PHARMACOGNOSTICAL, PHYTOCHEMICAL STUDIES AND EVALUATION OF ANTIDIABETIC POTENTIAL OF BIOPHYTUM SENSITIVUM DC. AND BIOPHYTUM INTERMEDIUM WT.

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PHARMACOGNOSTICAL, PHYTOCHEMICAL STUDIES AND EVALUATION OF ANTIDIABETIC POTENTIAL OF BIOPHTUM SENSITIVUM DC. AND BIOPHTUM INTERMEDIUM WT.

INTRODUCTION

The most ancient and celebrated treatises on Hindu medicine are no doubt the Ayurveda. India also possesses a great heritage of other ancient systems of medicine such as Siddha, Unani and Homeopathy. In addition to these traditional systems, there also exists in India a vast knowledge of tribal and folk medicine, which utilize around 7,500 species of plants as medicine. Some of the ethno botanically important species have also provided leads for production of modern drugs by pharmaceutical companies. It is estimated that in India 90% of the prescriptions contain plant products. Ayurvedic and other traditional systems of Indian medicines fully depend on wild plants for preparation of drugs.

The World Health Organization (WHO) estimated that 80% of the population of developing countries still rely on traditional medicine, mostly plant drugs, for their primary health care needs. Demand for medicinal plant is increasing in both developing and developed countries due to growing recognition of natural products being non-toxic, having no side-effects, easily available at affordable prices. The medicinal plant sector has traditionally occupied an important position in the socio cultural, spiritual and medicinal area of rural and tribal families (WHO, 2002-2005)\(^1\). Traditional medicine still remains the main resource for a large majority of people treating health problems. Being a comprehensive knowledge system, traditional medicine encompasses the utilization of substances, dosages and practices based on socio-cultural norms and religious beliefs as well as witnessed experiences and observations of a specific group. This knowledge is handed down from generation in order to diagnose, prevent or eliminate a physical, social or spiritual imbalance.

In India approximately 1,800 plant species are used in Ayurveda, 600 for Siddha, about 400 for Unani and more than 400 for Homeopathic system of medicine, with
substantial overlap of common plants among these systems. Diabetes mellitus (DM), or simply diabetes, is a group of metabolic diseases in which a person has high blood sugar, either because the pancreas does not produce enough insulin, or because cells do not respond to the insulin that is produced. This high blood sugar produces the classical symptoms of polyuria (frequent urination), polydipsia (increased thirst) and polyphagia (increased hunger). In the recent years more than 500 herbal medicines have been reported to possess anti-diabetic property. An ethno-botanical survey of semi-structured questionnaire of medicinal plants in five districts of Logos State of Nigeria reputed for the treatment of diabetes has been reported.

The treatment of DM is based on oral hypoglycemic agents and insulin. However, DM is also treated in Indian traditional medicine using anti-diabetic medicinal plants. The oral hypoglycemic agents currently used in clinical practice have characteristic profiles of serious side effects. Hence, there is a need to search for newer anti-diabetic agents that retain therapeutic efficacy and are devoid of side effects that could be important sources of such agents.

Classification of diabetes based on etiology

Like other endocrine conditions, any defect along the pathway results in abnormal fuel metabolism, which will be manifested primarily as hyperglycemia. In 1997, the American Diabetes Association revised the nomenclature for the major types of diabetes and eliminated the terms insulin dependent diabetes mellitus and non-insulin-dependent diabetes mellitus and their acronyms, IDDM and NIDDM. The new classification of diabetes based on etiology is as follows:

1. Type 1a diabetes: pancreatic beta islet cell destruction leading to absolute insulin deficiency.
   - Autoimmune (most common)
   - Idiopathic (rare)

2. Type 1b presents like type 1 (with Diabetic Keto Acidosis), then behaves like type 2.

3. Type 2 diabetes: varying degrees of insulin resistance and insulin deficiency.
4. Gestational diabetes
5. Other specific types:

✓ Maturity onset diabetes of the young (MODY): Autosomal dominant pattern (Onset of hyperglycemia generally before age 25)
✓ Genetic defects in insulin action: Mutant insulin gene and mutation of insulin receptor and some forms of polycystic ovarian syndrome.
✓ Diseases of the exocrine pancreas: Includes trauma, infection, chronic necrotizing pancreatitis and pancreatic carcinoma, cystic fibrosis and hemochromatosis.
✓ Endocrinopathies: acromegaly, Cushing’s syndrome, glucagonoma and pheochromocytoma.
✓ Drug/chemical induced diabetes: glucocorticoids, cyclosporine A, nicotinic acid, interferon, pentamidine, occasionally thiazide diuretics.
✓ Infections: Congenital rubella virus, adenovirus, mumps and cytomegalovirus.

**Type 2 diabetes mellitus**

Type 2 diabetes is a chronic disease in which there is high blood glucose level. It is the most common form of diabetes (90% of diabetes cases). It is characterized by insulin resistance, which may be combined with relatively reduced insulin secretion. The defective responsiveness of body tissues to insulin is believed to involve the insulin receptor. However, the specific defects are not known.

The large number of plants clearly demonstrated the importance of herbal plants in the treatment of diabetes. It also shows the effort to isolate new potential antidiabetic agents. The plant families, including the species (sp), most studied for their confirmed hypoglycaemic effects include: Leguminosae (11 sp), Lamiaceae (7 sp), Liliaceae (8 sp), Cucurbitaceae (7 sp), Asteraceae (6 sp), Moraceae (6 sp), Rosaceae (6 sp), Euphorbiaceae (5 sp), Araliaceae (5 sp) and Oxalidaceae. The most studied species are: *Citrullus colocynthis* (Opuntia streptacantha. (Cactaceae), *Trigonella foenum graecum* L.
(Leguminosae), *Momordica charantia* L. (Cucurbitaceae), *Ficus bengalensis* L. (Moraceae), *Polygala senega* L. (Polygalaceae), and *Gymnema sylvestre* R. (Asclepiadaceae)\(^3\).

