"PHYTOPHARMACOLOGICAL EVALUATION OF BARK OF *SYMPLOCOS RACEMOSA ROXB*. FOR ITS ANTI DIABETIC ACTIVITY"

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PHYTOPHARMACEUTICALS AND NATURAL PRODUCTS

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CERTIFICATE

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DECLARATION

I hereby declare that the dissertation entitled **"Phytopharmacological evaluation of the bark of *Symplocos racemosa Roxb*. for its anti diabetic activity"**, *is based on the original work carried out by me under the guidance of Prof. Vimal Kumar, Professor & H.O.D, and Mrs. Nagja V. Tripathi, Assistant Professor, Department of Phytopharmaceuticals and Natural Products, Institute of Pharmacy, Nirma University. I also affirm that this work is original and has not been submitted in part or full for any other degree or diploma to this or any other university or institution.*

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Abbreviations

AChE	Acetyl Cholinesterase
AGEs	Advanced Glycation End Products
BHA	Butylated Hydroxyl Anisole
DM	Diabetes Mellitus
DNA	Deoxy Ribonucleic Acid
ECM	Extracellular Matrix
FSM	Forced Swim Test
GABA	Gama Amino Butyric Acid
GS•	Thiyl Radicals
GSH	Reduced glutathione
HDL	High Density Lipoproteins
HPTLC	High Performance Thin Layer Chromatography
HA•	Alloxan Radicals
ICA	Islet Cells Autoantibodies
IL	Interleukins
LDL	Low Density Lipoprotein
Ng	Nano Gram
μg	Micro Gram
μl	Micro Litre
MIC	Minimum Inhibitory Concentration
NADPH	Nicotinamide Adenoside Diphosphate
NO	Nitric Oxide
PKC	Protein Kinase C
PPAR	Peroxisome Proliferator Activated Receptors
ROS	Reactive Oxygen Species
VEGF	Vascular Endothelial Growth Factor
VLDL	Very Low Density Lipoproteins
STZ	Streptozotocin
TGF β	Transforming Growth Factor β
TNF	Tumour Necrosis Factor
WHO	World Health Organization

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INDEX

CHAPTER NO.		*TITLE*	*PAGE NO.*
A.	**LIST OF TABLES**		
B.	**LIST OF FIGURES**		
C.	**ABBREVIATIONS**		
1.	**ABSTRACT**		1
2.	**INTRODUCTION**		2-12
	2.1	Introduction to Herbal Medicine	2
	2.2	Introduction to Diabetes	4
	2.3	Pathogenesis of Diabetes	9
3.	**AIM & OBJECTIVES**		13
4.	**LITERATURE REVIEW**		14-31
	4.1	Introduction to Plant	14
	4.2	Estimation of Ellagic Acid in Lodhra Bark by HPTLC Analysis	18
	4.3	Pharmacological Activity	18
	4.4	Ethnopharmacology Uses	26
	4.5	Other Herbs Used as Anti Diabetics	27
	4.6	Screening Models for *Symplocos racemosa Roxb*	28
	4.7	Polyherbal Preparations Experimentally Investigated for Anti Diabetics Activity	29
5.	**MATERIALS AND METHODS**		32-42
	5.1	Morphology & Microscopy of Plant	32
	5.2	Physicochemical Parameter	33

	5.3	Preparation of Extracts	34
	5.4	Preliminary Phytochemical Screening	35
	5.5	Estimation of Phenolics and Flavonoids Substances	38
	5.6	Anti Diabetic Activity	39
	5.7	Estimation of Glucose (God-Pod Method)	41
6.	**RESULTS**		43-53
7.	**CONCLUSION**		54
8.	**REFERENCES**		55-64

A. LIST OF TABLES

Table number	Table title	Page number
2.1	Comparison of Type 1 and Type 2 Diabetes.	7
4.1	Medicinal Plants Experimentally Investigated for Anti Diabetic Activity	27
4.2	Polyherbal Preparations Experimentally Investigated for Anti Diabetics Activity	30
5.1	Experimental Plan for Anti-Diabetic Study	40
6.1	Macroscopic Characteristics of the Bark of *Symplocos racemosa Roxb*	44
6.2	Physicochemical Parameters of Bark of *Symplocos racemosa Roxb*	46
6.3	Phytochemical Screening of the Bark of *Symplocos racemosa Roxb*	47
6.4	Total Phenolics and Flavonoids Content of the Bark of *Symplocos racemosa Roxb*	49
6.5	Effects of Glucose Levels on Different Groups	49

B. LIST OF FIGURES

Figure Number	Figure Title	Page Number
2.1	Temporal Model for Development of Type 1 Diabetes	10
2.2	Causative Factors of Diabetes Mellitus	11
6.1	Outer and Inner Dried Bark of *Symplocos racemosa Roxb*	43
6.2	Powder Microscopy of *Symplocos racemosa Roxb*	45
6.3	TLC Analysis of *Symplocos racemosa Roxb*	48
6.4	Comparison Between Groups and Glucose on 7 Days	50
6.5	Comparison Between Groups and Glucose on 14 Days	50
6.6	Comparison Between Groups and Glucose on 21 Days	51
6.7	Comparison Between Groups and Glucose on 28 Days	51
6.8	Comparison Between Days and Glucose Level	52
6.9	Histopathology of Pancreas Section	53

ABSTRACT:

The phytochemical and pharmacological evaluation of *Symplocos racemosa* was carried out for its anti diabetic activity. The present study investigates the anti diabetic activities of methanolic and petroleum ether extract of *S. racemosa* bark in streptozotocin (STZ) induced diabetic rats. The preliminary phytochemical screening of methanolic extract showed the presence of glycosides, flavonoids, tannins, phenols, carbohydrate, alkaloids. The preliminary phytochemical screening of petroleum ether extract showed the presence of phytosterol and triterpenoids. Two doses of methanolic extract (150 and 300 mg/kg) and single dose of petroleum ether extract (100 mg/kg) of bark of *S. racemosa* was administered orally and compared with the standard drug Glibenclamide (0.4 mg/kg). The methanolic extract at the dose of 150 mg/kg showed potent anti diabetic activity. The petroleum ether extract at the dose of 100 mg/kg also showed potent effect. The results of the methanolic extract of the bark of *Symplocos racemosa* showed more potent anti diabetic activity against streptozotocin induces diabetic rats.

2. INTRODUCTION:

2.1 Introduction to Herbal Medicine:

Herbal medicine is still the mainstay of about 75–80% of the world population, mainly in the developing countries, for primary health care because of better cultural acceptability, better compatibility with the human body and lesser side effects. However, the last few years have seen a major increase in their use in the developed world. In Germany and France, many herbs and herbal extracts are used as prescription drugs and their sales in the countries of European Union were around $ 6 billion in 1991 and may be over $ 20 billion now. In USA, herbal drugs are currently sold in health food stores with a turnover of about $ 4 billion in 1996 which is anticipated to double by the turn of the century**.**

The human being exploited to alleviate his suffering from injuries of deceases utilizing plant growing around him. The plant kingdom still hold many species of plant containing substance of medicinal value which have yet to be discovered and the large no. of plant are constantly being screened for their possible pharmacological value in addition to already exploited plants. As the results of modern isolation technique and pharmacological screening procedure, new plant drugs usually find their way into modern medicines. Now a days maximum world' population depends on herbal medicines.

Medicinal plants often contain additional active principles other than the major active principles and physiologically inert substances like cellulose and starch. Unlike the chemical entities, which contains one active ingredient pulps a number of inert substances, which makeup the dosage form (like tablet, capsules and syrups). Indian system of medicines comprises of Ayurveda, Unani, Siddha, Homeopathy, Naturopathy, and Yoga. Each of which uses the herbal constituents in some or the other form, crude drug are not so effective because they have not been tested for efficacy according to rigid pharmacological standards. As the constituents derived from the medicinal plants proved the cure the human.

The constituents having particular therapeutic effect are identified and isolated. Natural product research has lead to a new physiological and pharmacological concept, particular when a new compound is found to have specific biological effects. Unlike synthetic drugs, which are synthesized in laboratory under the controlled conditions; the drug which are obtained from natural sources are showing

variability in concept of active constituents due to one or more of the following reasons : seasonal variability, soil requirements, altitude, rain fall, light, temperature, time of collection.

During the 40 years precise method for the evolution of herbal pharmacopoeia drug & their preparation have been devised based on their source constitution derived from wild and cultivated origin.

In this respect internationally several pharmacopoeia have been providing monograph stating quality parameter and standardization of many herbs and some product made out of these herbs. In India, the herbal drug market is about $ one billion and the export of plant based crude drugs is around $ 80 million**.**

In recent years WHO has emphasized the need to ensure standardization of medicinal plant product by using modern techniques. In some case thought the chemical nature of active constituents is not possible to isolate without changing its chemical integrity then only way to left quality assurance chemist is to standardize it biologically.

General protocol followed for the standardization of herbal materials are mainly authentication, i.e. proper botanical identity, foreign matter content i.e free from soil, stones, dust, insects and other contaminant, organoleptic evaluation, microscopical evaluation, volatile matter, ash value and extractive value and other protocol are like sampling preliminary examination, moisture content, loss on drying, swelling index, R_f values, microbial contamination toxic remedies etc. A Pharmacognostical study of a plant drug enables the scientist for proper identification of the drug and removes adulteration and provides a basis for authentication of the crude drug. The phytochemical investigation of a plant may involve the extraction of plant materials, separation and isolation of the constituents of the interest characterization of isolated compounds. The chemist produces several new compounds every year as potential drugs. This necessitates the development of a "screening program" for initial detection of more qualification as well as classification of biological activity. In standardization or evaluation of herbal drugs, assessment of biological efficacy is found to be most assuming method .In this method requirements are a suitable animal for testing and control, methodology for experiment assessment of result.

