**	19.13±0.30**
Diabetic+EAFBN (100mg/kg)	2.83±0.05**	9.46±0.27**	26.36±0.51**
Diabetic+EAFBN (200mg/kg)	3.17±0.11**	12.61±0.22**	23.53±0.50**
Diabetic+EAFBN (400mg/kg)	3.72±0.09**	15.83±0.44**	19.36±0.28**

Values are given in mean ±SEM for groups of six animals each. **$p < 0.01$,* $p < 0.05$ denotes when diabetic control and HFD control was compared with the normal control and treated groups were compared with the diabetic control.

Histopathology

The histopathological studies of the pancreatic tissues are shown in (Figure-29). Normal rats showed the normal architecture of the pancreas with the preserved islet of Langerhans cells (Figure-29A). Induction of diabetes using streptozotocin resulted in hyalinization of islets of langerhans cells with focal mild degenerative changes, mild fibrosis, dilated and congested vessels along with focal chronic inflammatory cell infiltrate in diabetic rats (Figure-29B). HFD control rats showed mild edema of islets of langerhans cells, thick walled and congested vessels and focal lymphocytic infiltrate exhibiting focal edematous changes (Figure-29C). The abnormal histopathology of the pancreas due to streptozotocin induced diabetes was reversed in the glibenclamide and EAFBN treated diabetic animals. The recovery of the standard glibenclamide treated group is evident as near normal architecture with preserved islet cells and mild edema (Figure-29D). The EAFBN (100mg/kg, 200mg/kg & 400mg/kg) treated groups resulted in mild hyalinization islets of langerhans cells with focal mild degenerative changes when compared to untreated diabetic rats. It can be noted that the islets of langerhans cells regenerated in the treatment groups (Figure-29 E, F&G).

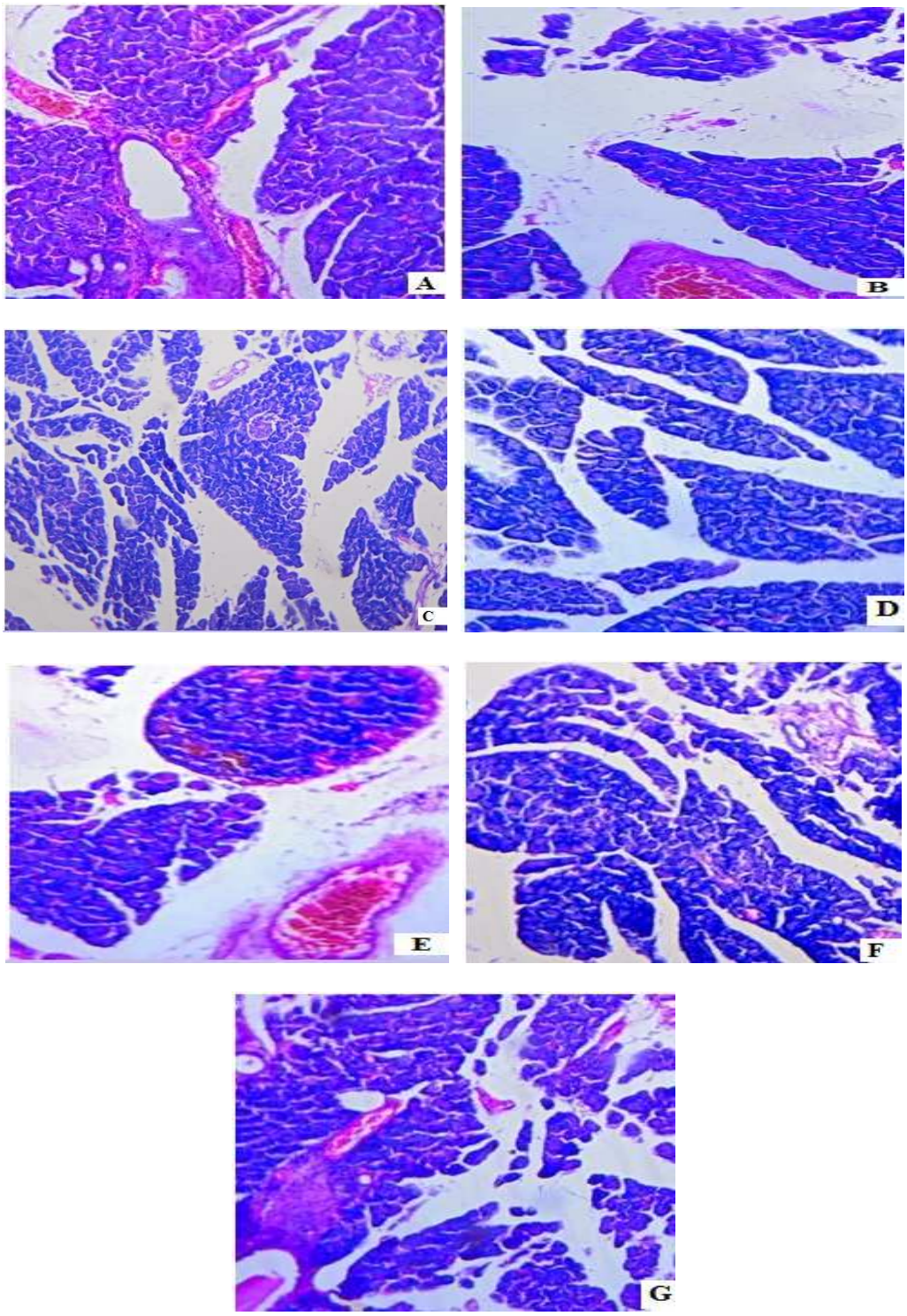


Figure 29: Histology of control and treated rat pancreas of antidiabetic studies. A) Control rat, B) Diabetic control, C) HFD control, D) Standard, E) EAFBN (100 mg/kg), F) EAFBN (200 mg/kg) and G) EAFBN (400 mg/kg).

7.6. WOUND HEALING ACTIVITY

In excision wound healing studies, significant activity was observed with EAFBN and standard drug of nitrofurazone comparable to that of the diabetic control. In EAFBN (10% w/w & 5% w/w) groups significant (P<0.01) percentage contraction of the excision wound area was 96.6±0.10 and 95.8±0.27, while the standard drug of nitrofurazone was 99 ±0.25 during the period of 16 days (Table-24 and Figure-30). Wound percentage contraction was significantly delayed in diabetic control 86.93±0.27 compared to normal control 90.1±0.27. The epithelization period of days observed in EAFBN (10% w/w & 5% w/w) and standard drug was 15.83 ± 0.16, 17.5 ± 0.34 and 15.16 ± 0.16 respectively. The complete epithelization was observed in the same day of EAFBN (10% w/w) and standard drug, hence diabetic control rats were 5 days increase the period of epithelization of diabetic rats 23.66±0.55 compared to normal control 18.83 ± 0.16.

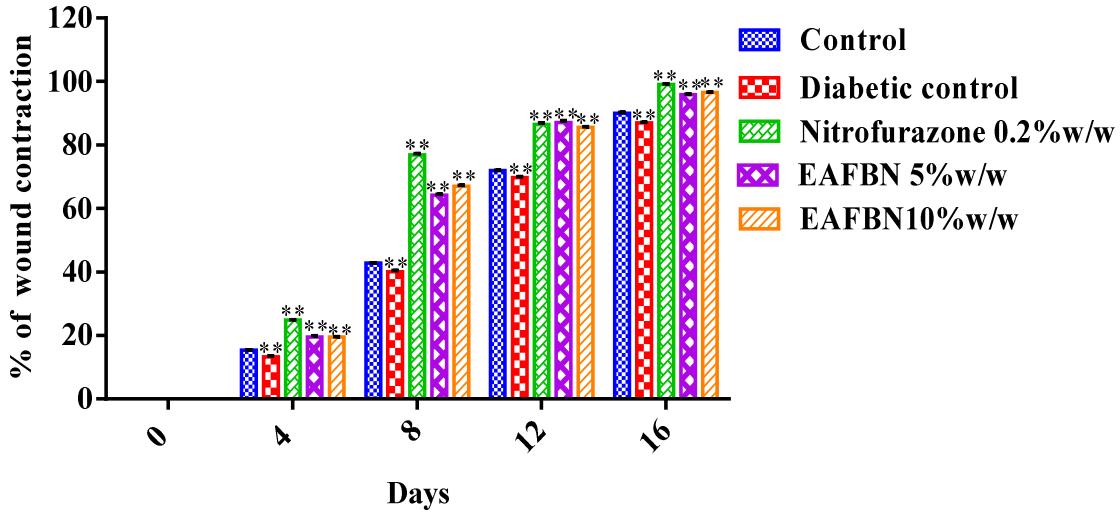


Figure 30: The effects of EAFBN on Excision wound model percentage of wound contraction of experimental group of rats.