The following bioactive phytochemicals have been isolated from the plant and herb species of India. These active principles are dietary fibres, alkaloids, flavonoids, saponins, amino acids, steroids, peptides and others. These have reported to possess potent hypoglycemic, anti-hyperglycemic and glucose suppressive activities\(^4\).

Based on the above consideration, the present study initially attempted to identify plants with antidiabetic property. It has been documented that plants of different species, but belonging to same genus may have the medicinal activity in common. Applying this factor, a preliminary investigation on the search for plants, revealed that plants belonging to Oxalidaceae family have been reported for antidiabetic property. They are *Biophytum sensitivum* DC. and *Biophytum intermedium* Wight. The *Biophytum* genus have been claimed to possess antidiabetic property. The antidiabetic activity was also planned to carried out on the plants *Biophytum sensitivum* DC. and *Biophytum intermedium* Wight which belong to the same genus *Biophytum* and claimed to possess anti diabetic property which is of rare occurrence. The selected rare medicinal plant *Biophytum intermedium* have no scientific reports for its antihyperglycemic and antioxidant properties and may be considered as a new drug for antidiabetic and antioxidant activities. The present study demonstrates the antihyperglycemic and antioxidant properties of *Biophytum sensitivum* and *Biophytum intermedium* with special reference to type 2 diabetes mellitus. For further study, *Biophytum sensitivum* DC. and *Biophytum intermedium* Wight belonging to the family Oxalidaceae were selected for antidiabetic property by *in-vivo* model.

*Biophytum sensitivum* described as a medicinal plant used for various purposes like conjunctivitis, cough, oedema and chest pain. The leaf taken with butter milk in dysentery. The roots ground and used to cure gonorrhea\(^5\). Leaves of *Biophytum sensitivum* traditionally used in vomiting and dysentery in tribal medicine.

Many synthetic drugs have been used for the treatment of diabetes and other ailments. But these are being replaced by the natural drugs due to the increased cost of
production and complicated technical procedures and long time consumption in the research and development of synthetic drugs used in the modern system of medicine.

Our aim is to focus on natural system of medicine, because of its low rate of side effects, easy availability, low cost and more over safe than other system of medicine and to develop herbal medicines for diabetes.

Thus it is clear that as we step into the new millennium there is a need to integrate the traditional herbal medical wisdom with the cutting edge scientific advances plant based anti diabetics for health promotion and disease prevention. There is an exigent need for the development of indigenous alternative anti diabetic agents for the effective treatment in the light of growing cases of type 2 diabetes mellitus to the time honored anti diabetics. The present proposed study focuses on herbal treatment and plants used in the treatment of type 2 diabetes mellitus, a major crippling disease in the world leading to huge economic losses. In order to study about the traditional uses of plants in Asian cultures and their emerging importance in the human health, we have selected the rare medicinal plants *Biophytum sensitivum* DC. and *Biophytum intermedium* Wight family Oxalidaceae, for the treatment of type 2 diabetes mellitus.

The present study included pharmacognostical, phytochemical, pharmacological studies with special reference to the antidiabetic activity for both selected medicinal plants. The findings of the present study may provide scope for lead molecules with proven antidiabetic property with least or no side effects or toxicity.
LITERATURE REVIEW

Diabetes mellitus, a chronic illness associated with substantial morbidity and mortality is a major public health problem worldwide. It is a group of metabolic disorder characterized by hyperglycemia and abnormalities in carbohydrate, fat and protein metabolism. It results from defects in insulin secretion, insulin sensitivity or both. Chronic micro vascular, macro vascular and neuropathy complications may ensure.

Metabolic imbalance causing diabetes mellitus is a characteristic of materialistic world. Differences in social structure, psychic stress, obesity, hormonal imbalance and heredity are optimizing the growth pandemic. Increasing population with diabetes has a huge requirement of effective remediation. The Indian flora has a vast variety of medicinal plants, which are used traditionally for their anti diabetic property. However, careful assessment including sustainability of such herbs, ecological and seasonal variation in activity of phyto-constituents, metal contents of crude herbal antidiabetic drugs, through toxicity study cost effectiveness is required for their popularity.

Diabetes mellitus, being a multifactorial disease, demands multiple therapeutic approaches. Global studies on diabetes mellitus have reiterated that primary prevention is necessary and drastic steps must be taken to diagnose the disease early on, provide effective management and also take steps to prevent the onset of disease in high-risk subjects. According to WHO, plant-based traditional system of medicine is still the mainstay of about 75-80% of the world population, mainly in the developing countries, for primary healthcare because of better cultural acceptability, better compatibility with the human body and lesser side effects.

The major complications of diabetes are both acute and chronic. In acute cases, dangerously elevated blood sugar, abnormally low blood sugar due to diabetes medications may occur. In chronic cases, disease of the blood vessels (both small and large) which can damage the eye, kidneys, nerves, and heart may occur.

The associated disadvantages with insulin and oral hypoglycemic agents have lead to stimulation in the research for locating natural resources showing anti diabetic activity. Many studies have been carried out in search of a suitable plant drug that would be effective in Diabetes mellitus. Herbal remedies for diabetes have been recorded in ancient medical literature. Plants hold definite promises in the management of diabetes mellitus.
Despite considerable progress in the treatment of diabetes by oral hypoglycemic agents, search for newer drugs continues because the existing synthetic drugs have several limitations. In recent times there has been renewed interest in the plant remedies. In the Ayurvedic treatment, medicines consists of plant products, either single drug or in combination with others which are considered to be less toxic and free from side effects compared to synthetic drugs.