Plants have a long history of medicinal use. In countries such as India and China they are a central part of medicinal treatment. Herbalism is the use of plant material, based on tradition and folk lore to treat or prevent disease. A herbal practitioner (or herbalist)

treats by selecting a herb or combination of herbs specific for the set of symptoms of a particular patient. Herbal medicine (phytotherapy, phytomedicine) uses standardized extracts prepared from plants or plant parts for which there is documentation of therapeutic activity. India is one of the 12 mega biodiversity centers having 45,000 plant species; its diversity is unmatched due to the 16 different agro climatic zones, 10 vegetative zones, and 15 biotic provinces. The country has a rich floral diversity. Traditional medicine is the synthesis of therapeutic experience of generations of practicing physicians of indigenous systems of medicine. Traditional preparation comprises medicinal plants, minerals and organic matters etc.

Herbal drug constitutes only those traditional medicines that primarily use medicinal plant preparations for therapy. The ancient record is evidencing their use by Indian, Chinese, Egyptian, Greek, Roman and Syrian dates back to about 5000 years. About 500 plants with medicinal use are mentioned in ancient texts and around 800 plants have been used in indigenous systems of medicine. Indian subcontinent is a vast repository of medicinal plants that are used in traditional medical treatments, which also forms a rich source of knowledge. The various indigenous systems such as Siddha, Ayurveda, Unani and Allopathy use several plant species to treat illness.

In India around 20,000 medicinal plant species have been recorded recently, but more than 500 traditional communities use about 800 plant species for curing different diseases. Currently 80% of the world population depends on plant-derived medicine for the first line of primary health care for human alleviation because it has no side effects. Plants are important sources of medicines and presently about 25% of pharmaceutical prescriptions in the United States contain at least one plant-derived ingredient (Rao, N., et. al., 2011).

2.2 Introduction to Diabetes:

Diabetes mellitus (DM) is a chronic disease caused by inherited and/or acquired deficiency in production of insulin by the pancreas, or by the ineffectiveness of the insulin produced. Such a deficiency results in increased concentrations of glucose in the blood, which in turn damage many of the body’s systems, in particular the blood vessels and nerves (Nagappa, *et al.,* 2003).

Diabetes is a serious illness with multiple complications and premature mortality, accounting for at least 10% of total health care expenditure in many countries (King *et al.,* 1998). Diabetes mellitus is a metabolic disorder characterized by

hyperglycemic, glycosuria and negative nitrogen balance and it is mainly due to lack of insulin secretion in beta cells of pancreas and desensitization of insulin receptors for insulin. It causes number of complications like retinopathy, neuropathy, and peripheral vascular insufficiencies (Chehade., 2000).

Diabetes is one of the most prevalence chronic diseases in the world. This is a chronic incurable condition due to insulin deficiency that affect 10% of the population. The number of diabetic people is expected to rise from present estimate of 150 million to 230 million in 2025. For a long time, diabetes has been treated with several medicinal plants or their extract based on the folklore medicine. Nowadays herbal medicines are highly recommended for the treatment of diabetes inspite of other therapeutic option, which can produce serious side effects & in addition they are not safe during pregnancy. Therefore the search for the more effective and safer hypoglycemic agents has continued to be an important area of active research. Furthermore, after the recommendation made by WHO on diabetes mellitus, investigation on hypoglycemic agent from medicinal plants has become more important. (Kumar, S., et. al., 2008; Venkatesh, S., et. al., 2008.)

Diabetes mellitus (DM) currently is a major health problem for the people of the world and is a chronic metabolic disorder resulting from a variable interaction of hereditary and environmental factors and is characterized by abnormal insulin secretion or insulin receptor or post receptor events affecting metabolism involving carbohydrates, proteins and fats in addition to damaging liver, kidney and β cells of pancreas. (Baynes, JW., 1991). The number of people suffering from the disease worldwide is increasing at an alarming rate with a projected 366 million peoples likely to be diabetic by the year 2030 as against 191 million estimated in 2000. (Wild, SG., et.al., 2004). From literature review it has been revealed that 15 - 20% of diabetic patients are suffering from insulin-dependent diabetes mellitus (IDDM) or type-I. (Wilson, JD., et.al., 1998). In Type-I diabetes mellitus, there is completely destruction of pancreatic β cells and patient is unable to release insulin for maintaining the blood glucose. In Type-II diabetes mellitus pancreatic β cells partially destruct and/or formation of such proteins opposing the insulin action. The IDDM is noted both in adult and child hood. It is characterized by elevation of both fasting and post-prandial blood sugar levels. Chronic hyperglycemia during diabetes causes glycation of body proteins that in turn leads to secondary complications affecting eyes, kidneys, nerves and arteries. (Galadari, EO., et.al., 1993). These may be delayed, lessened or prevented by maintaining blood glucose values close to normal in modern medicine; no satisfactory effective therapy is still available to cure the diabetes mellitus. Though insulin therapy is also used for the management of diabetes mellitus, but there are several drawbacks like insulin resistance, (Piedrola, G., et. al, 2001), anorexia, nausea, brain atrophy and fatty liver after chronic

treatment. Besides the use of insulin for the treatment of insulin dependent diabetes mellitus (IDDM), other approaches for the control of hyperglycemia include the use of amylin analogues which regulate gastric emptying and inhibitors of intestinal alpha glucosidases like acarbose, miglitol and voglibiose which delay postprandial hyperglycemia. Sulphonylureas, the most widely used class of drugs act by closure of ATP dependent channel. Metformin, a biguanide oral antidiabetic limits intestinal glucose absorption. These drugs have certain effects like causing hypoglycemia at higher doses, liver problems, lactic acidosis and diarrhea. It is apparent that due to the side effects of the currently used drugs, there is a need for a safe agent with minimal adverse effects, which can be taken for long durations. Though biguanides and sulfonylureas are valuable in treatment of diabetes mellitus, their use is restricted by their limited action, pharmacokinetic properties, secondary failure rates and accompanying side effects.(Bailey, CJ., et.al., 1989). Moreover, these therapies only partially compensate for metabolic derangements seen in diabetics and do not necessarily correct the fundamental biochemical lesion (Taylor, R., 1988).

Symptoms of Diabetes:

In both types of diabetes, signs and symptoms are more likely to be similar as the blood sugar is high, either due to less or no production of insulin, or insulin resistance. In any case, if there is inadequate glucose in the cells, it is identifiable through certain signs and symptoms. These symptoms are quickly relieved once the Diabetes is treated and also reduce the chances of developing serious health problems.

A. Diabetes Type 1:
   - Nausea and vomiting
   - Ketoacidosis
   - Fast weight loss
   - Dehydration

B. Diabetes Type 2:
   - Increased fatigue
   - Polydipsia
   - Polyuria
   - Polyphagia
   - Weight fluctuation
   - Blurry vision
   - Irritability

Infection
Poor wound healing

Classification:

Diabetes is classified by underlying cause. The categories are: type 1 diabetes—an autoimmune disease in which the body's own immune system attacks the pancreas rendering it unable to produce insulin; type 2 diabetes—in which a resistance to the effects of insulin or a defect in insulin secretion may be seen; gestational diabetes; and “other types”.

Table 2.1: Comparison of type 1 and type 2 diabetes.

	Type 1 Diabetes	**Type 2 Diabetes**
Etiology	Autoimmune	Peripheral resistance
Formerly known as	IDDM	NIDDM
Age of onset	Younger	Older
Obesity	Rare	Common
Family History/ Twin concordance	Rare	Common
HLA association	Yes	No
Ketosis	Yes	No
Insulin resistance	No	Yes
Endogenous insulin	No	Yes
Respond to Oral Agents	No	Yes
Metabolic liability	Labile	Not labile

Type 1 Diabetes:

Diabetes is a chronic disease that occurs either when the pancreas does not produce enough insulin or when the body cannot effectively use the insulin it produces. Type 1 diabetes (previously known as insulin-dependent, juvenile or childhood-onset) is characterized by deficient insulin production and requires daily administration of insulin. More than 220 million people worldwide have diabetes. In 2004, an estimated 3.4 million people died from consequences of high blood sugar. Common consequences of diabetes are heart disease, stroke, neuropathy, retinopathy and nephropathy. 50% of people with diabetes die to heart disease and stroke. After 15 years of diabetes, approximately 2% of people become blind, and about 10% develop

severe visual impairment. 10-20% of people with diabetes die to kidney failure. Diabetic neuropathy affects up to 50% of people with diabetes.

Generally, the deleterious effect of diabetes are through macro vascular complications (coronary artery disease, peripheral arterial disease, and stroke) and micro vascular complications (diabetic nephropathy, neuropathy, and retinopathy). The underlying mechanisms for the development of micro and macro vascular complications are the increased oxidative stress, activation of polyol pathway flux, increased advanced glycation end products (AGEs) formation, increased protein kinase c (PKC) isoforms activation and activation of hexosamine pathway flux.

Increased oxidative stress causes the stimulation of polyol pathway flux, hexosamine pathway flux, increased AGEs formation and activation of PKC isoforms. These all further proceed toward the deleterious effects of hyperglycemia. Reactive oxygen species (ROS) also affects transcription, decreases nitric oxide production and stimulates transforming growth factor-β (TGF- β) (Brawnwald et al., 2005). Stimulation of polyol pathway increases sorbitol formation which eventually induces osmotic stress, decreases Na+/K+ ATPase activity, decreases cytosolic NADPH, activates PKC, decreases glutathione and ↓other antioxidant defenses. These all lead to tissue damage and defined structural changes in the retinal vasculature (Balasubramanyam, Rema & Premanand, 2002).

Increased AGEs modify gene transcription; modify extracellular matrix (ECM) molecules and leads to cellular dysfunction. AGEs increase inflammatory cytokines and growth factors via NFκB (nuclear factor kappa B) production through ROS production and causes vascular pathology (Brawnwald et al., 2005). AGEs deactivate NO leading to coronary vasodilation myocardial inflammation and endothelial dysfunction. In eyes, they cause neovascularization, ECM degradation, formation of micro aneurysms and pericyte loss and chronic hyper permeability (Balasubramanyam, Rema & Premanand, 2002).