Table 24: The effects of EAFBN on Excision wound model percentage of wound contraction of an experimental group of rats

TREATMENT	PERCENTAGE (%) OF WOUND CONTRACTION AREA (Original wound area 200 mm^2)				Period of Epithelialization (days
	4th Day	8th Day	12th Day	16th Day	
Control	15.35±0.07	42.86±0.07	71.92±0.08	90.1±0.27	18.83±0.16
Diabetic control	13.23±0.35**	40.15±0.40**	69.77±0.33**	86.93±0.27**	23.66±0.55**
Nitrofurazone 0.2%w/w	24.81±0.14**	76.93±0.42**	86.45±0.50**	99 ±0.25**	15.16±0.16**
EAFBN 5% w/w	19.5±0.42**	64.2 ±0.34**	87.1±0.55**	95.8±0.27**	17.5±0.34**
EAFBN 10%w/w	19.5±0.18**	67±0.32**	85.5±0.23**	96.6±0.10**	15.83±0.16**

Values are given in mean ±SEM for groups of six animals each. **$p < 0.01$, * $p < 0.05$ denotes when diabetic control was compared with the normal control and treated groups were compared with the diabetic control.

In the incision wound model the animal treated with the topical application of EAFBN 5% w/w treated animals skin breaking strength (gm) was significantly 290.83 ± 3.0, EAFBN (10% w/w) and standard drug have shown more or less same significant skin breaking strength (342.5 ± 3.81and 350 ± 2.88). But it was less in diabetic control 199.33 ± 3.94 and normal control 217.5 ± 3.81 (Table -25 and Figure-31).

Table 25: The effects of EAFBN on Incision wound model Breaking strength of an experimental group of rats

TREATMENT	Breaking strength (gm)
Control	217.5±3.81
Diabetic control	199.33±3.94**
Nitrofurazone 0.2%w/w	350±2.88**
EAFBN 5% w/w	290.83±3.0**
EAFBN 10%w/w	342.5±3.81**

Values are given in mean ±SEM for groups of six animals each. **p < 0.01, * p < 0.05 denotes when diabetic control was compared with the normal control and treated groups were compared with the diabetic control.

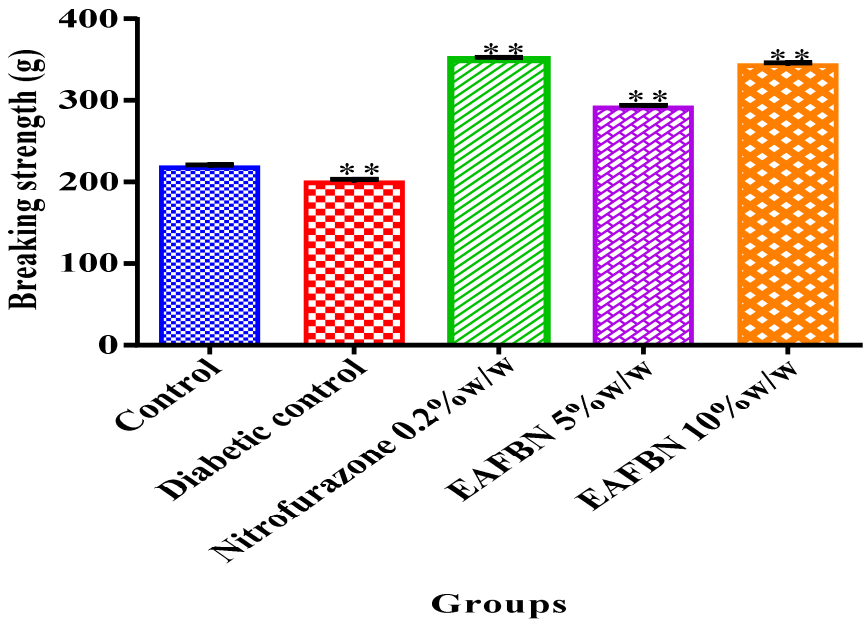


Figure 31: The effects of EAFBN on Incision wound model Breaking strength of experimental group of rats.

7.7. ISOLATION OF PHYTOCONSTITUENTS OF *BARLERIA NOCTIFLORA* BY USING COLUMN CHROMATOGRAPHY

7.7.1. Compound C1

The ethyl acetate fraction of *Barleria noctiflora* on column chromatography gave a compound. The physical and spectral characteristic of the compound is given below.

1. Homogeneity

The homogeneity of compound C1 was proved by a single spot on TLC using silica gel G as an adsorbent and solvent mixture of different polarities as developers. The solvent system used and the corresponding Rf values are given in Table-26.

2. Colour reactions

Compound C1 exhibited a positive response for Libermann-Burchard's test and Salkowski test, indicating its steroid nature.

3. Melting point of compounds C1 was found to be 170°C

Table 26: TLC profile of compound C1

Mobile phase	Solvent ratio	Rf value of the spot
Chloroform: ethyl acatate	80:20	0.58
Hexane: methanol	90:10	0.79
Chloroform: benzene	92:8	0.8

IR spectrum

The IR spectrum Figure-32 showed absorption for O-H stretching 3520-3420, C-H strechting aliphatic 2924, 2852, C-H strechting aromatic 1643, C=O stretching of flavones 1643, C=C aromatic stretching 1458, 1400, 1590, C-O stretching 1080, Substituted aromatic and finger print bands 597, 563.

^{1}H NMR spectrum (δ ^{PPM} $CDCl_3$)

Aromatic proton (7.287 – 6.952), Hydroxyl proron (4.527), Methine proton (2.756 – 2.578), Methylene proton (2.317 – 2.033) Methyl proton (1.895 – 0.819) Figure- 33.

^{13}C NMR spectrum (δ ^{PPM} $CDCl_3$)

Acetic carbon C=O (173), Aromatic carbon C=C (126-122), C-O (76-80), C-C (30-29), α&β C-O (20-29), R-CH_3 type carbon (11-20) Figure- 34.