Flavonoids fraction from *Pterocarpus marsupium* has been shown to cause pancreatic β-cell regranulation. Marsupin, pterosupin and liquiritigenin obtained from this plant showed antihyperlipidemic activity. (-) epicatechin, its active principle, has been found to be insulinogenic, enhancing insulin release and conversion of proinsulin to insulin *in vitro*. Like insulin, (-) epicatechin stimulates oxygen uptake in fat cells and tissue slices of various organs, increases glycogen content of rat diaphragm in a dose dependent manner\(^8\,9\).

Terpenoid type quinines represent a new class of compounds of potential use in the treatment of type 2 diabetes\(^10\).

Tannins enhance glucose uptake and inhibit adipogenesis, thus being potential drugs for the treatment of NIDDM. Tannins can improve the pathological oxidative state of a diabetic situation. It is found in abundance in the tree bark, wood, fruit, fruit pod, leaves, and roots and also in plant gall. They have been observed to enhance the glucose uptake through mediators of the insulin-signalling pathways, such as P13K (Phosphoinositide 3-Kinase) and p38 MAPK (Mitogen-Activated Protein Kinase) activation and GLUT-4 translocation\(^11\).

Coumarins are phenolic substances made of fused benzene and a pyrone ring. The medicinal plants, besides having natural therapeutic values against various diseases and considerable works have been done on these plants to treat diabetes mellitus. Terpenes can be used for the prevention and/or treatment of diabetes type 2, obesity and neuropathy\(^12\).

Berberine a quarternary ammonium salt from the protoberberine group of isoquinoline alkaloids is used successfully in experimental and human diabetes mellitus. The mechanism of action include inhibition of aldose reductase, inducing glycolysis, preventing insulin resistance through increasing insulin receptor expression. Berberine, the
main constituents of traditional Chinese medicine Coptidis rhizome, display a good prospect in the prevention and treatment of T2DM\textsuperscript{13}.

Lectin has insulin like activity due to its nonprotein specific linking together to insulin receptors. This lectin lowers blood glucose level by acting on peripheral tissues. Lectin is a major contributor to hypoglycemic effect\textsuperscript{7}. Insulin like polypeptides responsible for hypoglycemic properties\textsuperscript{14}. Polypeptide isolated from fresh fruit juice and dried powder of \textit{Momordica charantia} have hypoglycemic effect with single treatment for 6-14 weeks\textsuperscript{15}.

The genus \textit{Biophytum} contain insulin-like principle and are recommended in diabetes\textsuperscript{5}. The study also showed rise in serum insulin levels in the treated animals, suggesting a pancreatic mode of action (i.e. insulinotropic effect) of genus \textit{Biophytum} may be mediated through stimulating the synthesis/release of insulin from the \(\beta\)-cells of Langerhans.

Based on the above consideration, the present study initially attempted to identify plants with anti diabetic property. It has been documented that plants of different species, but belonging to same genus may have the medicinal activity in common. They are \textit{Biophytum sensatium} DC. and \textit{Biophytum intermedium} Wight.

\textbf{\textit{Biophytum sensatium} DC.}

\textit{Biophytum sensatium} DC is a member of the genus \textit{Biophytum}, and is commonly known as Life plant. It is called Nilaccurunki, Tintaanaalee in Tamil and Mukkuty in Malayalam\textsuperscript{5}. Oxalidales (Order), Oxalidaceae (Family), \textit{Biophytum} (Genus), \textit{B. sensatium} (Species)\textsuperscript{16}.

The whole plant of \textit{Biophytum petersianum} was extracted with a mixture of water-alcohol (1:1) to evaluate its relaxant effect on aorta rings. These results indicate that hypotensive effect of \textit{Biophytum petersianum} may result from inhibition of calcium influx via both voltage and receptor-operated calcium channels\textsuperscript{17}. A polysaccharide isolated from \textit{Biophytum sensatium} has been found to enhance complement fixation\textsuperscript{18}. Whole plant of \textit{Biophytum sensatium} used for snake bite treatment in any form i.e venom neutralization, oral form for pain relief and local application form for pain relief\textsuperscript{19,20}.
Amentoflavone and polysaccharide present in the inflorescence of *Biophytum sensitivum* possessed antiarthritic and anti-inflammatory activities\(^\text{21}\).

Among the plants tested for DPPH scavenging ability, *Biophytum sensitivum* showed high activity but less than the activity of ascorbic acid, which has the IC\(_{50}\) value of 35.7\(^\text{22}\). The levels of glutathione-S-transferase and glutathione reductase increased and that of glutathione and that of glutathione peroxidase decreased after administering the *Biophytum* extract\(^\text{23}\). Amentoflavone (13’, II8-biapigenin) was isolated from the roots of *B.sensitivum* DC. (Oxalidaceae) and proved to be a selective inhibitor of cyclooxygenase (COX)-1, an enzyme which is the part of the prostaglandin synthesis cascade and thus involved e.g. in inflammatory reactions and pains\(^\text{24}\). *Biophytum sensitivum* showed that methanolic extracts of *Biophytum sensitivum* have apoptotic effect on B16F-10 cells, and regulatory effects on NO- and cytokine production on tumor-associated macrophages. They also found that the methanolic extract stimulates the immune cell system in mice, leading to immune cell proliferation and that this, in turn, can stimulate NK cell-mediated tumor lysis. The anti-inflammatory activity of aqueous and methanol extracts of aerial parts, an aqueous extract of roots as well as ultrafiltration fractions of a methanol extract of roots of *Biophytum sensitivum* were evaluated in the carrageenan induced rat paw oedema model. All the extracts except the methanol extract of aerial parts exhibited anti-inflammatory activity, but inhibition of edema was found to be maximum with aqueous extracts\(^\text{25}\).