Increased PKC activation results in decreased endothelial nitric oxide synthase (eNOS) activity and increased endothelin-1 level causing blood flow abnormalities; increased vascular endothelial growth factor (VEGF) causing vascular permeability and angiogenesis; increased TGF activation causing collagen and fibronectin accumulation leading capillary occlusion and collagen deposition in heart; and increase in plasminogen activator inhibitors (PAIs) leading to vascular occlusion (Brawnwald et al., 2005). It also increases oxidants which causes glomerular sclerosis consequently resulting in increased tubular fibrosis, increased

blood urea nitrogen (BUN) and creatinine and finally results in renal failure (Ohshiro, Lee & King 2005).

Stimulation of hexosamine pathway flux causes increase in PAIs and activation of TGF-β1 leading to vascular and capillary occlusion (Brawnwald et al., 2005). Increased hexosamine directly shows perturbation of the neuroprotective effect of insulin & induction of apoptosis of retinal neurons (Balasubramanyam, Rema & Premanand 2002).

Current treatment for diabetes is not much satisfactory and the identification of new classes of anti diabetic agents is a clinical imperative.

2.3 Pathogenesis of Diabetes:

Individuals with a genetic susceptibility have normal beta cell mass at birth but begin to lose beta cells secondary to autoimmune destruction that occurs over months to years. This autoimmune process is thought to be triggered by an infectious or environmental stimulus and to be sustained by a beta cell–specific molecule.

In the majority of individuals, an immunologic marker appears after the triggering event but before diabetes becomes clinically over. Beta cell mass then begins to decline, and insulin secretion becomes progressively impaired, although normal glucose tolerance is maintained. The rate of decline in beta cell mass varies widely among individuals, with some patients progressing rapidly to clinical diabetes and others evolving more slowly.

Features of diabetes do not become evident until a majority of beta cells are destroyed (~80%). At this point, residual functional beta cells still exist but are insufficient in number to maintain glucose tolerance. The events that trigger the transition from glucose intolerance to frank diabetes are often associated with increased insulin requirements, as might occur during infections or puberty. After the initial clinical presentation of type 1A DM, a ―honeymoon phase may ensue during which time glycemic control is achieved with modest doses of insulin or, rarely, insulin is not needed. However, this fleeting phase of endogenous insulin production from residual beta cells disappears as the autoimmune process destroys the remaining beta cells, and the individual becomes completely insulin deficient.

Pathologically, the pancreatic islets are infiltrated with lymphocytes. Beta cells seem to be particularly susceptible to the toxic effect of some cytokines [tumor necrosis factor (TNF-α), interferon γ, and interleukin-1 (IL-1)]. The precise mechanisms of beta cell death are not known but may involve formation of nitric oxide metabolites, apoptosis, and direct CD8 T cell cytotoxicity. After all beta cells are destroyed, the inflammatory process abates, the islets become atrophic, and immunologic markers disappear.

Individuals with a genetic predisposition are exposed to an immunologic trigger that initiates an autoimmune process, resulting in a gradual decline in beta cell mass. The downward slope of the beta cell mass varies among individuals. This progressive impairment in insulin release results in diabetes when ~8o% of the beta cell mass is destroyed. A —honeymoon phase may be seen in the first **1** or **2** years after the onset of diabetes and is associated with reduced insulin requirements. Type 1A DM develops as a result of the synergistic effects of genetic, environmental, and immunologic factors that ultimately destroy the pancreatic beta cells.

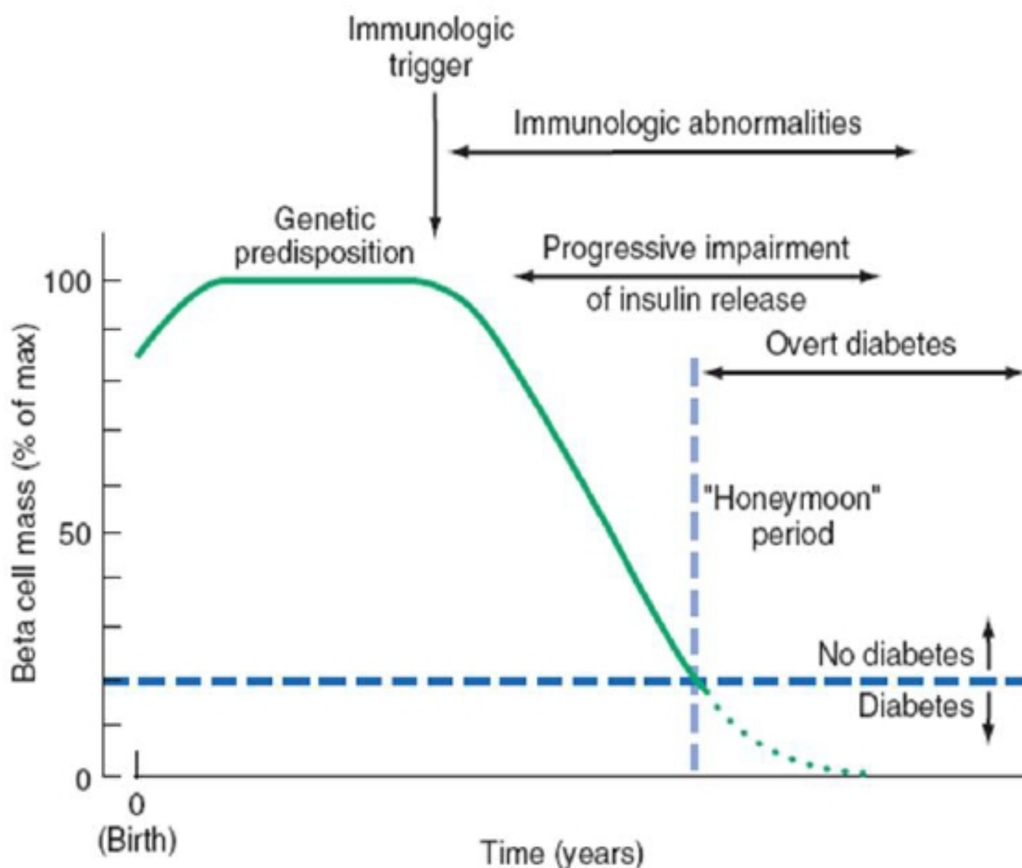


Figure 2.1: Temporal model for development of type 1 diabetes

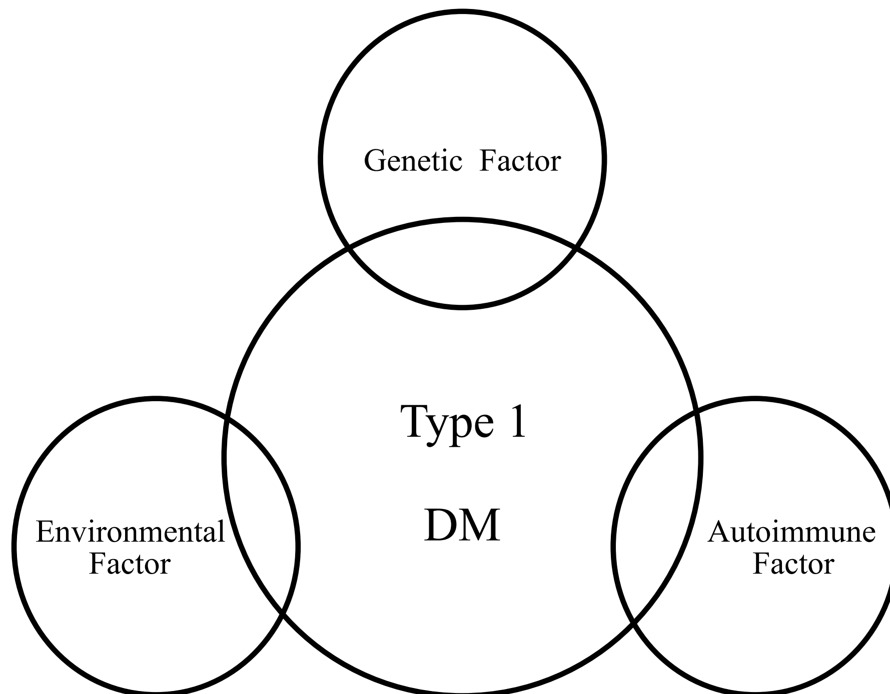


Figure 2.2: Causative factors of Diabetes Mellitus:

2.3.1 Genetic Factors:

The major susceptibility gene for type 1A DM is located in the HLA region on chromosome 6. Polymorphisms in the HLA complex account for 40 to 50% of the genetic risk of developing type 1A DM. Most individuals with type 1A DM have the HLA DR3 and/or DR4 haplotype. The risk of developing type 1A DM is increased tenfold in relatives of individuals with the disease.

2.3.2 Autoimmune Factors:

Pancreatic islet molecules targeted by the autoimmune process include insulin, glutamic acid decarboxylase (GAD, the biosynthetic enzyme for the neurotransmitter GABA), ICA-512/IA-2 (homology with tyrosine phosphatases), and phogrin (insulin secretory granule protein). Other less clearly defined autoantigens include an islet ganglioside and carboxy peptidase H. With the exception of insulin, none of the autoantigens are beta cell specific, which raises the question of how the beta cells are selectively destroyed. Current theories favor initiation of an autoimmune process directed at one beta cell molecule, which then spreads to other

islet molecules as the immune process destroys beta cells and creates a series of secondary autoantigens. The beta cells of individuals who develop type 1A DM do not differ from beta cells of normal individuals, since transplanted islets are destroyed by a recurrence of the autoimmune process of type 1A DM.

2.3.3 Immunologic Markers:

Islet cell autoantibodies (ICAs) are a composite of several different antibodies directed at pancreatic islet molecules such as GAD, insulin, IA-2/ICA-512, and an islet ganglioside and serve as a marker of the autoimmune process of type 1A DM. Testing for ICAs can be useful in classifying the type of DM as type A and in identifying non diabetic individuals at risk for developing type 1A DM.