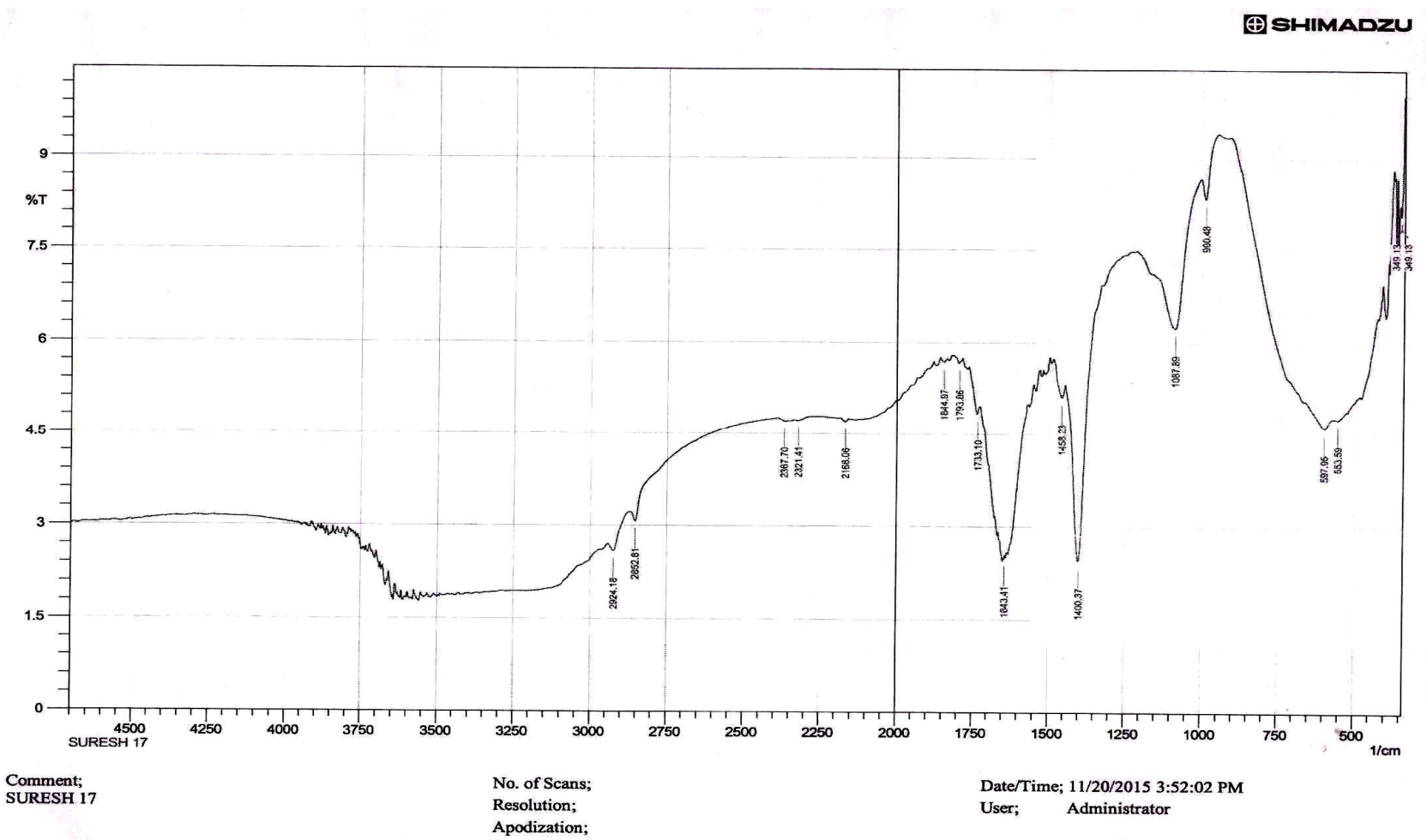


Figure 32: IR Spectrum of compound 1

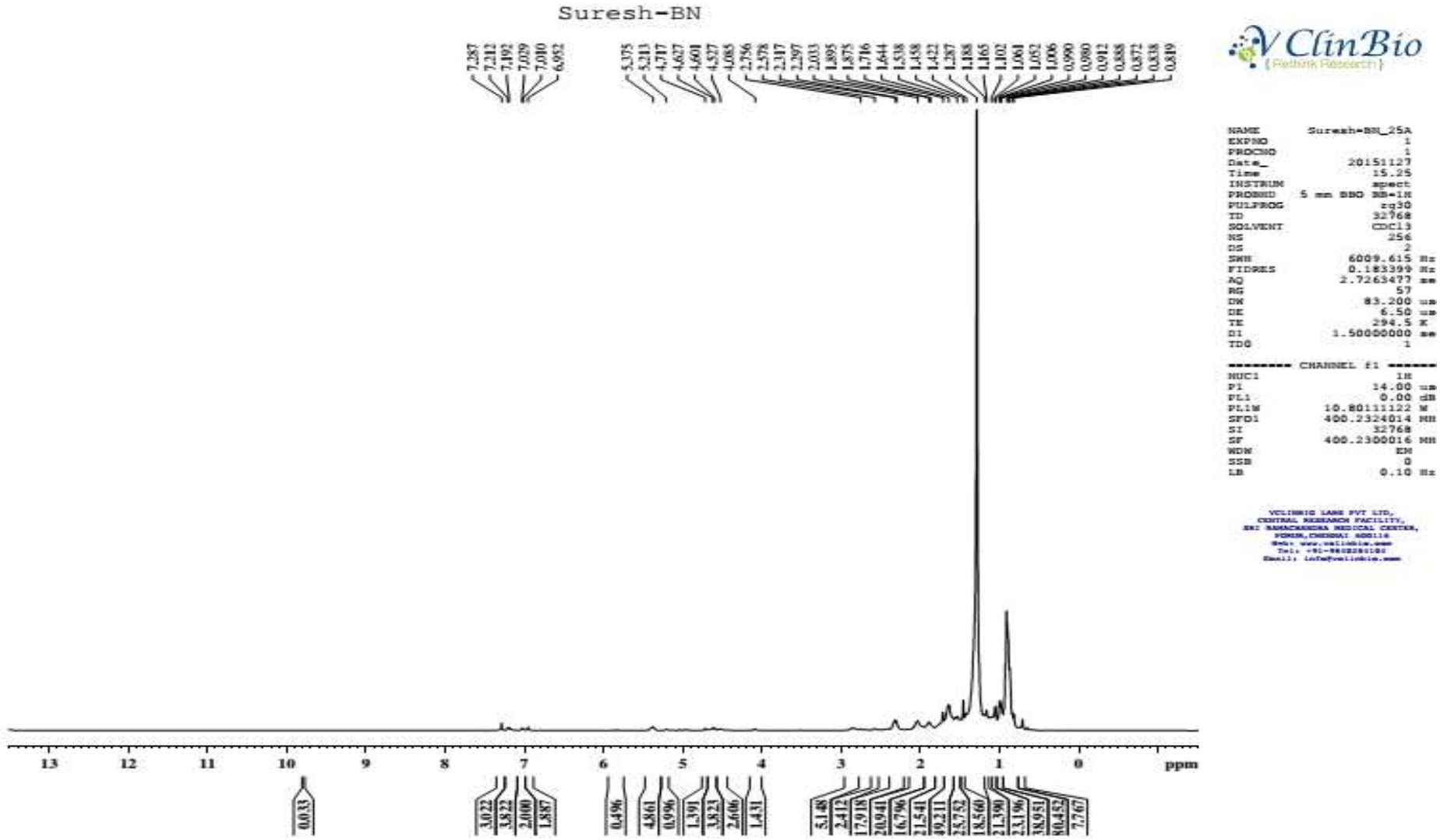


Figure 33: ^{1}H NMR spectrum (δ^{PPM} $CDCl_3$)

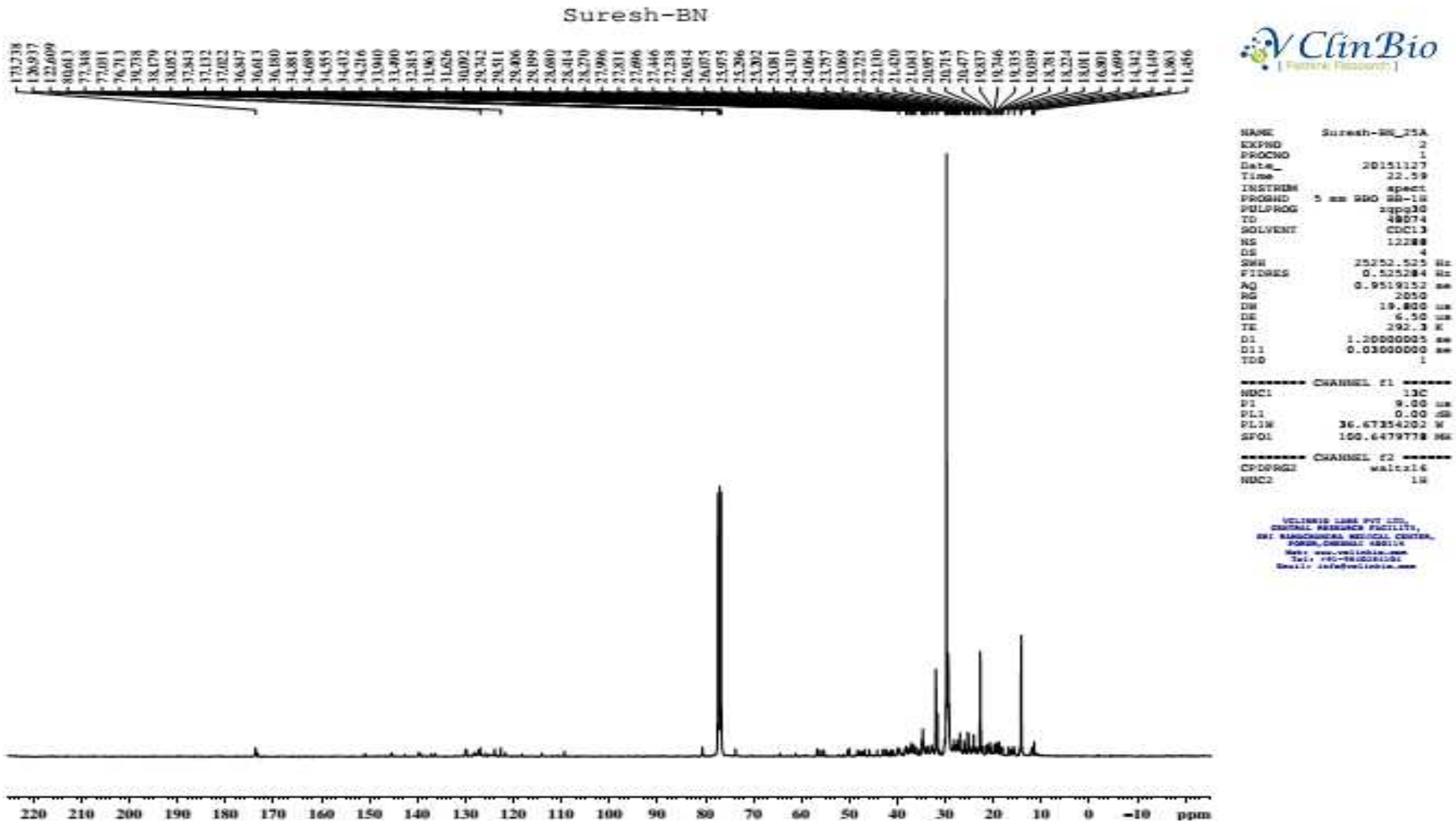


Figure 34: ^{13}C NMR spectrum (δ^{PPM} $CDCl_3$)

8. DISCUSSION

Traditions are dynamic entities of unchanging knowledge. Traditional medicine is an evolutionary process as communities and individuals continue to discover new techniques that can transform practices. It has been confirmed by WHO that herbal medicine serves the health needs of about 80 % of the world's population. Meanwhile, consumers in developing countries are becoming disillusioned with modern health care and seeking alternatives. The recent resurgence of plant remedies results from several factors, such as the effectiveness of the plant medicines, the side effects of most modern drugs and the development of science and technology.