*Biophytum sensitivum* treatment significantly reduced lung tumor nodule formation, accompanied by reduced lung collagen hydroxyproline, hexosamine, and uronic acid levels. *Biophytum sensitivum* treatment down-regulated the expression of matrix metalloprotease-2 and -9 and at the same time up regulated the lung tissue inhibitor of metalloprotease-1 and -2 expression. *Biophytum sensitivum* treatment could alter proinflammatory cytokine production and could inhibit the activation and nuclear translocation of p65, p50, activated transcription factor-2, and cyclic adenosine monophosphate response element-binding protein in B16F-10 melanoma cells\(^\text{26}\). *Biophytum sensitivum* displayed antiangiogenic activity in both in vitro and in vivo models. Intraperitoneal administration of methanol extract of *Biophytum sensitivum* at a concentration of 50 mg/kg inhibited the tumor-directed capillary formation induced by B16-F10 melanoma cells and increased the level of proinflammatory cytokines such as IL-1\(\beta\), IL-6, TNF-\(\alpha\), GM-CSF, and VGEF (Vascular Endothelial Growth Factor), the
direct endothelial cell-proliferating agent\textsuperscript{27}. Wound healing properties of polysaccharides from \textit{Biophytum petersianum} Klotzsch were reported to be related to their effects on the complement system\textsuperscript{28}.

Leaves of \textit{Biophytum sensitivum} used in the treatment of Cataract\textsuperscript{29}. The larvicidal activity of acetone extract of leaves of \textit{Biophytum sensitivum} at different doses were analyzed on fourth instar \textit{Aedes aegypti} larvae which is a vector for the arboviruses responsible for yellow fever and dengue fever, both of which are endemic to Asia and Africa at three different concentrations (200, 300 and 500 mg/L) were used to determine larvicidal and consequent effects on adult emergence. Pupicidal activities of acetone extract showed the highest effect\textsuperscript{30,31}. Antimicrobial activity of aqueous, ethanol and acetone extracts of leaves of \textit{Biophytum sensitivum} were tested on clinical isolates of urinary pathogens viz., \textit{Escherichia coli}, \textit{Klebsiella} and \textit{Proteus} and by well diffusion method. Ethanolic and acetone extracts showed greater inhibitory action than the aqueous ones. The effect of these plants may delay the development of urinary disease\textsuperscript{32}. \textit{Biophytum petersianum} Klotzsch traditionally used in cerebral malaria. The plant is also used against stomach ache in Nigeria, and in Gabon the roots and seeds are considered to be purgative. The powdered seed mixed with shea butter is applied to wounds in Nigeria.

\textit{Biophytum intermedium} Wight

\textit{Biophytum intermedium} Wight is generally known as Paraainellipachalai\textsuperscript{33} in Tamil. It is a member of the genus \textit{Biophytum}. Oxalidales (Order), Oxalidaceae (Family), \textit{Biophytum} (Genus), \textit{B. intermedium} (Species).

Plant paste is consumed with water for the treatment of stomach disorder\textsuperscript{33}. The selected rare medicinal plant \textit{Biophytum intermedium} Wight have no scientific reports on pharmacological activity and may be considered as a new drug for antidiabetic and antioxidant activity.
AIM AND OBJECTIVES

Aim

To scientifically validate the selected rare medicinal plants *Biophytum sensitivum* DC. and *Biophytum intermedium* Wight in terms of

- Pharmacognostical,
- Phytochemical and
- Pharmacological investigations with special reference to anti-diabetic property.

Objectives

- To fix the pharmacognostical standards for the selected rare medicinal plants with special reference to the macroscopical and microscopical evaluation including powder microscopy, quantitative microscopy and proximate analysis.
- To isolate and characterize the phyto constituents responsible for exhibiting anti diabetic property.
- To give scientific evidence for the treatment of anti diabetic property.
- To show the potential of different diet pattern of people with normal food and high fat diet.
MATERIALS AND METHODS

Materials

The selected medicinal plants, *Biophytum sensitivum* DC. and *Biophytum intermedium* Wight are distributed in Tuticorin and Kuttralam respectively and they were freshly collected in the month of December and November 2012 respectively. They were authenticated (Specimen no. PARC 2013/2013 and PARC 2013/2012 respectively) by botanist Dr. P. Jayaraman, Director, Plant Anatomy Research Centre (PARC), Chennai, Tamilnadu, India. The whole parts of both plants were taken for the present study.

PHARMACOGNOSTICAL STUDIES

Macroscopical studies of the whole plants of *Biophytum sensitivum* DC. and *Biophytum intermedium* Wight

Taxonomy, synonym, vernacular names, geographical distribution, habit and habitat, description of plant like leaflets, flowers, fruits, and seeds were discussed in detail to identify the both plants with the support of photographs as an establishment of authenticity by botanist.

Microscopical studies of *Biophytum sensitivum* DC. and *Biophytum intermedium* Wight

The microscopical studies of leaflet, stem and root to ascertain the arrangement of tissues of both selected medicinal plants were performed. The T.S. of leaflet through midrib, crystal distribution around the vascular bundle of the midrib, leaf lamina, leaf margin, stomata, T.S. of stem, T.S. of root and the powder analysis of both plants of *Biophytum sensitivum* DC. and *Biophytum intermedium* Wight were performed.