2.3.4 Environmental Factors:

Putative environmental triggers include viruses (coxsackie and rubella most prominently), bovine milk proteins, and nitro urea compounds. Identification of an environmental trigger has been difficult because the event may precede the onset of DM by several years (Braunwald et al., 2005).

3. AIM & OBJECTIVES:

- To perform phytochemical and pharmacological evaluation of bark of *Symplocos racemosa* for its anti diabetic activity.
- To validate the traditional claims of bark of *Symplocos racemosa* using scientific methodology which can be further utilized for chronic ailments like Diabetes.

4. Literature Review:

4.1 Introduction to *Symplocos racemosa Roxb*:

4.1.1 Taxonomical Classification:

Kingdom: Plantae
Genus: Symplocos
Class: Angiospermae
Order: Ericales
Family: Symplocaceae
Genus: Symplocos
Species: *Symplocos racemosa*
Botanical name: *Symplocos racemosa Roxb*

It is known by variety of common name(s) and synonym(s) such as

Sanskrit:

Aksibhaisajya, Aksibhesaja, Balabhadra, Balipriya, Bhillataru, Bhilli, Galava, Hastilodhraka, Hemapushpaka, Kandakilaka, Kandanila, Laktakarma, Lodhra, Lodhrah, Lodhraka, Lodhravriksha, Mahalodhra, Marjana, Nayanousadha, Rodhra, Rodhrah, Savara, Savaraka, Savarakarodhra, Savararodhra, Savura, Shahara, Shaharalodhra, Shambara, Shavaraka, Shukla, Srinata, Sthulavalkala,

Hindi:

Lodhra, Lodhra Pathani

Bengali:
Lodhra

Marathi:
Lodhra

Urdu:
Ludh Pathani, Pathan Lodh, Lodh Pathani, Lodh Pathani Sayida, Pathani Lodh

Tamil:
Vellattippattai, Vellilottiram, Velli-Lodhram, Kacacankai, Kaya Vilai

Telgu:
Lodduga, Lodhuga-Chettu, Sabaramu, Sapara, Erralodduga

4.1.2 Description of the Plant:

Distribution:

It is large genus of trees and shrubs, widely distributed in the tropics and subtropics of Asia, Australia, and America. It consists of almost 290 species, about 68 species are found in India, of which only a few are of economic importance. In Pakistan only two species are found, namely *Symplocos racemosa* and *Symplocos chinensis* (Rao, N., et. al., 2011).

Macroscopic:

Trees or shrubs, usually glabrous, leaves often turning yellow when dry, alternate, coriaceous or membranous, toothed or entire. Flowers are usually white, in axillary spikes or racemes, sometimes reduced to few-flowered fascicles or to a single flower; bracts usually solitary at the base of each pedicel, caduceus; bracteoles are 1-3 beneath the flower. Calyx-tube is adnate, short when in flower, often enlarged in fruit; lobes 5, imbricate. Petals 5 in 1 series, or 6-10 in 2 series, free almost or entirely to the base, or obscurely connate (rarely connate into a tube), imbricate. Stamens are usually numerous, many seriate, adnate to the corolla-tube or to the petals, the outer the longer; filaments filiform or flattened at the base; anthers short dehiscing longitudinally. Ovary inferior (in Indian species), 3- (rarely 2- or 4-) celled; ovules

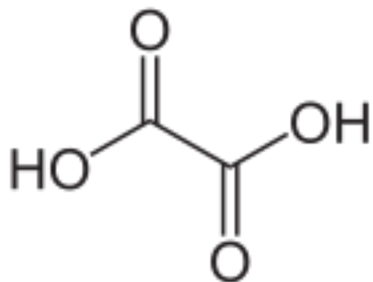
2, pendulous from the inner angle of each cell; style usually filiform; stigma capitate or small, scarcely lobed. Drupe is ellipsoid or subglobose; stone usually woody, often ribbed, 1-3-seeded. Seeds oblong; embryo terete, straight or curved, in the centre of fleshy albumen; cotyledons much shorter than the radicle. Mature stem bark occurs in channelled or curved pieces, few fiat pieces also occur in thickness upto 1cm, outer surface uneven and rough due to fissures and cracks, grayish brown to grey externally, pale to whitish- brown internally, fracture short and granular in cortical region and somewhat fibrous in inner region, taste, astringent and feebly bitter (Rao, N., et. al., 2011).

Microscopic :

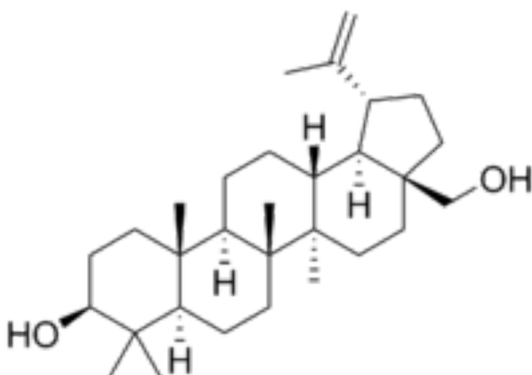
Transverse section of mature bark shows a wide cork of thin walled, rectangular cells arranged in radial rows, cork cambium 1-3 layered, secondary cortex consists of thin-walled, oval and tangentially elongated parenchymatous cells towards outer side and rounded cells towards inner side, a number of stone cells, in singles or in groups present, scattered throughout the region having highly thickened walls with distinct pits, prismatic and cluster crystals of calcium oxalate, and starch grains, mostly simple present in a number of cortical cells, secondary phloem wide consisting of sieve elements, phloem parenchyma, phloem fibres and stone cells, phloem parenchyma thin walled, oval to rectangular, containing prismatic crystals of calcium oxalate scattered in phloem parenchyma, phloem fibres lignified and present in singles or in groups, crystals not present in fibres, isolated fibres spindle shaped with pointed ends, groups of stone cells as rounded patches distributed throughout phloem region, medullary rays unit multiseriate consisting of rectangular cells having brown colouring matter in some cells, broader medullary rays dialating towards outer phloem region, a number of phloem cells also contain starch grains, mostly arranged in groups, rarely solitary, simple and rounded. Powder-Greyish-brown, under microscope shows fragments of cork, stone cells, fibres, prismatic and cluster crystals of calcium oxalate and starch grains (Rao, N., et. al., 2011).

Chemical Constituents:

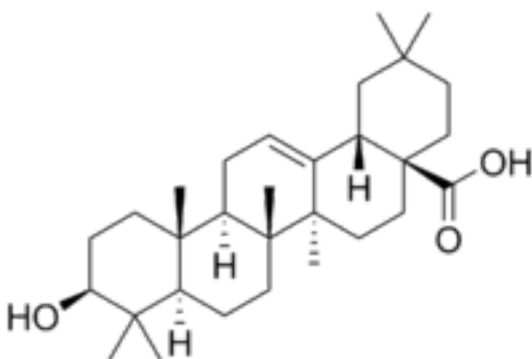
Monomethyl pelargonidin glucosides
Loturine (Alkaloid)
Colloturine (Alkaloid)
Loturidine (H_1 Histamine antagonist)



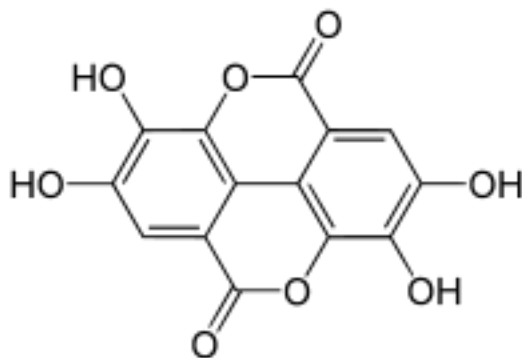
Oxalic acid (Organic Compound)
Phytosterol (Sterols)
3-monoglucofuranoside



Betulin (Triterpenoid)
Acetyloleanolic (Triterpenoid)



Oleanolic acid (Triterpenoid)



Ellagic acid (Tannins)

4.2 Estimation of Ellagic Acid in Lodhra Bark by HPTLC Analysis was done using Hexane: Ethyl acetate (8:2 v/v) at 254 nm (Rao, N., et al., 2011).

4.3 Pharmacological Activity:

In vivo Animal Models of Diabetes Mellitus:

Diabetes can be induced by pharmacologic, surgical or genetic manipulations in several animal species. Most experiments in diabetes are carried out on rodents, although some studies are still performed in larger animals. The classical model employed by Banting and Best was pancreatectomy in dogs (Bliss, 2000). It is also described prone strains to diabetes mellitus that have been employed in several researches (Chen and Wang, 2005; Rees and Alcolado, 2005; Masiello, 2006). Currently, the murine model is one of the most used due to the availability of over 200 well- characterized inbred strains and the ability to delete or over- express specific genes through knockout and transgenic technologies (Rees and Alcolado, 2005; Masiello, 2006).

Pharmacological Induction of Diabetes:

The induction of experimental diabetes in the rat using chemicals which selectively destroy pancreatic ß cells is very convenient and simple to use. The most usual substances to induce diabetes in the rat are alloxan and streptozotocin. The understanding of changes in ß cells of the pancreas as well as in the whole organism after alloxan or streptozotocin treatment is essential for using these compounds as diabetogenic agents. The metabolic disturbances in alloxan- and streptozotocin treated rats were described recently by Szkudelski et al. 2001. This review focuses on

the elucidation of the mechanism of cytotoxic action of alloxan and streptozotocin in ß cells of the rat pancreas.

The Mechanism of Alloxan Action:

Alloxan (2,4,5,6-tetraoxypyrimidine; 5,6-dioxyuracil) was first described by Brugnatelli in 1818. Wöhler and Liebig used the name “alloxan” and described its synthesis by uric acid oxidation. The diabetogenic properties of this drug were reported many years later by Dunn, Sheehan and McLethie (1943), who studied the effect of its administration in rabbits and reported a specific necrosis of pancreatic islets. Since then, alloxan diabetes has been commonly utilized as an animal model of insulin-dependent diabetes mellitus (IDDM).