The drug discovery and ethnopharmacology using natural products are remaining essential issues in the current target-rich and lead-poor scenario.[124] Many modern drugs have their origin in ethnopharmacology. Globally, there is a positive trend in favor of integrative and traditional health sciences in both practice and research. There are regular approaches to the discovery of drugs, including the use of chemical synthesis, serendipity, chemical biology, combinatorial chemistry and genomics. However, the innovative approaches engage in ethnopharmacology, reverse pharmacology, systems biology, holistic and personalized medicine. There are clear trends, shows mainstream in pharmaceutical research is moving away from single molecule or single target approach to multiple target and combination approaches.[125] The ethnopharmacology knowledge and experiential base permit drug research from 'clinics to laboratories' is a true reverse pharmacology approach. In this process, 'safety' remains the important preliminary point and the efficiency becomes a matter of validation. A golden triangle consisting of modern medicine,

modern science and traditional knowledge with systems orientation will collect to form an innovative discovery of newer, affordable, safe and effective therapies.[126]

An analysis the origin of drugs developed 1981-2012 showed that natural products or natural product-derived drugs comprised 28% of all new chemical entities launched into market.[127] In addition, 24% of these new chemical entities were synthetic or natural mimic compounds, based on the study of pharmacophore related to natural products.[128] This combined percentage (52% of all new chemical entities) suggests that natural products are important sources for new drugs and are also good lead compounds suitable for further modification during drug development.

Current drug discovery from plants has mainly relied on bioactivity guided isolation methods; use of natural products has been the single most successful strategy for the discovery of new medicines and leads. Plants represent a vast resource of untapped chemicals. The chemical diversity of plants is far greater than that of microbes. Hence, it is utmost important to involve in herbal drug research. Herbal medicine has been improved in developing countries as an alternative solution to health problems and costs of pharmaceutical products.

Barleria noctiflora is presumed for its medicinal properties in indigenous medicine in India. Most of the *Barleria* species were potent anti inflammatory, analgesic, anti leukemic, antitumor, anti-hyperglycemic, anti-amoebic etc and many of the Acanthaceae family are used as medication for asthma.[70] The whole part used in the treatment of antidiabetic and antioxidant.[84] So far no biological and phytochemical investigations were carried out on this plant. Hence, in the present study, we were interested in carrying out a systematic biological and phytochemical

investigation of *Barleria noctiflora*. The aim of the plant is pharmacognostical, pharmacological evaluation and isolate compound from *Barleria noctiflora* used in the *in vitro* and *in vivo* studies.

Pharmacognostical standardization was carried out on the basis of detailed botanical evaluation of the leaves and stem of *Barleria noctiflora*, which includes morphology and microscopy as well as WHO recommended physicochemical studies. The results of the standardization may throw an immense light on the botanical identity of the leaves and stem of *Barleria noctiflora*. This may furnish a basis of judging the authenticity of the plant and also to differentiate the drug from its adulterants and other species. The macroscopic characters were examined to identify the right crude drug.

Transverse section of the leaves showed a fairly prominent midrib, lateral veins and dorsiventral lamina. The vascular strand is omega shaped with an abaxial arc and two laterals out curved wings. The lamina has smooth, even surface with two layers of palisade cells, the marginal part of the lamina is slightly curved down and bluntly conical. The stem has thin continuous epidermal layer, a wide cortex with homogenous layers of parenchyma cells, vascular cylinder consists of a thin, discontinuous layer of sclerenchyma abutting in phloem and a wider zone of xylem elements.

The powder characters of a drug are mainly used in the identification of the drug in the powder form. The *Barleria noctiflora* powder was dark greenish gray in color with pungent odour, slightly sweet and sour taste. A microscopical examination the powder showed numerous unicellular covering trichomes, which are slightly curved. Diacytic stomata made up of rectangular or polygonal epidermal

cells with thin anticlinal walls were seen. Quantitative microscopic data were especially useful for identifying the different species of genus and also helpful in the determination of the authenticity of the plant. The physicochemical parameters were mainly used in judging the purity and quality of the drug. An ash value of a drug gives an idea of the earthy matter or inorganic composition or other impurities present along with the drug.

The ash values were important since ash may be derived from the plant itself (physiological or natural ash) as well as from the extraneous matter, especially sand and soil adhering to the surface of the drug (non physiological ash). The ash values varied in cases of many crude drugs and its study give an idea about the quality and purity of the drug. The determination of physiological and non physiological ash together is called as total ash. The total ash may vary within wide limits from a specimen of genuine drug due to variable natural or physiological ash, in such cases the ash obtained is treated with acid, which most of the natural ash is soluble leaving the silica as acid- insoluble ash which represents most of the ash from the contaminating soil.

Extractive values give an idea about the chemical constituents present in the drug as well as useful in the determination of exhausted or adulterated drugs. The results suggest that the powdered have high water soluble extractive value. The loss on drying reveals the percentage of moisture present in the drug, since moisture facilitates the enzyme hydrolysis or growth of microbes which leads to deterioration. The quantitative microscopy parameters are identification and standardization of crude drugs. The crude fiber content which was studied can be implied to determine the nutritive value. Fluorescence analysis of powdered leaves was studied in both

UV and day light. The powder showed green fluorescence in UV light, which indicates the presence of chormophore in the drug. Preliminary phytochemical analysis revealed the secondary metabolites are known to support bioactivity in this plant.

Determination of preliminary phytochemical analysis is of paramount importance for the purpose of evaluation of crude drugs. In the phytochemical studies, the plant extracts showed the presence of flavonoids, alkaloids, phenols, saponins, steroids, glycosides, tannins, protein and carbohydrate were present.

The Total phenol and flavonoids is important for the purpose of evaluation of crude drugs. Phenols occurring in nature and the environment are of interest from many viewpoints (antioxidants, astringency, bitterness, color, oxidation substrate, protein constituents, etc.). Phenols are responsible for the majority of the oxygen capacity in most plant-derived products[129, 130] with few exceptions, such as carotene, the antioxidants in foods are phenols. The total phenol content of the extracts which is majorly responsible for antioxidant activity was estimated using the Folin-Ciocalteu method. The ethanolic and aqueous extract of *Barleria noctiflora* was found to have the highest total phenol content among the plant extracts studied. The result shows total phenolic content was higher in the ethanolic extract compared to the aqueous extract. It shows the ethanolic extract of *Barleria noctiflora* posseses high antioxidant ability.

The flavonoids are responsible for the various biological activities like hypoglycemic, free radical scavenging, coronary heart disease prevention, anti inflammatory and hepatoprotective etc. The total flavonoides content was obtained with rutin equivalent. The potent flavonoids content was present in ethanolic and

aqueous extract of *Barleria noctiflora.* So the presence of phenolic and flavonoids were responsible for the biological activities.

In the last two decades, there has been deep interest in the role of oxygen free radicals, commonly known as "Reactive Oxygen Species" (ROS) and of "Reactive Nitrogen Species" (RNS) in experimental medicine. ROS/RNS play a dual role in biological systems, since it can be either beneficial or harmful to living systems.[41] The effects of beneficially ROS involve physiological roles in cellular reaction to noxious, as for example in protection against infectious agents and in the function of cellular signaling systems. The further benefit of ROS at low concentrations is the induction of a mitogenic response. In high concentrations, ROS can be essential mediators of damage to cell structures, including membranes, lipids and proteins and nucleic acids.

Oxygen Free Radicals (OFR) are continuously generated in cells exposed to an aerobic environment. Antioxidant defense systems have co-evolved with aerobic metabolism to counteract oxidative damage from OFR.[131] Despite the antioxidant defense, OFR-related damage of proteins and DNA accumulates during life and has been postulated to lead such age dependent diseases as atherosclerosis, arthritis, neurodegenerative disorders and diabetes. The oxidative stress increases ROS and RNS.[132] The reactive oxygen species include charged species such as hydroxyl and superoxide radical and unchanged species hydrogen peroxide and singlet oxygen. The potential sources of oxidative stress in diabetes strength include auto oxidation of glucose, shrink tissue concentration of low molecular mass antioxidant. The ROS generated by increase glucose level is linked to high glucose and other metabolic irregularity significant to the development of diabetic complications.[133] In the past

few decades, increase the evidence has connected oxidative stress to variety of pathological condition include cardiovascular diseases, chronic inflammatory disease, ischemic organ damage, drug toxicity, arthritis and diabetes mellitus.[134] The source of oxidative stress is cascade of ROS leaking from mitochondria. This process has been associated with β- cells of the pancreas and the onset of type 1 diabetes mellitus via the apoptosis cell death of pancreatic beta cells, and the onset of type 2 diabetes provide insulin resistance. The underlying mechanism in onset of diabetes is complex, because of the increase the glucose level could be due to the cause effective relationship of increased oxidative stress.[135]

In the DPPH method, the data observed for the extracts revealed that extracts were likely to have the effects of scavenging free radicals. From the results we observed that dose dependent in DPPH radical scavenging activity. ABTS radical scavenging activity, crude ethanolic extract of *Barleria noctiflora* showed the less IC_{50} values compared to other methods. The radical scavenging activity showed a direct role of its phenolic compounds in free radical scavenging. In hydrogen peroxide method, the extract of *Barleria noctiflora* demonstrated potent antioxidant activity. In the lipid peroxidation method, the extracts of successive and crude ethanolic extracts exhibit potent activity compared to other extracts. The extracts showed potent scavenging activity by inhibition of p-NDA. The alkaline DMSO method, the extracts moderately inhibits the superoxide radical generation. The phenolic compounds of extracts may be responsible in scavenging radical activity.