Quantitative microscopy for the leaflets of *Biophytum sensitivum* DC. and *Biophytum intermedium* Wight

Leaf constants

The vein islet number, vein terminal number, stomatal number and stomatal index were determined on fresh leaflets using standard procedures.
Physico - chemical parameters for whole plant powders of *Biophytum sensitivum* DC. and *Biophytum intermedium* Wight\(^{45}\)

In physico-chemical evaluation, total ash value, acid insoluble ash, water soluble ash, sulphated ash, crude fibre content, and loss on drying were determined by using recommended procedures.

**Extractive values**

Extractive values for whole plant powders of *Biophytum sensitivum* DC. and *Biophytum intermedium* Wight by using solvents of increasing order of polarity and successively as per recommended procedures.

**Fluorescence analysis of powdered drug and extracts\(^{46}\)**

The fluorescence analysis of whole plant powders of *Biophytum sensitivum* DC. and *Biophytum intermedium* Wight were performed by using UV chamber at 254 nm as per recommended procedures.

**PHYTOCHEMICAL STUDIES**

**Extraction**

The whole plants were shade dried to control temperature, humidity and damage of active constituents. The dried whole plant materials were powdered separately by using grinder except leaflets which were manually grinded and defatted with petroleum ether separately. Defatted 500 gm of each powder was extracted by 95% ethanol in a Soxhlet apparatus for 72 h followed by concentration in rotary evaporator under reduced pressure at temperature 40-50\(^0\)C and then lyophilized to get a dry residue. The percentage yield of extracts were calculated with reference to air dried powder. Some part of the total extracts were used for qualitative phytochemical evaluation and rest of the extracts were used for pharmacological screening. The shade dried coarse powdered material (500 gm) separately extracted in Soxhlet apparatus using various solvents according to their polarity\(^{47}\).
Identification of phytoconstituents by preliminary phytochemical screening

All the extracts of both plants were subjected to qualitative chemical tests separately for the detection of various plant constituents. As per literatures, we have selected ethanolic extracts of both plants for further study. The ethanolic extracts of both plants showed the presence of plant constituents like flavonoids, alkaloids, carbohydrates, glycosides, phytosterol, saponins, tannins and phenolic compounds, proteins and free amino acids, lignins, volatile oils.48

Separation and isolation of plant constituents by chromatographic methods

Thin Layer Chromatography

The various extracts of whole plant powders of *Biophytum sensitivum* DC. and *Biophytum intermedium* Wight were subjected to thin layer chromatography using different mobile phases that are suitable for detecting various phytoconstituents like alkaloids, glycosides, flavonoids, steroids and essential oils, only ethanolic extracts have maximum flavonoid content and gave characteristic spots and the Rf values were calculated. As per literatures, various trials have been made for few solvent systems for flavonoids are given below.

Ethyl acetate : Acetic acid : Formic acid : Water (100:11:11:26)49,50

Butanol : Acetic acid : Water (4:1:5)51

Toluene : Methanol : Acetic acid (20:2:1)52

Chloroform : Ethyl acetate : Methanol (14:3:3)53

n-Butanol : Chloroform : Acetic acid : Water (7:3:1:1)54

Out of the various trials made in TLC for the mobile phase, Toluene : Methanol : Acetic acid (20:2:1) was found to be effective.

High Performance Thin Layer Chromatography55

The ethanolic extract of *Biophytum sensitivum* DC. (EEBS) and ethanolic extract of *Biophytum intermedium* Wight (EEBI) were subjected to HPTLC for the separation of phytoconstituents. HPTLC was performed on silica gel 60F254 TLC plates (E, Merck,
Germany), Toluene: methanol: Glacial acetic acid (20:2:1), as mobile phase. Sample and standard at different aliquots of 1,2,3,4,5µl were applied to the plates as 5 mm wide from the bottom, by means of pressurised nitrogen gas (150 kg/cm²) through CAMAG Linomat V fitted with a 100 µl syringe. Ascending development, with the mobile phase consisting of solvent was performed in a twin-trough glass chamber (10x10cm) obtained from CAMAG, with previously saturated mobile phase for 30 minutes at room temperature (25±2°C) and relative humidity (60±5%). Subsequent to the development, the TLC plates were dried in air flow. Plates were then scanned at 254 nm (deuterium lamp) with the CAMAG TLC scanner 3 (slit dimension : 3mm x 0.45mm, scanning speed : 20mm s⁻¹). The peak areas and peak heights were recorded.

**Column chromatography**

The EEBS and EEBI were subjected to column chromatography for the separation of phytoconstituents. A column of suitable size (1m X 1.5 inch) was chosen and packed with silica gel 60-120 mesh by adding slurry of the adsorbent in petroleum ether. EEBS and EEBI were dissolved in ethanol, and mixed with silica gel (60-120 mesh) and fed to the column through a funnel. Petroleum ether was added to the column and kept aside without disturbance for overnight for the settlement of the extracts. Maximum precautions were taken to remove the air bubbles. The column was eluted with different organic solvents in the order of increasing polarity (petroleum ether, chloroform, acetone, ethyl acetate and ethanol). All the fractions were subjected to TLC studies by using above mentioned solvent systems used for TLC of extracts. Fractions showing similar Rf value, melting point and identification test, were pooled together and solvents evaporated to get residues. The residues were named as BSC 1 (Biophytum sensitivum compound 1) and BIC 2 (Biophytum intermedium compound 2).