Alloxan exerts its diabetogenic action when it is administered parenterally: intravenously, intraperitoneally or subcutaneously. The dose of alloxan required for inducing diabetes depends on the animal species, route of administration and nutritional status. Human islets are considerably more resistant to alloxan than those of the rat and mouse. The most frequently used intravenous dose of this drug to induce diabetes in rats is 65 mg/kg b.w. When alloxan is given intraperitonealy or subcutaneously its effective dose must be 2-3 times higher. The intraperitoneal dose below 150 mg/kg body weight may be insufficient for inducing diabetes in the rat. Fasted animals are more susceptible to alloxan whereas increased blood glucose provides partial protection.

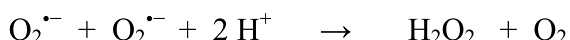
The mechanism of alloxan action has been intensively studied, predominantly in vitro, and is now characterized quite well. Using isolated islets and perfused rat pancreas it was demonstrated that alloxan evokes a sudden rise in insulin secretion in the presence or absence of glucose. This phenomenon appeared just after alloxan treatment and was not observed after repetitive exposure of islets to this diabetogenic agent. The sudden rise in blood insulin concentration was also observed in vivo just after alloxan injection to rats. Alloxan-induced insulin release is, however, of short duration and is followed by complete suppression of the islet response to glucose, even when high concentrations (16.6 mM) of this sugar were used.

Alloxan is a hydrophilic and unstable substance. Its half-life at neutral pH and 37°C is about 1.5 min and is longer at lower temperatures. On the other hand, when a

diabetogenic dose is used, the time of alloxan decomposition is sufficient to allow it to reach the pancreas in amounts that are deleterious.

The action of alloxan in the pancreas is preceded by its rapid uptake by the ß cells. Rapid uptake by insulin-secreting cells has been proposed to be one of the important features determining alloxan diabetogenicity. Another aspect concerns the formation of reactive oxygen species. A similar uptake of alloxan also takes place in the liver. However, the liver and other tissues are more resistant to reactive oxygen species in comparison to pancreatic ß cells and this resistance protects them against alloxan toxicity. The formation of reactive oxygen species is preceded by alloxan reduction. In ß cells of the pancreas its reduction occurs in the presence of different reducing agents. Since alloxan exhibits a high affinity to the SH-containing cellular compounds, reduced glutathione (GSH), cysteine and protein-bound sulfhydryl groups (including SH-containing enzymes) are very susceptible to its action. However, other reducing agents such as ascorbate may also participate in this reduction. Lenzen *et al.* (1996) proposed that one of the SH-containing compounds essential for proper glucose-induced insulin secretion is glucokinase (EC 2.7.1.2), being very vulnerable to alloxan. Alloxan reacts with two -SH groups in the sugar-binding side of glucokinase resulting in the formation of the disulfide bond and inactivation of the enzyme. Glucose can protect glucokinase against the inactivation hindering the access of alloxan to the -SH groups of the enzyme.

Dialuric acid is formed as a result of alloxan reduction. It is then re-oxidized back to alloxan establishing a redox cycle for the generation of superoxide radicals. The reaction between alloxan and dialuric acid is a process in which intermediate alloxan radicals (HA•) and an unidentified "compound 305" (maximum absorption at 305 nm) is formed. The latter appears when alloxan is reduced by GSH. Superoxide radicals are able to liberate ferric ions from ferritin and reduce them to ferrous ions. Fe3+ can also be reduced by alloxan radicals. Moreover, superoxide radicals undergo dismutation to hydrogen peroxide:



$$O_2^{\bullet-} + O_2^{\bullet-} + 2H^+ \rightarrow H_2O_2 + O_2$$

This reaction may occur spontaneously or may be catalyzed by superoxide dismutase (EC 1.15.1.1)

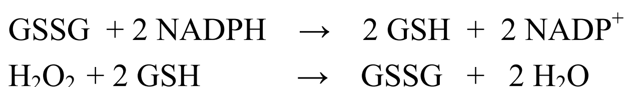
One of the targets of the reactive oxygen species is DNA of pancreatic islets. Its fragmentation takes place in ß cells exposed to alloxan. DNA damage stimulates poly

ADP-ribosylation, a process participating in DNA repair. Some inhibitors of poly ADP-ribosylation can partially restrict alloxan toxicity. This effect is, however, suggested to be due to their ability to scavenge free radicals rather than to a restriction of poly ADP- ribosylation initiated by alloxan. Superoxide dismutase, catalase (EC 1.11.1.6) and non-enzymatic scavengers of hydroxyl radicals were also found to protect against alloxan toxicity. Therefore, chemicals rendering anti-oxidative properties and inhibiting poly ADP-ribosylation can attenuate alloxan toxicity.

It has been argued that glucose counteracts alloxan cytotoxicity *in vitro* and *in vivo*. This ability, however, is not only the result of the protection of glucokinase. The protective effect of glucose against necrotic death of ß cells may be due to interaction of the sugar with the glucose transporter GLUT2 resulting in limited alloxan uptake. In the presence of Fe^{2+} and hydrogen peroxide, highly reactive hydroxyl radicals are then formed according to the Fenton reaction:

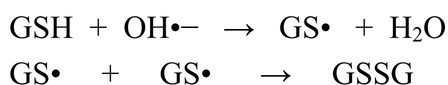
$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{-} + OH^{\bullet -}$$

It has been previously proposed that the action of glucose is also related to its metabolism and to the increased generation of reducing equivalents (NADH and NADPH) accelerating the recirculation of glutathione. GSH is known to provide protection against free radicals. It may thus divert hydrogen peroxide from the pathway leading to the formation of hydroxyl radicals:



$$GSSG + 2\,NADPH \rightarrow 2\,GSH + 2\,NADP^{+}$$
$$H_2O_2 + 2\,GSH \rightarrow GSSG + 2\,H_2O$$

Moreover, Sakurai and Ogiso (1991) observed that the *in vitro* generation of hydroxyl radicals in the presence of alloxan strongly depends on GSH concentration. GSH in low concentrations potentiated the formation of these radicals, whereas the oxygen consumption, autoxidation of dialuric acid and formation of hydroxyl radicals were significantly inhibited in higher concentrations. GSH at high concentrations can also inhibit HA• generation and directly neutralize hydroxyl radicals. Thiyl radicals (GS•) formed in this reaction are
then converted to GSSG:



$$GSH + OH^{\bullet -} \rightarrow GS^{\bullet} + H_2O$$
$$GS^{\bullet} + GS^{\bullet} \rightarrow GSSG$$

Indeed, in rat islets incubated with alloxan the GSH content and GSH/GSSG ratio were decreased, whereas glucose evoked the opposite effect.

In the *in vivo* experiment, glucose given to rats 20 min prior to alloxan partially restricted alloxan- induced increase in the activity of glutathione peroxidase (EC 1.11.1.9) and mitigated the drop of liver nonprotein -SH groups (especially reduced glutathione). The protective action of this sugar is, however, strongly glucose and alloxan dose-dependent.

It has been proposed that disturbances in intracellular calcium homeostasis constitute an important step in the diabetogenic action of alloxan. This concept was confirmed by *in vitro* and *in vivo* experiments demonstrating that alloxan elevates cytosolic free Ca^{2+} concentration in pancreatic ß cells. This effect arises from several events: alloxan-induced calcium influx from extracellular fluid, exaggerated calcium mobilization from intracellular stores and its limited elimination from the cytoplasm. The calcium influx may result from the ability of alloxan to depolarize pancreatic ß cells. Depolarization of the cell membrane opens voltage-dependent calcium channels and enhances calcium entry into cells. Alloxan was also found to exert a stimulatory effect on mitochondrial Ca^{2+} efflux with simultaneous inhibitory action on Ca^{2+} uptake by mitochondria. The restriction of calcium removal from the cells due to alloxan-induced inhibition of liver plasma membrane Ca^{2+}-ATPase was also reported. The effect of alloxan on intracellular calcium concentration seems to be mediated, at least partially by H_2O_2, since hydrogen peroxide itself exerts a similar effect on calcium concentration in ß cells.

Thus, the previously mentioned sudden rise in insulin release from ß cells treated with alloxan may be one of the effects of alloxan-induced augmentation in cytosolic Ca^{2+} concentration. The exaggerated concentration of this ion contributes to supraphysiological insulin release and, together with reactive oxygen species, causes damage of pancreatic ß cells.

The results of experiments with calcium channel antagonists have confirmed the important role of cytosolic calcium in the cytotoxic action of alloxan. Pretreatment of rats with verapamil prevented the alloxan-induced increase in ß cell Ca^{2+} concentration and abolished the stimulatory effect of alloxan on insulin release. The calcium channel antagonists (verapamil and diltiazem) also suppressed hyperglycemia and the onset of alloxan diabetes in rats.

Summing up, the toxic action of alloxan on pancreatic ß cells, described many years ago by Dunn *et al.* 1943, are the sum of several processes such as oxidation of essential -SH groups, inhibition of glucokinase, generation of free radicals and disturbances in intracellular calcium homeostasis.

Many investigators suggested that the selectivity of alloxan action is not quite satisfactory. Recent experiments confirmed this objection. The diabetogenic dose of alloxan was found to decrease -SH groups accompanied by a simultaneous rise in glutathione peroxidase activity in the rat liver two minutes after its administration. At the same time, the blood insulin concentration rose dramatically. This exaggerated insulinemia did not evoke, however, any significant reduction of blood glucose suggesting impaired peripheral insulin sensitivity in the short time after alloxan treatment. It was also observed that alloxan intensified basal and epinephrine-induced lipolysis in isolated rat adipocytes and insulin failed to restrict this effect.

Thus, using alloxan to evoke diabetes, animals should be examined after proper period of time to minimize side effects of alloxan action. It should also be emphasized that the range of the diabetogenic dose of alloxan is quite narrow and even light overdosing may be generally toxic causing the loss of many animals. This loss is most likely due to kidney tubular cell necrotic toxicity, in particular when too high doses of alloxan are administered (Szkudelski, T., 2001).