Among the different extraction of plant tested for *in vitro* antioxidant activity using different methods, the crude ethanolic extract of *Barleria noctiflora* was found to be more potent in DPPH, ABTS, hydrogen peroxide, lipid peroxidation, p-NDA

and alkaline DMSO hydroxyl radical methods. A large number of phenolic compounds are known to possess potent antioxidant activity. The preliminary phytochemical tests of the extract indicated the presence of flavonoids, tannins and phenolic compounds. The total phenol content of the plant ethanolic extract was found to be higher than all the other extracts. In the *in vitro* antioxidant studies, the ethanol extract of *Barleria noctiflora* exhibited potent antioxidant activity. Hence, the study of *in vitro* antioxidant activity shows a potent effect in ethanol extract and it was selected and fractioned with different solvents, from the ethyl acetate and n-butanol fractions were tested for *in vitro* anti diabetic activity.

The *in vitro* α-amylase and α- glucosidase inhibitory studies established EAFBN had inhibitory activity of intestinal digestive enzyme. The percentage inhibition showed a concentration dependant reduction. These enzymes are accountable in hydrolyzing dietary starch into maltose and then split down into glucose prior to absorption. Since α-amylases an important role in the breakdown of starch in human beings and animals, the presence of such inhibitors in food material may be responsible for impaired starch digestion. The α-amylase inhibitor may be valued as novel therapeutic dietetic agents.[136]

Acarbose like drugs, that inhibit α- glucosidase present in the small intestine epithelium, have been verified to decrease postprandial hyperglycemia and improve damaged glucose metabolism without promoting insulin secretion in type 2 diabetic patients. These medications are useful for people who have blood glucose levels are above the level considered serious for diabetes[137] and also useful for people taking sulfonyl urea medication. Therefore, the breaking and delay of carbohydrate absorption with a natural based α-glucosidase inhibitor offers a prospective

therapeutic approach for the management of type 2 diabetes mellitus. Hence EAFBN was potent inhibition of α-amylase and α- glucosidase intestinal digestive enzyme; it may be the beneficial effect of the type 2 diabetes.

In present times, growing and increasing interest in herbal medicines, Consequently herbal medicines have established greater attention as an alternative to clinical therapy leading to increasing demand.[138] The exclusive use of herbal drugs, prepared and dispensed by unscientifically trained herbalists, for treatment of diseases is very common in some rural communities. The experimental screening method is important in order to ascertain the safety and efficacy of herbal products as well as to establish the active component of these herbal remedies. The use of herbal medicines is gradually more popular nowadays, the majority of herbal products is marketed without reliable scientific evidence and mandatory safety and evaluation of toxicity in most countries due to the evidence regarding side effects of herbal medicine.[139] FDA has published about guidance documents controlling the herbal products. There is a rising demand of toxicological evaluation of herbal medicines. *In vivo* studies are considered as the testing of standard in toxicology. However, have arise the ethical issues, particularly in the case of chronic and subacute toxicity testing.

The rats were used in the toxicity study as *Barleria noctiflora* is taken mainly by humans for its aphrodisiac effects. So no death was recorded in the acute toxicity study and no moderate changes in animal behavior. Based on our toxicity study, the oral LD_{50} for ethyl acetate fraction of *Barleria noctiflora* (EAFBN) was more than 2000 mg/kg.

The subacute study was observed no changes of body weight and organ weight at all doses, so the EAFBN can be claimed to be non-toxic. The prolonged exposure to elevated glucose levels lead to toxic effects in a variety of cell types, there is a strong relationship between chronic hyperglycemia and impaired function of the vascular endothelium, with particular damage observed in retinal endothelial cells and renal glomerulus. Managing elevated glucose levels is crucible to reduce the risk of diabetes mellitus related complications.[140] The significant reduction of serum glucose level, there might be due to the presence of hypoglycemic components in the EAFBN. This observation gives credence to the use of the herbal product as a hypoglycemic agent.

Many studies have indicated an important predictive role of increased serum triglyceride, low HDL-Cholesterol and raise LDL-Cholesterol levels regularly co-exist, which are important factors for Coronary Heart Disease (CHD).[141] The postprandial metabolism of lipids is particularly in patients with hypertriglyceridemia. It has been shown to cause endothelial dysfunction and predisposing to atherogenesis.[142] The significant decreases were found between the levels of total cholesterol, triglyceride and LDL-Cholesterol, hence HDL-Cholesterol level was significantly increased. So the EAFBN fraction showed prevent and reduced the risk factors of CHD and lipids induced disorder like diabetes, atherogenesis etc.

The liver performs multiple diverse functions essential to life, synthesis, excretion and detoxification. The liver function tests are providing useful information about the presence and severity of hepatobiliary injury or impairment of liver function. The biochemical parameter is in liver function test are AST and ALT,

etc. The serum ALT & AST are responsible for the integrity of the hepatocytes and sensitive index of hepatocellular damage. The liver parameter of AST was showed no significant changes but the ALT level were significant changes, but the values are in between of the normal values. So the fraction had some beneficial effects by reducing cardiovascular risk factors**.** This specific enzyme for liver injury usually peaks for one or two days only and then drops back to basal level.[143] In a study on the liver enzymes, the fraction were not damaged the liver cell. *Barleria noctiflora* was found to have no effects on the human Cytochrome P450 system[144] and fraction may contain compounds which are non toxic. There is no significant change haematological parameter like RBC, WBC, Hb and PCV. So the result reveals, there was no pathological indication in toxicity study. Histopathological studies of EAFBN treated animal, no degenerative changes and exhibited normal architecture with glomerular and tubular structures in the kidney. The histology of liver exhibited normal liver parenchyma with no distortion of architecture, with normal lobules, acini, and no bile stasis kuffer cell prominence. Overall, these findings support the hypothesis of EAFBN reduce the risk of toxicity in the long duration of drug administration and reduce the risk of diabetes and cardiovascular patients.

The pancreas is a mixture of exocrine and endocrine tissue. The predominant exocrine part consists of graph like clusters secretary cells that form sacs known as acini, which connect to ducts that eventually empty into duodenum. The smaller endocrine part consists of isolated island of endocrine tissue, the islet of langerhans, which are dispersed throughout the pancreas. It synthesizes a number of components, including glucagon (α cell), insulin (β cell), somatastatin and pancreatic polypepdide; these hormones are involved in the regulation of plasma glucose level. Some chemicals were used to induce the diabetes, namely alloxan

monohytrate and streptozotocin. Both inducing drugs are acting on destruction of β cells, but the alloxan monohydrate in the pancreas is preceded rapid uptake by β cells and formation of reactive oxygen species. However, the liver and other tissues are more resistant to reactive oxygen species. This resistance protects against alloxan toxicity, so now a days are not used to induce the diabetes. Hence streptozotocin is used to induce the both type 1 and type 2 diabetes. As a human type 2 diabetes mellitus, diet has a great influence on the development of overt diabetes as well as hypertension, hyperlipidaemia and eventually nephropathy in experimental models.[145] So in the present study, rats were fed with high fat diet and then induced with low dose of streptozotocin; high fat diet fed rats showed a higher plasma lipid profile compared to normal rats. The clinical symptoms of type 2 diabetes rats are closer to those of diet and obesity related diabetes.

Type 2 diabetes mellitus is now taking its place as one of the main threats to human health in the twenty first century.[146] The impact of type 2 diabetes mellitus is increasingly felt around the world, with its prevalence rising dramatically over recent decades. The WHO estimated that there were 150 million people aged 20 years and older living with diabetes in 2000 and this will have risen to 300 million in 2020. There will be a 42% increase; from 51 to 72 million, in developed countries and a 170% increase, from 84 to 228 million, in developing countries.[147] In the past two decades, there have been several important developments, which have significant impact on the definition of diabetes and thereby on the assessment of its magnitude.