**Spectral characterization of isolated plant compounds BSC 1 and BIC 2**

**Fourier Transformed Infra Red spectroscopy**

IR spectra of BSC 1 and BIC 2 were recorded using Perkin Elmer spectrometer FT-IR SPECTRUMONE. The samples were mixed with 3 mg of KBr procured from Merck chemicals which were previously dried in a oven at 120°C for overnight and
compressed into a thin transparent pellet using a hydraulic press under 10t/cm$^2$ pressure and 20mbar vacuum. The pellet placed directly in the standard sample mount of a FT-IR spectrometer. The spectrums were measured by wavelength ranging from 4000cm$^{-1}$ to 400cm$^{-1}$ at a resolution of 4cm$^{-1}$. IR is used to probe bond vibrations and bending in molecules and to reveal the types of functional groups present in compound.

**Nuclear Magnetic Resonance spectroscopy**

NMR spectra of BSC 1 and BIC 2 were recorded using a Bruker, 300MHz spectrometer 9.4 Tesla super-conducting magnet equipped with a BBO 300MHz, with Z-gradient nucleus probe, operating temperature range 20°C. NMR is an important spectroscopic method and a premier organic spectroscopy to determine the detailed chemical structure of the chemicals that are isolated from natural products. The structures of isolated compounds were elucidated by $^1$H-NMR and $^{13}$C-NMR (BRUKER Avance 111 300MHz) analysis. The samples were dissolved in D$_2$O and values were measured in δppm. NMR spectra may also be used for compound identification, by a fingerprint technique and sometimes as a specific method of assay for the individual components of a mixture.$^{58}$

**Liquid Chromatography-Mass Spectroscopy (LCMS)$^{59}$**

**Method:**

BSC 1 and BIC 2 were analyzed using UHPLC + Focused with reverse-phase Acclaim 120, RP-C18 120 Å, 2.1 × 150mm, 3.0μm column (Dionex, USA). Mass analyzer Quadrupole II and Detector TOF (Bruker, Germany). Experimental conditions used for LC-MS/MS analysis were given below.

**LC conditions:** LC-MS analysis was set with UV at 325nm, at the flow rate of 0.2mL/min, gradient mobile system start with 0.2 min at 99% CAN (1% acetic acid) and 1% water to 75% water at 16th minute, this was brought to 99% water in 19th minute and was maintained at same condition until run ends for another at 23rd minute.
**MS conditions:** MS analysis was performed using ESI in the negative mode, Nebulizer pressure was 30.5psi with 6.0 l/min N₂ flow, m/z range: 50-1000m/z, Capillary voltage was 4500V, dry heat temperature at 280°C.

Mass spectroscopy provides molecular weight for the isolated compounds. It relies of production of ions from a parent compound and the subsequent characterization of the pattern that are produced.

**PHARMACOLOGICAL STUDIES**

**Experimental animals**

Healthy adult male Spraque Dawley rats (250-260 gm) were used for this study. The animals were stabilized for 1 week, housed in polypropylene cages, maintained under standard conditions (12hr light and 12hr dark cycle, 25±30°C), the animals were fed with standard pellet diet and water *ad-libitum* throughout the course of the study. The animals were handled gently to avoid giving them too much stress, which could result in an increased adrenal output. The experimental protocol was approved by Institutional Animal Ethics Committee (IAEC No. SVCP/IAEC/Ph.D/01/2013) after scrutinization. The normoglycemic animals were selected for this experiment having the fasting blood glucose level around 80 mg/dL. The hyperglycemic animals were selected having the fasting blood glucose concentration around 200-300 mg/dL.

**Preparation of the dose**

The suspension of ethanolic extracts of selected medicinal plants were freshly prepared in 0.1% CMC and suspended in distilled water and Glibenclamide 0.5 mg/kg/p.o was prepared in 0.1% CMC suspended in distilled water.
Acute oral toxicity study (OECD guidelines, 2001)

Principle

The acute toxicity test aims at establishing the therapeutic index. The acute toxicity study was done according to OECD guidelines (Organization for Economic Co-operation and Development, Guideline - 423, 2001).

Procedure

The suspension of ethanolic extracts of selected medicinal plants were administered orally to overnight fasted male Sprague Dawley rats (n=6) at a dose of 2000 mg/kg body weight respectively. The animals were observed continuously for the initial 4h for behavioral changes and mortality and intermittently for the next 6h and then again at 24h and 48h after dosing for 14 days. The parameters observed were sensitivity response, piloerection, locomotion, salivation, defecation and urination.

Selection of doses:

In this study dose of 2000 mg/kg was found to be safe, so dose 1/10\textsuperscript{th} and 1/5\textsuperscript{th} i.e. 200 mg/kg and 400 mg/kg were chosen for the experimentation.

Determination of Oral Glucose Tolerance Test (OGTT) in normal rats

Procedure

OGTT experiment was performed as explained by Bonner-Weir\textsuperscript{60}. The normal rats were divided into six groups (n=6) and were fasted overnight (18h). The next day the rats were administered either drinking water or Glibenclamide (0.5 mg/kg/p.o., used as the standard drug) or 200 and 400 mg/kg of the extracts. Glucose (2g/kg) was administered 30 minutes after the feeding of the extract. Blood was withdrawn from the tail vein under ether inhalation anesthesia at 240 minutes of glucose administration and estimated by using glucose oxidase strip.