The Mechanism of Streptozotocin Action:

Streptozotocin (STZ, 2-deoxy-2-(3-(methyl-3-nitrosoureido)-D-glucopyranose) is synthesized by *Streptomycetes achromogenes* and is used to induce both insulin-dependent and non-insulin-dependent diabetes mellitus (IDDM and NIDDM, respectively).

The range of the STZ dose is not as narrow as in the case of alloxan. The frequently used single intravenous dose in adult rats to induce IDDM is between 40 and 60 mg/kg b.w., but higher doses are also used. STZ is also efficacious after intraperitoneal administration of a similar or higher dose, but single dose below 40 mg/kg b.w. may be ineffective. For instance, when 50 mg/kg b.w. STZ are injected intravenously to fed rats, blood glucose (determined 2 weeks after treatment) can reach about 15 mM.

STZ may also be given in multiple low doses. Such treatment is used predominantly in the mouse and the induction of IDDM is mediated by the activation of immune mechanisms. However, Ziegler *et al.* (1984) and Wright and Lacy (1988) demonstrated that the non-specific activation of the immune system *via* complete Freund's adjuvant prior to STZ injections allows to reduce its diabetogenic dose even in the rat.

NIDDM can easily be induced in rats by intravenous or intraperitoneal treatment with 100 mg/kg b.w. STZ on the day of birth. This method of NIDDM induction was described for the first time by Portha *et al.* (1974). At 8-10 weeks of age and thereafter, rats neonatally treated with STZ manifest mild basal hyperglycemia, an impaired response to the glucose tolerance test and a loss of ß cell sensitivity to glucose.

Streptozotocin action in ß cells is accompanied by characteristic alterations in blood insulin and glucose concentrations. Two hours after injection, the hyperglycemia is observed with a concomitant drop in blood insulin. About six hours later, hypoglycemia occurs with high levels of blood insulin. Finally, hyperglycemia develops and blood insulin levels decrease. These changes in blood glucose and insulin concentrations reflect abnormalities in ß cell function. STZ impairs glucose oxidation and decreases insulin biosynthesis and secretion. It was observed that STZ at first abolished the ß cell response to glucose. Temporary return of responsiveness then appears which is followed by its permanent loss and cells are damaged.

STZ is taken up by pancreatic ß cells *via* glucose transporter GLUT2. A reduced expression of GLUT2 has been found to prevent the diabetogenic action of STZ. Wang and Gleichmann (1995, 1998) observed that STZ itself restricts GLUT2 expression *in vivo* and *in vitro* when administered in multiple doses.

Intracellular action of STZ results in changes of DNA in pancreatic B cells comprising its fragmentation. Recent experiments have proved that the main reason for the STZ-induced ß cell death is alkylation of DNA. The alkylating activity of STZ is related to its nitrosourea moiety, especially at the O_6 position of guanine. After STZ injection to rats, different methylated purines were found in tissues of these animals.

Since STZ is a nitric oxide (NO) donor and NO was found to bring about the destruction of pancreatic islet cells, it was proposed that this molecule contributes to STZ-induced DNA damage. The participation of NO in the cytotoxic effect of STZ was confirmed in several experiments. Pancreatic ß cells exposed to STZ

manifested changes characteristic for NO action, i.e. increased activity of guanylyl cyclase and enhanced formation of cGMP. STZ is, however, not a spontaneous nitric oxide donor. This molecule is liberated when STZ is metabolized inside cells, but NO synthase is not required for this effect. On the other hand, the lowering of NO concentration in pancreatic islet cells by inhibition of the inducible form of nitric oxide synthase partially counteracted DNA cleavage induced by STZ. A similar effect can be attained by NO scavengers. However, the results of several experiments provide the evidence that NO is not the only molecule responsible for the cytotoxic effect of STZ. STZ was found to generate reactive oxygen species, which also contribute to DNA fragmentation and evoke other deleterious changes in the cells. The formation of superoxide anions results from both STZ action on mitochondria and increased activity of xanthine oxidase (EC 1.1.3.22). It was demonstrated that STZ inhibits the Krebs cycle and substantially decreases oxygen consumption by mitochondria. These effects strongly limit mitochondrial ATP production and cause depletion of this nucleotide in ß cells. Restriction of mitochondrial ATP generation is partially mediated by NO. This molecule was found to bind to the iron-containing aconitase inhibiting enzyme activity.

Augmented ATP dephosphorylation increases the supply of substrate for xanthine oxidase (ß cells possess high activity of this enzyme) and enhances the production of uric acid – the final product of ATP degradation. Then, xanthine oxidase catalyses reaction in which the superoxide anion is formed. As a result of superoxide anion generation hydrogen peroxide and hydroxyl radicals are formed. The inhibition of xanthine oxidase by allopurinol restricts the cytotoxic effect of STZ *in vitro*. Pretreatment of ß cells with this inhibitor prevented the STZ-induced decrease of insulin secretion.

It can be stated that potent alkylating properties of STZ are the main reason of its toxicity. However, the synergistic action of both NO and reactive oxygen species may also contribute to DNA fragmentation and other deleterious changes caused by STZ. NO and reactive oxygen species can act separately or form the highly toxic peroxynitrate. Therefore, intracellular antioxidants or NO scavengers substantially attenuate STZ toxicity.

STZ-induced DNA damage activates poly ADP-ribosylation. This process leads to depletion of cellular NAD+, further reduction of the ATP content and subsequent inhibition of insulin synthesis and secretion. The concept of unfavorable consequences of augmented poly ADP-ribosylation as a result of STZ action was confirmed by experiments revealing that the inhibition of this process prevents the toxicity of

this diabetogenic agent. It was found that 3-aminobenzamide, a strong inhibitor of poly(ADP-ribose) synthase, protected against the action of STZ in rats, even when this substance was administered 45-60 min after STZ. Another inhibitor of poly (ADP-ribose) synthase, nicotinamide, which is also scavenging oxygen free radicals, exerted best protection when it was administered shortly after STZ. The failure of protective action of nicotinamide administered after STZ is probably due to a potent reduction of the cellular ATP content by STZ since nicotinamide uptake is ATP-dependent. The protective effect of 3-aminobenzamide and nicotinamide was also confirmed *in vitro*.

It has been suggested that some inhibitors of poly ADP-ribosylation may also exert a protective effect due to their hydroxyl radical scavenging properties. However, in the case of STZ, recent investigations in poly(ADP-ribose) polymerase-deficient mice demonstrated that the inhibition of poly ADP-ribosylation itself prevents STZ-induced ß cell damage and hyperglycemia. Thus, it can be stated that the activation of poly ADP-ribosylation is of greater importance for the diabetogenicity of STZ than generation of free radicals and DNA damage *per se*.

Calcium, which may also induce necrosis, does not seem to play a significant role in the necrosis evoked by STZ since calcium channel antagonists do not protect ß cells against streptozotocin, as they do in the case of alloxan (Szkudelski, T., 2001).

4.4 Ethnopharmacological Uses:

Traditional Uses:

Symplocos racemosa (Lodhra) is a medicinal plant widely used by the traditional practitioners against various diseases as single or in compound drug. It has a wide range of usage in Ayurveda and Unani medicines. Its bark is described as an emmenagogue tonic for the persons of plethoric constitution and is useful in bowel complaints and ulcers. Its decoction is used as a gargle for giving firmness to bleeding and spongy gums. It cures watery eyes, opthalmia and is good for all diseases of the eye. It also cures "Kapha" biliousness, diseases of the blood, dysentery, inflammations, vaginal discharges, leprosy, elephantiasis, filaria, and is useful in abortions, miscarriages and ulcers in the vagina. The bark in 20-grain doses mixed with sugar, is given in menorrhagia due to relaxation of the uterine tissue; it is given two or three times a day, for three or four days. It is also used for leucorrhoea. The bark is also prescribed in the treatment snake-

bite and scorpion-sting. In snake bite it is given internally in powder form or in the form of a decoction [Joshi, 2000].

4.5 Other Herbs Used as Anti-Diabetics:

Natural remedies have been used for centuries for a wide variety of ailments by various cultures. Herbal medicines are still the mainstay of about 75 – 80 % of world population, mainly in the developing countries , for primary health care because of better cultural acceptability , better compatibility with the human body and lesser side effects. However , the last few years have seen a major increase in their use in the developed world. The World Health Organization (WHO) has recently defined traditional medicine (including herbal drugs) as comprising therapeutic practices that have been in existence , often for hundreds of years , before the development and spread of modern medicine and are still in use today.

The chemical constituents present in the herbal drugs are a part of the physiological functions of living flora and hence they are believed to have better compatibility with human body. These drugs are made from renewable resources of raw materials by eco – friendly processes and bring economic prosperity to the masses growing these raw materials.