The ability of the body to utilize glucose may be ascertained by measuring its glucose tolerance. Oral glucose tolerance test is indicated by the nature of the blood

glucose curve following administration of glucose. When the rats were subjected to OGTT, blood glucose level was found to be raised with time and was gradually decreased until 120 min in control rats. Treatment with EAFBN significantly developed glucose tolerance, as indicated by a reduction in the peak blood glucose level. It's indicating increased glucose tolerance due to augmented glucose transport and utilization, which might be due to potentiating of insulin and augmented glucose transport.

During the 28-day experimental period the body weight was reduced in HFD-STZ induced diabetic rats. STZ treatment induces body weight loss related to diabetes severity. The decrease body weight might be due to the low utilization of uptake blood sugar in the cell. While there was a significant gain of body weight in EAFBN treated diabetic rats. The administration of EAFBN restores body weight seems to be a result of its facility to reduce hyperglycemia.[148] Diabetic rats treated with EAFBN 400 mg/kg showed an enhanced body weight compared to diabetic control. This may be due to the shielding effect of the extract in managing muscle wasting i.e. reversal of gluconeogenesis.[149]

The liver plays a central and a crucible role in the carbohydrate regulation metabolism. Its normal performance is necessary for the maintenance of blood glucose levels and continuous supply of organs that require glucose as a source of energy. An appreciation role of the liver regulation of carbohydrate homeostasis is necessary to understand the many biochemical and physical alterations. In the liver presence of diabetes to understanding how liver disease may affect glucose metabolism. It utilizes glucose as a fuel and then to store as glycogen and synthesize (gluconeogenesis). The majority of the absorbed glucose is retained by the liver, so

rises in peripheral glucose concentration reflects only minor component of postprandial absorbed glucose. Hence liver plays a possible and more significant role than peripheral tissue in the controlling of systemic blood glucose, most absorbed glucose is not taken up the liver, but is metabolized via glycolysis in the peripheral tissues.[150]

In the study of 28 days in every 7 days monitoring the blood glucose level of diabetic animal was significantly increasing the blood glucose level compared to normal animal. The increase level of glucose to the concept of glucose toxicity, which envisions chronic exposure to abnormality, high levels of glucose over as a pathogenic force leads to a toxic effect on β-cells. Hence EAFBN treated animals were significant reduction in all the doses. So the EAFBN drug produces beneficial effects in the controlling of blood glucose level, its efforts to stimulate the β-cells promote by insulin, and the metabolites are involved ATP, NADH and glucose -6-phosphate respond to regulation of carbohydrate metabolism.

Lipid abnormalities are common in people with type 2 diabetes and are a major contributor to the increase in cardiovascular risk. In type 2 diabetes elevated total and LDL-cholesterol, additional diabetes related abnormalities like elevated triglycerides and reduce HDL-cholesterol. Insulin resistance and central obesity are two closely linked factors that are important determining in type 2 diabetes mellitus. Impaired insulin action allows greater free fatty acid release from an increase mass of intra abdominal adipose tissue, promoting hepatic triglyceride synthesis at the same time lipoprotein lipase activity, therefore triglyceride clearance is reduced by insulin resistance.[151] Reduction of HDL cholesterol levels has been attributed to triglycerides enrichment by cholesterol ester transfer protein and increase of hepatic

triglyceride lipase activity, related to insulin resistance. The lipid profile of EAFBN drug treated animals were significant changes in diabetic animals. It may valuable effect of diabetic patients in Coronary Heart Disease (CHD). The cholesterol, triglyceride and LDL cholesterol levels were significantly reduced and increase HDL- cholesterol level deals with no risk of atherosclerotic vascular disease and CHD. These results indicate that EAFBN has hypoglycemic and hypolipidemic effects on the diabetic rats. The hypolipidemic activity might be due to the increase in insulin secretion which ultimately led to inhibition of lipolysis and prevention of dyslipidemia. The lipid lowering activity of EAFBN might be due to the multiple therapeutic benefits such as potentiating of insulin, prevention of cholesterol accumulation and inhibition of lipid peroxidation.

Liver dysfunction is a known association with diabetes. Liver Function Tests (LFT) is indices of hepatic structure, cellular integrity and function. The LFT based on the measurements of blood components that give an idea of existence, extent and type of liver damage. The main biochemical parameters are in LFT Alanine Aminotransferase (ALT), Aspartate Aminotransferase (AST) and Alkaline Phosphatase (ALP). The ALT and AST were indicating the integrity of the hepatocytes and sensitive index of hepatocellular damage. The ALP are indicating the indices of cholestasis and blockage of bile flow.[152] AST is high in heart muscle, liver and skeletal muscle, hence damage tissues are released the AST in blood. The AST levels are elevated in blood generate CHD, chronic hepatocellular disease, hepatitis, cirrhosis and acute hepatic obstruction. The ALT is found mainly in liver, less quantity in heart, skeletal muscle and kidney. The ALT level was more in the liver specific than AST; therefore ALT levels are sensitive and specific indicator of liver disease.[153] The elevation of ALT was associated with the onset of diabetes and

a gluconeogenesis enzyme whose gene transcription is suppressed by insulin. The impairment of insulin signaling rather than purely hepatocytes injury, hence found elevated ALT is a risk factor for type 2 diabetes mellitus. The ALP is highest in liver, biliary tract, bone and placenta. The plasma ALP level is used to detect the disorders in liver and bone.[154]

The hepatic tumors and hepatitis may cause the elevation in serum ALP level. In the present study, the HFD-STZ treated animals showed a significant increase in the levels of ALT, AST and ALP in serum, when compared to the normal animals. The treatment with the EAFBN caused a significant reversal of these values towards the normal, indicating stabilization of plasma membrane as well as repair of hepatic tissue damage caused by HFD-STZ toxicities. The stabilization of these enzyme levels by the EAFBN was a clear indication of the improvement of the functional status of the liver. The results produced suggest that pharmacological effective may find therapeutic applications in the management of hepatic disorders in addition to their insulin secretogogue and anti hyperglycemic effects. So the EAFBN was accounted to no risk factors CHD, liver damage and bone disorder of type 2 diabetic patients.

Insulin is a precursor molecule, of preproinsulin, which is proinsulin further maturation into insulin. Insulin is metabolized by insulinase in the liver, kidney and placenta. About 50% of insulin secreted by the pancreas β-cells and its removed by first pass extraction in the liver. It promotes glycogenesis in the liver and glycogenolysis. It also inhibits hepatic gluconeogenesis, stimulate glycolysis and inhibit ketogenesis.[155] In the present study the HFD-STZ treated animals showed a significant decrease insulin level in serum evident of diabetic rats had increased the

glucose levels and decrease insulin level, when compared to the normal animals. The treatment with the EAFBN caused a significant reversal of insulin level indicating the anti- hyperglycemic effect through insulin secretion from the remnant and regenerate β-cells or due to increased peripheral glucose utilization.[156]

HbA1c can be used as a diagnostic test for diabetes providing that stringent quality assurance tests are standardized to criteria aligned, which preclude its accurate measurement. The fasting plasma glucose levels were performing an OGTT and day to day variability in glucose, hence HbA1c has now recommended by an international committee, although it gives equal or almost equal sensitivity and specificity to fasting or post load glucose measurement.[157] Whether HbA1c is better for predicting the development of microvascular complications. In the present study HFD-STZ induced diabetic rats were shown the increase the HbA1c level than control. The treatment of EAFBN rats was shown significant decreases towards normal, its indicated the therapeutic effect of the preventing secondary microvascular complications of diabetes. It is a strong predictor of no risk cardiovascular risk factor, cardiovascular events and strokes in diabetic patients.[158]

When the blood sugar is increased, it can stress on kidney causing serious damage to vascular, leading to kidney disorder. The kidney excretes metabolic waste products and regulates the serum level of various substances. Serum creatinine and urea level change inversely with changes in glomerular filtration rate and are helpful in gauging the degree of renal dysfunction. Serum creatinine may provide as a surrogate marker of muscle mass and potential relationship between low serum creatinine and type 2 diabetes mellitus.[159] The Blood Urea Nitrogen (BUN) and creatinine levels are helpful in attaining the BUN to creatinine ratio. As kidney fail,

BUN levels will increase, as well as level of creatinine in blood, it tends to indicate a more chronic condition of kidney damage. In the present study, diabetic rats significant increase the serum creatinine and urea level, it shows the renal damage and frequent urine output. An EAFBN treatment rat shows the significant reduction of serum creatinine and urea level. The fall in creatinine level may be due to increased creatinine clearance indicating the normal glomerular filtration rate. It reveals that, there are no changes in kidney function and its preventing diabetic nephropathy and hyperglycemia induced oxidative stress.

The liver glycogen synthesis is impaired in diabetes due to defective glycogen synthase activation. In chronic diabetes, glycogen accumulation and postulated long standing insulin deficiency, it may facilitate synthase activity. The glucose uptake of the diaphragm was studying the effect of glucose transporters. The diabetic rats were GLUT4 content decreased; expression is down regulated, there is an insulin deficiency.[64] In diaphragms of diabetic rats, insulin did not restore glucose utilization. GLUT4 is the regulation of insulin, glucose transporter found primarily in adipose tissue and striated muscle. In the present study increase the glycogen in the liver can be brought about by an increase in glycogenesis and decrease in glycogenolysis. Hemidiaphragms taken from rats treated with EAFBN showed a significant enhancement of the glucose uptake and significant inhibitory effect on glucose transport (glycogenolysis) in liver compared to diabetic control.