Group I  – Normal control
Group II  – Glibenclamide (Standard) (0.5 mg/kg b.w., p.o)
Group III – Animals received EEBS (200 mg/kg/p.o)
Group IV – Animals received EEBS (400 mg/kg/p.o)
Group V – Animals received EEBI (200 mg/kg/p.o)
Group VI – Animals received EEBI (400 mg/kg/p.o)

Evaluation of anti diabetic potential of *Biophytum sensitivum* DC. and *Biophytum intermedium* Wight

Experimental design and Development of High Fat Diet (HFD) fed and Streptozotocin (STZ) treated type 2 diabetic rats

Rats were divided into seven groups of six animals each. Group I (normal control) - administered vehicle alone (Carboxy Methyl Cellulose CMC 0.1%; 1ml/kg/b.w); Group II - diabetic control rats fed with HFD (40% fat, 18% protein and 41% carbohydrate as a percentage of total kcal) for 2 weeks and injected with low dose STZ (35 mg/kg b.w., i.p., in citrate buffer; pH 4.5); Group III (Standard) - diabetic rats administered Glibenclamide (0.5 mg/kg b.w., p.o); Group IV (HFD+STZ+EEBS (200 mg/kg b.w., p.o); Group V (HFD+STZ+EEBS (400 mg/kg b.w., p.o); Group VI (HFD+STZ+EEBI (200 mg/kg b.w., p.o); Group VII (HFD+STZ+EEBI (400 mg/kg b.w., p.o) rats were fed with HFD for 2 weeks and then injected with STZ and then supplemented with ethanolic extracts of selected medicinal plants with the dose of 200 and 400 mg/kg b.w., p.o for 4 weeks. The development of hyperglycemia was confirmed by fasting blood glucose (FBG) estimation after 6 days of STZ injection. The animals that maintained fasting blood glucose concentration around 200 - 300mg/dl were considered diabetic and selected for studies. The test extracts treatment was started after diabetes was confirmed and dose was determined from previous study. After the 28 days of treatment, blood was collected from tip of the tail vein and allowed to clot and the serum was separated by centrifugation at 3500 rpm for 10 minutes. Serum was assayed either immediately or stored at -20°C for biochemical estimation.
Experimental animal groups

Group I - Normal control (CMC 0.1%; 1ml/kg/b.w)
Group II - Diabetic control (HFD+STZ 35 mg/kg b.w., i.p)
Group III - Standard (HFD+STZ+Glibenclamide, 0.5 mg/kg b.w., p.o)
Group IV - HFD+STZ+EEBS (200 mg/kg/p.o)
Group V - HFD+STZ+EEBS (400 mg/kg/p.o)
Group VI - HFD+STZ+EEBI (200 mg/kg/p.o)
Group VII - HFD+STZ+EEBI (400 mg/kg/p.o)

Effect of EEBS and EEBI on various parameters of High Fat Diet (HFD) and low dose Streptozotocin (STZ) induced type 2 diabetic rats

Evaluation of changes in body weight

After the 28 days of treatment, the body weight of experimental animals were recorded on 0th and 28th day of the experiment.

Collection of blood and serum samples

After the 28 days of treatment, blood was collected from the heart by cardiac puncture and allowed to clot and the serum was separated by centrifugation at 3500 rpm for 10 minutes. Serum was assayed either immediately or stored at -20°C for biochemical estimation.

Biochemical estimation

Estimation of blood glucose levels: Blood glucose concentration was measured by end point colorimetric enzymatic test using glucose oxidase and peroxidase (GOD-POD) method.

Estimation of lipids in serum: Serum was analyzed for serum total cholesterol (TC), serum triglycerides (TG), high density lipoprotein (HDL) were estimated using standard enzymatic colorimetric kits (S.K. Chemical and surgical distributors., Chennai), low
density lipoprotein (LDL) and very low density lipoprotein (VLDL) were calculated using formula.

**Estimation of serum Total Cholesterol:** Span diagnostic kit (S.K. Chemical and surgical distributors., Chennai) was used for the estimation of total cholesterol, which followed cholesterol oxidase/peroxidase (CHOD-POD) method\(^ {64} \).

**Estimation of serum triglycerides:** Span diagnostic kit (S.K. Chemical and surgical distributors., Chennai) was used for the estimation of serum triglycerides, which followed end point colorimetric enzymatic test using glycerol-3-phosphate oxidase\(^ {64} \).

**Estimation of serum High-Density Lipoprotein Cholesterol (HDL-C):** Span diagnostic kit (S.K. Chemical and surgical distributors, Chennai) was used for the estimation of HDL cholesterol, which followed cholesterol oxidase/peroxidase (CHOD-POD) method.

**Estimation of serum Low-Density Lipoprotein Cholesterol (LDL-C):** Using the data obtained including total cholesterol, HDL cholesterol and VLDL, the LDL cholesterol levels were calculated using the empirical equation of Friede Wald\(^ {65} \).

**Estimation of serum Very Low-Density Lipoprotein Cholesterol (VLDL-C):** Using the data obtained including triglycerides, the VLDL cholesterol levels were calculated using the empirical equation of Friede Wald\(^ {66} \).

**Estimation of serum total protein (Biuret method):** Serum total protein levels were estimated by using Span diagnostic kit procured from S.K. Chemical and surgical distributors, Chennai\(^ {67} \).

**Estimation of serum creatinine (Modified jaffe’s kinetic method):** Serum creatinine levels were estimated by using test kit (S.K. Chemical and surgical distributors, Chennai)\(^ {68} \).

**Estimation of serum urea:** Serum urea levels were estimated by using test kit (S.K. Chemical and surgical distributors, Chennai).
Estimation of Serum Glutamate Pyruvate Transaminase (SGPT): Serum glutamate pyruvate transaminase, SGPT was determined by using Reitman’s and Frankel (1957) method\textsuperscript{69}.

Estimation of Serum Glutamate Oxaloacetate Transaminase (SGOT): Serum oxaloacetate transaminase, SGOT was determined by using Reitman and Frankel method (1957) method\textsuperscript{69}.