Table 4.1: Medicinal Plants Experimentally Investigated for Anti Diabetic Activity

Biological name	Plant Part used	Extract/ Active Constituent	Animal model	Reference
Ananas comosus	Leaf	Ethanolic extract	Alloxan induced dyslipidemic rats	Xie et al., 2005
Boerhavia diffusa	Leaf	Aqueous extracts	Alloxan induced diabetic rats	Pari et al., 2004
Caesalpinia bonducella	Seed	Ethanolic extracts	STZ induced diabetic rats	Sharma et al.,1997
Capparis deciduas	Fruit	Ethanolic extract	STZ induced diabetic rats	Purohit et al.,2005
Capparis spinosa	Fruit	Aqueous extracts	STZ induced diabetic rats	Eddouks et al.,2005
Casearia esculenta	Root	Aqueous extracts	STZ induced diabetic rats	Prakasam et al.,2003

Coccinia indica	Leaf	Ethanolic extracts	STZ induced diabetic rats	Pari et al.,2003
Cuminum cyminum	Seed	Raw	Alloxan induced diabetic rats	Dhandapani et al., 2002
Eugenia jabolana	Seed Seed Kernel	Ethanol extract Ethanol extract	Alloxan induced diabetic rabbits STZ induced diabetic rats	Sharma et al., 2003 Ravi et al., 2005
Hibiscus rosa sinensis	Flower	Ethanol extract	STZ induced diabetic rats	Sachdeva et al., 2003
Kalopanax pictus	Stem Bark	Kalopanax saponin A hedearagenin	STZ induced diabetic rats	Park et al., 1998
Mangifera indica	Leaf	Mangiferin	STZ induced diabetic rats	Muruganandan et al., 2005
Momordica charantia	Fruit	-	STZ induced diabetes rats	Ahmed et al., 2001
Tinospora cordifolia	Root	Aqueous extract	Alloxan induced diabetic rats	Prince et al., 1998
Vaccinium myrtillus	Leaf	Alcoholic extract	STZ induced diabetic rats	Cignarella et al., 1996

4.6 Pharmacological Review of *Symplocos racemosa Roxb*:

Use of Antioxidant in cancer treatment is a rapidly evolving area. Antioxidants have been extensively studied for their ability to prevent cancer in humans. *Symplocos racemosa Roxb* is an indigenous plant having ample medicinal application. Ethanolic extract of *Symplocos racemosa Roxb* was subjected to various phytochemical screening tests. Antioxidant activity of the plant was evaluated against swiss albino mice. Result indicates that plant contains flavonoids, steroids, terpenoid, saponins, tannins, proteins and essential oil. Moreover, ethanolic extract of *Symplocos racemosa Roxb* had exhibited very good antioxidant activity. (Devmurari, V., 2010).

Symplocos racemosa is used in Indian System of Medicine (ISM) for various female disorders. Aqueous extract on oral administration significantly stimulated serum FSH

level (*P*<0.016) along with the rise in serum LH level (*P*<0.001). Moreover, histopathological studies revealed enhanced folliculogenesis, presence of mature follicles and detached oocytes, which are result of increased FSH and LH levels. Further, an increase in the ovary weight of treated animals was found due to observed FSH surge. These results was in concordance with the traditional use of the drug for female disorders. (Bhutani, K.K., et al., 2004).

Three new benzylated glycosides, locoracemosides A, B and C (1–3) were isolated from the bark of the stem of *Symplocos racemosa Roxb*. Their structures were determined by spectroscopic and chemical evidences. They displayed in vitro inhibitory activity against a-chymotrypsin. (Muhammad, A. R., et al.; 2008).

4.7 Polyherbal Preparations Experimentally Investigated for Anti Diabetic Activity:

Major formulations used in Ayurveda are based on herbs used as decoctions, infusion, tinctures and powders. Drug formulation in Ayurveda (As mention in Ayurvedic treatise like Charaka Samhita, Sushruta Samhita) is based on two principles: (a). Use as single drug, and (b). Use of more than two drugs. When two or more herbs are used in formulation they are known as polyherbal formulation. Sometimes herbs are combined with mineral preparation. The concept of polyherbalism is peculiar to Ayurveda although it is difficult to explain in term of modern parameter. Ayurveda has fundamental aspects for drug formulation.

The herbs are selected according to disease other herbs are used to prevent side effect arising from chief herb. It is evident that there are many herbal formulations of varying potency since these preparation act by different mechanism, it is theoretically possible that different combination of these extract will do better job in producing the effect. In the traditional system of plant medicine it is usual to use plant formulation and combined extract of plant are used as a drug of choice rather than individual ones to get the benefit of synergism and to find suitable anti hyperlipidemic and antioxidant combination therapy. Different polyherbal formulations those are scientifically investigated for their potential are listed below. All are appeared to be most effective , relatively nontoxic and have substantial documentation of efficacy.

Table 4.2: Polyherbal Preparations Experimentally Investigated for Anti Diabetics Activity.

Name of Preparation	Herbal Components	Model studies/ Mechanism of Action	Reference
Diacure	*Syzigium cumini* *Azardiricta indica* *Ocimum tenuiflorum* *Abitulon indicum* *Cassia auriculata* *Ficus bengalensis* *Tinospora cardifolia* *Phyllanthus emblica* *Trigonella foena graceum* *Curcuma longa* *Phyllanthus niruri*	STZ induced Diabetic rats	Arun N. et al., 2011
Dihar	*Syzygium cumini* *Momordica charantia* *Embelica officinalis* *Gymnema sylvestre* *Enicostemma littorale* *Azadirachta indica* *Tinospora cordifolia* *Curcuma longa*	STZ induced diabetic rats	Patel et al., 2009
Glyoherb	*Gymnema sylvestre* *Picrorrhiza kurroa* *Swertia chirata* *Momordica charantia* *Holarrhena antidysenterica* *Phyllanthus embilica* *Tribulus terrestris* *Terminalia chebula* *Euginea jambolana* *Trigonella foenumgraecum* *Azadirachta indica*	STZ induced Diabetic rats	Patel et al.,2009

	Cedrus deodara *Terminalia belletica* Bang bhasma		
Sugnil	*Aristolochia bracteata* *Shorea roxburghii* *Cassia auriculata* *Casearia esculanta* *Coscinium fenestratum* *Curcuma longa* *Eugenia jambolana* *Gymnema sylvestre* *Triphala*	STZ induced diabetic rats	Paranthaman et al ., 2011

5. MATERIALS AND METHODS:

Procurement of Herbs:

The dried bark of *Symplocos racemosa* was procured from LVG enterprise, Ahmedabad, Gujarat in the month of January, 2012.

Authentication of Bark:

The authentication of the bark of *Symplocos racemosa Roxb.* was carried out by Dr. B.L. Punjani, Ethanobotanist, P.G. Centre in Botany, Smt. S.M. Panchal Science College, Talod, Gujarat, India. The authentication of the bark of *Symplocos racemosa Roxb* was done by comparing the barks morphology and microscopy as mentioned in different standard texts.

Chemicals:

All the solvents were procured from CDH chemicals and enzymatic kits were acquired from Labcare diagnostic (India) Pvt. Ltd.

Glibenclamide and Streptozotocin were acquired from Torrent Pharma Ltd.(India) and M.P.Bio (India) respectively.

5.1 Morphological Study:

The morphological study of the dried sample of *Symplocos racemosa Roxb* was done for color, appearance, taste, fracture etc.

Microscopical Study:

The microscopical study of the powder of the dried bark of *S. racemosa* was done. The powder sample was treated with chloral hydrate and heated and treated with Phloroglucinol : HCl solution and dilute iodine solution. Powder was mounted on a slide and covered with the help of cover-slip. The slide preparations were examined and photographs were taken with monocular microscope with digital video eye piece (Lawrance Ltd. India) (Anonymous, 2005).

5.2 Physicochemical Parameters:

Determination of Extractive Value:

Alcohol Soluble Extractive Matters:

Macerated 5 g of powdered bark of *S. racemosa* with 100 ml of distilled water in a closed flask for 24 hours, shaking frequently during the first 6 hours and allowing to stand for 18 hours. Add the 5 ml of the chloroform to prevent the fermentation. Filter and evaporate 25 ml of the filtrate to dryness in shallow dish. Dry the residue at 105°C and weigh. The % of water soluble extractive with reference to the air dried drug has to calculated.

Water Soluble Extractive Matters:

(I) Cold Water Extractive Value:

5 g of the air-dried powdered bark of *S. racemosa* was macerated with 100 ml of distilled water of the specified strength in a closed flask for 24 hours, shaking at an interval of six hours. It was then allowed to stand for 18 hours. The macerate was filtered rapidly to prevent any loss of solvent. Twenty five ml of the filtrate was evaporated to dryness in a tarred flat bottomed shallow dish and dried at 105°C to a constant weight and finally weighed. The percentage of water-soluble extractive was calculated to the air-dried drug.

(ii) Hot Water Extractive Value:

About 4.0 g of coarsely powdered drug (air-dried) was placed & accurately weighed in glass stoppered conical flask. Added 100 ml distilled water and weighed to obtain total weight including flask. Shake well and allowed to stand for 1 hour. Attached a reflux condenser to the flask and boil gently for 1 hour. Cool and weighed. Readjusted to the original weight with the solvent specified in the test procedure for the plant material concerned. Shake well and filtered rapidly through a dry filter. Transferred 25 ml of filtrate to a tarred flat-bottomed dish and evaporate to dryness on a water-bath. Dried at 105˚C for 6 hrs, cool in a desiccator for 30 min, then weigh without delay. Calculate the content of extractable matter in mg per g of air-dried material.

Determination of Ash Values:

Total Ash:

Weighed accurately 2 g of the air-dried powdered bark of *S. racemosa* in crucible and incinerated at a temperature not exceeding 450°C in muffle furnace until free from carbon, cooled and weighed. Calculated the % of ash with reference to the air dried drug. Readings of analysis is mentioned as average of triplicates.

Acid Insoluble Ash:

Boiled the ash obtained from the total ash procedure with 25ml of 2M HCl for 5 min, filtered the insoluble matter on ashless filter paper, washed with hot water, incinerated at a temperature not exceeding 450°C in muffle furnace, cooled in a desiccators and weighed. Calculated the % of acid insoluble ash with reference to the air dried drug. Readings of analysis was mentioned as average of triplicates.

Water Soluble Ash:

Boiled the ash obtained from the total ash procedure with 25ml of water, the insoluble matter collected on an ashless filter paper, washed with hot water and ignited for 15 min. at a temperature not exceeding 450°C. The weight of the insoluble matter was subtracted from the weight of the ash. The difference in weight was the water soluble ash. The percentage of water-soluble ash was calculated with reference to the air-dried drug.

Loss on Drying:

Weighed a completely dry petri dish. Taken 5 g of powdered material in to the petri dish, covered it and accurately weigh the petri dish with the sample. Removed the lid of the petri- dish and placed the petri dish in an oven at 105°C, dried the sample to constant weight. Cooled to room temperature in desiccator before weighing.