In histopathological studies of the pancreas, STZ administration results in DNA damage in β-cells of pancreatic islets due to its potent alkylating properties.[160] Histopathologically the damage was seen in untreated diabetic rats. There was the regeneration of β-cells in EAFBN and glibenclamide treatment groups since the

cells recovered from the initial injury. The regeneration of the β-cells destructed by STZ are probably due to the fact that pancreas contains stable cells which have the capacity to regenerate. Therefore, the surviving cells can proliferate to replace the lost cells.[161]

Wound healing is a highly complex physiological process involving different phases such as contraction, epithelialization, granulation, collagenation, etc. Wound healing deficits in diabetes are multifactorial, complex and inter related. This defect was believed to cause by impaired flow of blood and release of oxygen from elevated blood glucose, reduce collagen and fibronectin synthesis from protein malnutrition, impaired immune and cell defenses. Collagen, keratin and fibrin accumulate glycation which affect binding of regulatory molecules, proteolysis and decrease the ability for protein linkage.[162] The hyperglycemia affects the whole range of neutrophil functions, which include migration, chemotaxis, adherence and phagocytic bactericidal activity.[163] In the present investigation the EAFBN promotes significant wound healing activity by increasing proliferation, formation of granulation tissue, synthesis of collagen and increase the rate of wound contraction compared with diabetic control. In incision wound study increase the breaking strength was indicative of improved collagenation, which significantly contributes to better effective wound healing.

In all the above studies, the EAFBN may cause first person to evaluate the toxicity studies, hypoglycemic effect and wound healing activity. Based on all these studies, it can be concluded that EAFBN posses strong hypoglycemic, antioxidant and wound healing activity. The preliminary phytochemical studies of *Barleria noctiflora* indicate the presence of some phytoconstituents like flavonoides,

alkaloids, saponins, steroids, glycosides, tannins, phenols etc. The observed hypoglycemic, antioxidant and wound healing activity might be due to the presence of any of these compounds. Plant constituents may be isolated and used as starting materials for the synthesis of modified structures or they may serve as model for biologically active compounds in natural products synthesis.

The ethyl actate fraction of *Barleria noctiflora* was subjected to column chromatographic separation to isolate its phytoconstituents.

The compound may be a flavonoid, aromatic, unsaturatyed, oxygenated ketonic. From the spectral data the compound was identified as Acyclic α, β unsaturated ketone this is the first report on the isolation of *Barleria noctiflora*. Due to the small quantities of the isolated compounds mass spectra was not performed and structure could not be confirmed and they were not tested further for any of the biological properties. The observed potent biological properties of the plant extracts may be due to the presence of any of these compounds flavonoides, alkaloids, saponins, steroids, glycosides, tannins, phenols. However, our efforts to isolate the flavonoids and phenolic compounds present in the extract were not successful due to the high polar nature of the compounds.

9. SUMMARY AND CONCLUSION

The herbal medicines are the major remedy in traditional medicinal system and are being used in medicinal practice for thousands of years. These have made a great contribution in maintaining human health. Diabetes mellitus is a metabolic disorder of hyperglycemia with disturbances of carbohydrate, lipid and protein metabolism resulting from defects of insulin secretion, insulin action and both. Management of diabetes mellitus without any side effects is still a challenge to the medical system. This is the increasing demand by patients to use natural products with hypoglycemic activity, because insulin and oral hypoglycemic drugs have undesirable side effects. Medicinal plants are good sources of natural antioxidants believed to exert their effects. In order to identify the herbal based plant drug, the present study was focused on pharmacognostical, phytochemical and pharmacological studies of *Barleria noctiflora.*

Barleria noctiflora was collected during the winter season in and around Erode District, Tamilnadu, India. It was identified and authenticated by Prof. P. Jayaraman, Director, National Institute of Herbal Science, Chennai-45, Tamilnadu, India.

This is the first report of pharmacognostical studies on the leaf and stem parts of *Barleria noctiflora.* Pharmacognostical studies that microscopical, macroscopical, physicochemical constituents and fluorescent analysis were carried out; these are valuable tools for selected plant and identification of powdered drugs.

The dried plant materials were subjected to extraction by Soxhlet process separately. All the extracts were concentrated to dryness under reduced pressure and controlled temperature. The ethanol extract was further fractionated with ethyl

acetate and n-butanol. The extract was used to *in vitro* antioxidant and the fractions were used *in vitro and in vivo* pharmacological studies.

The phytochemical studies of extracts showed the presence of flavonoides, alkaloids, saponins, steroids, glycosides, tannins, phenols, protein and carbohydrates in various extracts. The crude drugs of powder presence of vitamin C, vitamin D and inorganic elements like sodium, iodine, phosphate, and chloride.

The total phenolic and flavonoids content of *Barleria noctiflora* of crude ethanolic and aqueous extract was obtained using the regression calibration curve and is expressed as tannic acid and rutin equivalents. The total phenolic and flavonoids content was higher in the ethanolic extract compared to the aqueous extract. It shows the ethanolic extract of *Barleria noctiflora* posse's high antioxidant ability.

The IC_{50} values were found to be more than 100 µg/ml for all the extracts indicating the nontoxic nature of the extracts. Among, the five extracts of *Barleria noctiflora* tested for *in vitro* antioxidant activity, the ethanol extract of *Barleria noctiflora* exhibited potent antioxidant activity when compared to other extracts.

Hence, the study of *in vitro* antioxidant activity shows potent in ethanol extract selected and fractioned with different solvents from the ethyl acetate and n-butanol fractions were tested for *in vitro* antidiabetic activity by inhibition of α-amylase and inhibition of α-glucosidase. The results were expressed as IC_{50}, which is the concentration of the fraction to inhibit enzyme by 50%. The *in vitro* α-amylase and α- glucosidase inhibitory studies demonstrated EAFBN had inhibitory activity of intestinal digestive enzyme. Hence, ethyl acetate fraction of *Barleria noctiflora*

(EAFBN) was selected for *in vivo* evaluat of acute toxicity, sub acute toxicity, anti diabetic activity and wound healing activities.

In acute toxicity studies, the LD_{50} was determined as per OECD guidelines for fixing the dose for biological evaluation. The LD_{50} of the ethyl acetate fractions of *Barleria noctiflora* (EAFBN) falls under category 4 values with no death and no signs of acute toxicity at doses of 2000 mg/kg.

The subacute studies, the treatment of EAFBN 100, 250 and 500mg/kg body weight doses was no changes of body and organ weight was observed at all doses. The significant ($p<0.01$) reduction of serum glucose level, total cholesterol, triglyceride and LDL-Cholesterol, hence HDL-Cholesterol level was significant ($p<0.01$) increases. There might be due to the presence of hypoglycemic components in the ethyl acetate fraction of *Barleria noctiflora*. The liver parameter of AST was showed no significant changes and the ALT level was significant ($p<0.01$) changes, but the values are in between of the normal values. There is no significant change in hematological parameter like RBC, WBC, Hb and PCV. This observation gives credence to the use of the herbal product as a hypoglycemic agent and showed reduced the risk factors of CHD. So the result reveals, there was no pathological indication in toxicity study.

Histopathological studies of EAFBN treated 100, 250 and 500 mg/kg body weight doses of animal no degenerative changes and exhibited normal architecture with glomerular and tubular structures in the kidney. The histology of liver exhibited normal liver parenchyma with no distortion of architecture, with normal lobules, acini, and no bile stasis kuffer cell prominence. Overall, these findings support the hypothesis of EAFBN reduce the risk of toxicity in the long duration of

drug administration and reduce the risk of diabetes and cardiovascular patients. The treatment with the EAFBN at the dose of 500 mg/kg body weight showed the normal structure of kidney and liver tissue.

In an OGTT, the EAFBN doses of 100, 200 & 400 mg/kg have shown increase the tolerance for glucose and blood glucose levels were significantly reduced in the dose dependent after oral administration (2g/kg). The EAFBN tested drug showed significant activity, according to the dose dependent manner.

In hypoglycemic activity, the decrease body weight might be due to the low utilization of uptake blood sugar in the cell. While there was a significant ($p<0.01$) gain of body weight in EAFBN treated diabetic rats when compared to diabetic control. The administration of EAFBN restores body weight seems to be a result of its facility to reduce hyperglycemia.

The treatment with the EAFBN significant ($p<0.01$) decrease in blood glucose level was observed in HFD-STZ induced diabetic rats, when compared to diabetic rats. EAFBN at 100, 200 and 400 mg/kg caused a significant ($p<0.01$) reduction in serum lipid profile of total cholesterol, triglycerides, LDL- Cholesterol and significant ($p<0.01$) increase in HDL-Cholesterol level was observed when compared to diabetic rats. The liver parameters were ALT, AST and ALP significant decrease in EAFBN treated rats when compared to diabetic rats.

A significant ($P<0.01$) increase the level of insulin and significantly decreases the level of HbA1c was observed in EAFBN (100, 200 and 400mg/kg) body weight dose when compared to diabetic control. The renal parameters like serum urea and creatinine level shows significant ($p<0.01$) decrease was observed in for EAFBN (100, 200 and 400mg/kg) body weight dose when compared to diabetic

control. There was a marked significant ($p<0.01$) reduction in the liver glycogen levels of diabetic rats and EAFBN (100, 200 and 400mg/kg) treatment showed significant ($p<0.01$) increase in liver glycogen levels when compared with the diabetic rats.

The glucose uptake by hemidiaphragms showed a significant reduction of diabetic rats when compared to control group. The glucose uptake were significant ($p<0.01$) enhancement of rats treated with EAFBN (100, 200 and 400mg/kg), when compared to diabetic control. The glucose transport showed a significant ($p<0.01$) enhancement in diabetic rats as compared to normal rats. Also, both EAFBN (100, 200 and 400 mg/kg) treated rats showed a significant ($p<0.01$) inhibitory effect on glucose transport in liver tissue. In histopathology of the pancreas, β-cells of pancreatic islets were damage in diabetic animals. There was the regeneration of β-cells in EAFBN treatment groups since the cells recovered from the initial injury. The treatment with the EAFBN at the dose of 400 mg/kg body weight showed slightly normal structure of pancreas tissue. These results suggest the potent hypoglycemic nature of the EAFBN and support the CHD risk and other complications in type 2 diabetes.

The EAFBN was assessed for the wound healing activity in rats using excision and incision wound models. The percentage closure of the excision wound area for the control rats was found to be 15.35±0.07 to 90.1±0.27 between 4 to 16 days of the experiment. The treatment with the EAFBN at 5% w/w and 10% w/w showed a percentage closure, 19.5 ± 0.42 to 95.8 ± 0.27 and 19.5 ± 0.18 to 96.6 ± 0.10 between 4 to 16 days of the treatment. All the values were found in significant ($p<0.01$), when compared to diabetic control animals. The complete

epithelization was observed in the same day of EAFBN (10% w/w) and standard drug, hence diabetic control rats (23.66 ± 0.55) were 5 days increase the period of epithelization compare to normal control (18.83 ± 0.16).

In the incision wound model, the animal treated with the topical application of EAFBN 10% w/w treated animals skin breaking strength and standard drug have shown more or less same skin breaking strength. All the values were found in significant ($p<0.01$), when compared to diabetic control animals. In the present investigation the *Barleria noctiflora* fraction promotes significant wound healing activity by increasing proliferation, formation of granulation tissue, synthesis of collagen and increase the rate of wound contraction compared with diabetic control. Based on the results, we can conclude the plant of *Barleria noctiflora* could be used for diabetes and to prevent the complications in type 2 diabetes.

The preliminary phytochemical tests of the extract indicated the presence of flavonoides, alkaloids, saponins, steroids, glycosides, tannins. Many of these are known to possess antioxidant, hypoglycemic and wound healing activities. These activities observed in the fraction may be due the presence of flavonoids, tannins and phenolic in the EAFBN fraction.

The ethyl acetate fraction of *Barleria noctiflora* was subjected to column chromatography and isolation of active constituents. The compound was isolated and their purity was confirmed by TLC using different solvent systems. Based on the spectral analysis viz, IR, NMR and UV spectra, they were identified as Acyclic α, β unsaturated ketone.

In conclusion, the present study provides the phytochemical and biological investigation of the *Barleria noctiflora* plant. The plant extracts were shown to possess *in vitro* antioxidant properties. Among them, the ethanolic extract *Barleria noctiflora* was found to be most potent. The *in vivo* studies carried out ethyl acetate fraction of *Barleria noctiflora* rats proved the potent hypoglycemic activity. Detailed biochemical and histological evidence was provided to prove the activity. The ethyl acetate fraction of *Barleria noctiflora* was also found to possess *in vivo* wound healing activity in excision and incision models. In the phytochemical analysis, Acyclic α, β unsaturated ketone compound was isolated.

10. IMPACT OF THE STUDY

Objectives achieved

1. The present study for the first time pharmacognostical studies carried out to demonstrate the *Barleria noctiflora* for selection and identification.
2. Potent *in vitro* antioxidant activity observed in plant extracts in several methods
3. Potent *in vitro* antidiabetict activity observed in plant fractions.
4. This is the first time acute and subacute toxicological studies were observed in plant fraction, under the study did not exhibit any toxicity to the animal models.
5. The potent *in vivo* hypoglycemic activity in ethyl acetate fraction of *Barleria noctiflora* were proved undoubtedly based on the biochemical and histological evidences. The plant extract can be used in formulations containing such principles.
6. The study also proved the *in vivo* wound healing activity of ethyl acetate fraction of *Barleria noctiflora* against diabetes.
7. First time, Acyclic α, β unsaturated ketone compound was isolated from this plant

Scope for further research

There is further scope for research in isolating the other phytoconstituents and to carry out the other biological properties of the extract and its phytoconstituents. There is also a need to establish the mechanism of the observed activities. Further mass spectra of this compound may be obtained for confirmation of this compound structure.

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JKK Munirajah Medical Research Foundation College of Pharmacy
Committee for the Purpose of control and Supervision of Experiments on Animals (CPCSEA)
Institutional Animal Ethics Committee (IAEC)

CERTIFICATE

Name of the Investigator	: A. Suresh
Title of the Project	: Investigation of phytochemical and biological properties of Traditional medicinal plants for hypoglycaemic activity.
Proposal Number	: JKKMMRFCP/IAEC/2013/013
Date received after modification (if any)	: —
Date received after second modification	: —
Approval date	: 02/03/2013
Animals	: Wistar Rats / Albino Mice / Rabbits / Guinea Pigs
No. of animals requested	: 142 Rats, 54 Mice
No. of animals sanctioned	: 142 Rats, 54 Mice
Sex	: Male / Female - (For Mice) (Both sex for rat)
Expiry Date (Termination of the Project)	: 09/03/2014
Name of IAEC chairperson	: Dr.N.Senthil Kumar, M.Pharm., Ph.D Principal

CPCSEA Nominee.

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Poultry Disease Diagnosis & Surveillance Laboratory,
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Signature of Chairperson

Chairperson
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AUTHENTICATION CERTIFICATE

Based upon the organoleptic /macroscopic / microscopic examination of fresh / market sample, it is certified that the specimen given by..Mr. A. Suresh, Asst. Prof. Dept of pharmacology, JKKMMRF College of pharmacy, B. Kamarapalayam, Namakkal- 638183. is identified as below :

Binomial:.....Barleria noctiflora L.....

Family:.....Acanthaceae.....

Synonym(s):.....

.....

Regional names: .....

.....

Reg.No of the certificate:.....PARC/2011/1015.....

References : Nair, N.C & Henry, A.N. Flora of Tamilnadu, India 1: —— .1983.

Henry, A.N. *et al.* Ibid. —— II: P:142 .1987.

Ibid. —— III: —— .1989.

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RESEARCH PUBLICATIONS

Papers Published

1. **Suresh Arumugam, Senthilkumar Natesan**. Pharmacognostical Studies and Phytochemical Investigation of *Barleria noctiflora* Linn (Acantheceae). *International Journal of Pharmacognosy and Phytochemical Research*. 2015; **7(3);** 450-456. (Impact factor – 1.341).

2. **Suresh Arumugam, Senthilkumar Natesan**, Sekar Ganesan, Sumathi Kanagarajan. *In vitro* screening of various extract of *Barleria* noctiflora for their antioxidant and free radical scavenging activity. *International Journal of Pharmaceutical and Phytopharmacological Research*. 2015; **5(2):** 1-9. (Impact factor – 0.852).

Papers communicated

1. Suresh Arumugam, Senthilkumar Natesan. Hypoglycemic effects of *Barleria noctiflora* fractions on high fat fed with low dose Streptozotocin induced type-2 diabetes in rats. *International Journal of Pharmacy and Pharmaceutical Sciences.* **Accepted** (Impact factor – 0.55).

2. Suresh Arumugam, Senthilkumar Natesan. Acute and subacute toxicity of *Barleria noctiflora* linn (Acantheceae) ethyl acetate fraction used in treatment of diabetes. *Indian Journal of Pharmacology.*

3. Suresh Arumugam, Senthilkumar Natesan, Sekar Ganesan, Sumathi Kanagarajan. Wound healing activity of ethyl acetate fraction of *Barleria noctiflora* in experimentally induced diabetic rats. *Journal of Pharmaceutical Research.*