5.3 Preparation of Extracts:

The bark of *Symplocos racemosa* was dried under shade with occasional shifting and then made into a coarse powder with a mechanical grinder and stored in airtight

container for further use. The dried powder material of bark was first extracted with petroleum ether (60-80^oC) and the solvent was removed by rotary evaporator. After the extraction with petroleum ether the same plant material was dried and again extracted with methanol and dried using rotary evaporator.

5.4 Preliminary Phytochemical Screening:

The petroleum ether extract and methanolic extract were subjected to chemical tests to identify chemical constituent of the plant.

Test for Sugars:

Small quantity of extract was dissolved in 4 ml of distilled water and filtered and the filtrate was subjected to Molish's test.

Molish's Test:

Added 2-3 ml of extract to α-napthol and concentrated H_2SO_4, purple colour indicated positive test.

Test for Glycosides:

A few mg of residue was dissolved 4 ml of distilled water and filtrated and the filtrate was subjected to Legal Test and Borntrager's test.

Borntrager's Test:

Taken 2-3 ml of extract and hydrolysed with dilute HCl for a few min on water bath. To the hydrolysate, about 1.0 ml of benzene and 0.5 ml of dilute ammonia solution was added. Appearance of reddish-brown color at the junction of the two layers confirmed the presence of glycosides.

Test for Flavonoids:

Shinoda Test:

A small piece of magnesium and 3 to 4 drops of concentrated sulphuric acid was mixed

with 2-3 ml of extract and observed for formation of red colour indicates the presence of flavonoids.

Test for Sterols:

Salkowski Test:

10 mg of extract was dissolved in 2 ml of chloroform and 2ml of concentrated sulphuric acid was added from the side of the test tube. Test tube was shaken for few minutes. The development of red color in chloroform layer indicated the presence of sterols.

Liebermann–Burchard Test:

1 ml of concentrated sulphuric acid was added to 10 mg of extract in 1ml of chloroform. A reddish – blue color exhibited by chloroform layer and green fluorescence by the acid layer suggests the presence of sterols.

Test for Alkaloids:

Few mg of extract was taken in 5 ml of 1.5% v/v hydrochloric acid and filtered. These filtrates were then used for testing alkaloids.

Dragendorff's Test:

Dragendorff's reagent was added in 2ml of filtrate. Formation of orange-brown precipitate indicated the presence of alkaloids.

Mayer's Reagent:

To a 1ml of test filtrate in a watch glass, a few drops of mayer's reagent were added. If the formation of cream colored precipitate it shows the presence of alkaloids.

Test for Tannins:

The test extract was taken in water, warmed and filtered. 5 ml of filtrate was allowed to react with 1ml of 5% ferric chloride solution. If dark green or deep blue color is obtained, tannin is present.

Test for Saponins:

Foam Test:

1ml solution of extract was diluted with distilled water to 20 ml and shaken in a graduated cylinder for 15 minutes. Development of stable foam suggests the presence of saponins.

1ml extract was treated with 1% lead acetate solution. Formation of white precipitates indicates the presence of saponins.

Test for Terpenoids:

Knollar's Test:

5 mg of extract is treated with 2ml of 0.1% anhydrous stannic chloride in pure thionyl chloride. A deep purple color that changes to red indicates the presence of terpenoids.

Test for Protein and Amino Acid:

Small quantity of the extract was dissolved in few ml of water and filtered. Filtrate was subjected to Millon's test and Biuret test.

Millon's Test:

Take 2-3 ml of ME, add about 5 ml of distilled water and filtered. To 2 ml of the filtrate, 5-6 drops of Million's reagent (solution of mercury nitrate and nitrous acid) were added and observed for formation of red precipitates as an indication of the presence of proteins.

Test for Resins:

Few mg of extract was treated with caustic soda a red color was developed if resins are present.

TLC Profile of *Symplocos racemosa Roxb*

- **Stationary Phase**: Silica gel G
- **Mobile Phase**: Toluene : Ethyl acetate (9:1)
- **Detection**: Spray with Anisaldehyde sulphuric acid reagent.
- **Test solution**: 10 mg Petroleum Ether extract dissolved in 1ml Petroleum Ether
  20 mg Methanolic extract dissolved in 1 ml Methanol.

5.5 Estimations:

Estimation of Phenolic Substances:

1g of air-dried bark powder of *Symplocos racemosa Roxb* were extracted with 100 ml methanol by maceration for 24 hours and filtered. The final volume of the filtrate was adjusted to 100ml using methanol. 5ml of this extract was diluted with an equal volume of methanol and was used for the estimation of phenols. To 10ml of the each extract were added 10ml of distilled water and 1.5ml of diluted (1:2) Folin Ciocaulteu reagent and the mixture was kept for 5min. After adding 4ml of 20% Na_2CO_3 solution, the final volume was adjusted to 25ml using distilled water. The absorbance was measured at 765 nm at an interval of 30 min. up to 2 hours using distilled water as a blank. The data were compared with similarly prepared set of standard substance- Gallic acid, in concentration range of 50 µg to 300 µg per 25 ml (Singleton and Rosi, 1965).

Estimation of Flavonoids:

1g of air-dried bark powder of *Symplocos racemosa Roxb* were extracted with 100 ml methanol by maceration for 24 h and filtered. The final volume of the filtrate was adjusted to 100 ml using methanol. One ml of this extract was diluted up to 10ml with methanol and was used for the estimation of flavonoids. To 3 ml of the each extract, 3ml of methanolic $AlCl_3$ was added. After 10 min., absorbance was read at 430 nm (Baharam *et al.,* 1996).

5.6 Anti-Diabetic Activity:

Animals:

Wistar rats of both sexes (150-200 g) were maintained under standard animal house conditions, fed standard pellet diet and allowed access to water *ad libitum.* Fasted animals were deprived of food for at least 16 h, but were allowed free access to water. The study was approved by the Institutional Animal Ethics Committee of Institute of Pharmacy, Nirma University, Ahmedabad. **(Protocol No. IPS/PCOG/MPH11-12/2020).** Rats were preferable in preclinical anti-diabetic study because of reasonable constant blood sugar level and convenience in dosing and blood collection.

Chemicals & Materials:

- Streptozotocin
- Ether
- Test samples & Glibenclamide
- Glucose
- Anesthesia chamber
- Cotton & Capillaries
- Methanol
- Petroleum Ether

Streptozotocin Induced Diabetes Induction of Experimental Diabetes (T.S. Frodea & Y.S. Medeiros, 2008)

Diabetes was induced by administering intravenous injection of a freshly prepared STZ solution (45 mg/kg of body weight) in 0.1M cold citrate buffer to the overnight fasted rats. Because of the STZ instability in aqueous media, the solution is made using cold citrate buffer (pH 4.5) immediately before administration. Hyperglycemia was confirmed by the elevated blood glucose level determined at 48 hr after the dose. Animal that exhibited glycosuria after 48h was tested by urine test strips (Uristix, Bayer diagnostics Ltd, India) were considered as diabetic. The treatment was started after day 3 of diabetes induction and was considered as day 1 of treatment.

Details of Injections and Schedule:

Type 1 Diabetic Mellitus:

- Doses: Streptozotocin (45mg/kg)
  Site: Intravenous
  Volumes: Not more than 0.5 ml

- Doses: Glibenclamide (0.4 mg/kg)
  Sites: Orally
  Volumes: Not more than 0.5 ml

- Test Sample: Plant Extract (Methanolic extract)
  Low Dose: Test (150 mg/kg)
  High Dose: Test (300 mg/kg)
  Sites: Orally
  Volume: Not more than 0.5 ml

- Test Sample: Plant Extract (Petroleum Ether Extract)
  Dose: Test (100 mg/kg)
  Sites: Orally
  Volume: Not more than 0.5 ml

Blood Withdrawal:

Volumes: Not more than 1 ml
Sites: Retro- orbital plexus

Table 5.1: Experimental Plan for Anti-Diabetic Study

Sr No	Groups	Animal
1	Control (Normal)	6
2	Control (Diabetic) (STZ)(45 mg/kg)	6
3	Diabetic + Standard (Glibenclamide)(0.4 mg/kg)	6
4	Diabetic + Methanolic extract (150 mg/kg)	6
5	Diabetic + Methanolic extract (300 mg/kg)	6
6	Diabetic + Petroleum Ether extract (100 mg/kg)	6
	Total	36

Blood Sampling:

On day 0, day 7, day 14, day 21, day 28, blood samples (1 ml) were collected from the retro-orbital plexus of the eye under light ether anesthesia using capillary tubes. Blood was collected in to fresh vials and serum was separated in a refrigerated centrifuge at 10,000 rpm for 25 minutes at 4˚C temperature and was directly used for estimating serum glucose levels using glucose SLR reagent (Accucare, India). On day 28, blood samples (2 ml) were collected from all six animals of each group in specific vials. Estimation of blood glucose was done by the same method as mentioned in blood glucose lowering activity.

5.7 Estimation of Glucose (GOD-POD Method):

Principle:

Glucose was determined after enzymatic oxidation in the presence of glucose oxidase. The hydrogen peroxide formed reacts, under catalysis of peroxidase, with phenol and 4-aminophenazone to form a red-violet quinonemine dye as indicator.

Assay Procedure:

	Blank	Std	Serum sample
Sample	**-**	**-**	**10 µl**
Standard	**-**	**10 µl**	**-**
Glucose SLR reagent	**1000 µl**	**1000 µl**	**1000 µl**

Solution was put in the test tubes. Mixed & Incubated for 15 min. at R.T. Measure absorbance of Serum sample (AT) and standard (AS) against Reagent Blank (Glucose SLR reagent) at 505 nm.

Calculation:

Total Glucose (mg/dL) = Sample/Standard x conc. of standard

Statistical Analysis:

All the values are expressed as mean $\pm$ SEM. Statistics was applied using graph pad prism 5.0 version. Statistical significance between normal control and induce control,

induce control and treated, within the treated group was tested using one way ANOVA analysis followed by Tukey's multiple comparison test. Differences were considered to be statistically significant when $p<0.05$

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