Estimation of \textit{in-vivo} antioxidant activity

Preparation of Hepatic Post Mitochondrial Supernatant (PMS): Liver was perfused with ice cold saline (0.9\% w/v sodium chloride) and homogenized in chilled phosphate buffer (pH 7.4) using a glass homogenizer. The homogenates were centrifuged at 800g for 5 minutes at 4\textdegree{}C to separate the nuclear debris. The supernatant so obtained was centrifuged at 10,000g for 20 minutes at 4\textdegree{}C to get the Post-Mitochondrial Supernatant (PMS), which was used to assay the following \textit{in-vivo} antioxidant parameters\textsuperscript{70}.

Estimation of Lipid Peroxidation (LPO) from hepatic PMS: Oxidative stress is associated with peroxidation of cellular lipids, which is determined by measurement of Thio Barbituric Acid Reacting Substance (TBARS). The concentration of LPO products may reflect the degree of oxidative stress. The increased level of TBARS, results in attacks the polyunsaturated fatty acids in cell membranes and cause LPO. The malondialdehyde (MDA) content, a measure of lipid peroxidation was assayed in the form of TBARS\textsuperscript{70}.

Estimation of Super Oxide Dismutase (SOD) from hepatic PMS: Superoxide dismutase was assayed by the method of Marklund and Marklund (1974)\textsuperscript{71}.

Estimation of Catalase (CAT) from hepatic PMS: The Catalase activity was assayed by the method of Sinha (1972)\textsuperscript{72}.

Organ and fat pad weights: The animals were sacrificed by cervical dislocation and then different organ (liver, heart, kidney) and fat pads (mesenteric, left and right perirenal fat pads) were removed and weighed\textsuperscript{73}.
Estimation of liver triglyceride and total cholesterol

Extraction of liver lipids: 1gm of liver was distilled to a volume of 20ml with 2:1 chloroform-methanol mixture (v/v) and was homogenized for 3 minutes. The homogenate was filtered through a fat-free paper into a glass-stoppered vessel. The extract obtained corresponds to 0.05 its volume of tissue; i.e., 1ml of extract corresponds to 0.05 gm of tissue, 10ml of the extract was taken and was added with 2ml of water set to centrifugation for 20 minutes at 2400 rpm. The supernatant was discarded and the lower phase, which contains the lipid matter of liver, was used for the estimation of total cholesterol and triglyceride like in serum by span diagnostic kits.\(^7\)

Histopathological studies

At the end of the experimental period, the animals were sacrificed by cervical dislocation. The pancreas, kidney and liver samples were fixed for 48 hr in 10% formalin saline were dehydrated by passing successfully in different mixture of ethyl alcohol-water, cleaned in xylene, and embedded in paraffin. Sections of pancreas, kidney and liver were prepared and, then, stained with hematoxylin and eosin dye (H&E), which was mounted in neutral deparaffinated xylene (DPX) medium for microscopic observations.\(^5\)

Statistical analysis

The data were expressed as Mean ± SEM. The data of antidiabetic activity were analyzed by one-way analysis of variance (ANOVA) followed by Dunnet’s ‘t’ test. P value less than 0.01 was considered as statistically significant.
RESULTS AND DISCUSSION

In pharmacognostical studies, the macroscopical, microscopical studies of leaflet, stem and root were performed to ascertain the arrangement of tissues of both selected medicinal plants were evaluated. The T.S. of leaflet through midrib, crystal distribution around the vascular bundle of the midrib, leaf lamina, leaf margin, stomata, T.S. of stem, T.S. of root and the powder analysis of both plants were evaluated. Quantitative microscopy, physico-chemical evaluation, extractive values and fluorescence analysis for both plants were determined.

In phytochemical studies, the ethanolic extracts of both plants showed the presence of plant constituents like flavonoids, alkaloids, carbohydrates, glycosides, phytosterol, saponins, tannins and phenolic compounds, proteins and free amino acids, lignins, volatile oils by qualitative chemical tests.

The various extracts of whole plant powders of *Biophytum sensitivum* DC and *Biophytum intermedium* Wight were subjected to thin layer chromatography using different mobile phases that are suitable for detecting various phytoconstituents like alkaloids, glycosides, flavonoids, steroids and essential oils preliminarily. Only ethanolic extracts gave characteristic spots. As per literatures, *Biophytum* genus plants have been reported to contain flavonoids and flavonoids maximum extracted by ethanol also. So we have concentrated in ethanolic extracts of selected medicinal plants. As per literatures, various trials have been made for few solvent systems for flavonoids. Out of the various trials made in TLC, for the mobile phase, Toluene : Methanol : Acetic acid 20:2:1, (v/v) was found to be effective. The ethanolic extracts of both plants were subjected to thin layer chromatography using Toluene : Methanol : Acetic acid (20:2:1) as mobile phase, showed six spots with Rf values were calculated as 0.25, 0.36, 0.40, 0.51, 0.65 and 0.77 in EEBS and five spots with Rf values were calculated as 0.29, 0.38, 0.64, 0.71 and 0.76 in EEBI detected in UV 365nm.

The EEBS and EEBI were subjected to HPTLC for the separation of phytoconstituents using Toluene : Methanol : Glacial acetic acid, 20:2:1, (v/v) as mobile phase at 254nm. The extracts were run along with the standard flavonoid compound and
observed that the extracts showed the presence of flavonoids and confirmed from the chromatogram after derivatization. The \( R_f \) value of the different compounds of flavonoids present in the EEBS was found to be 0.02, 0.20, 0.26, 0.35, 0.42, 0.50, 0.63, 0.69 and 0.76 of peak 1, 2, 3, 4, 5, 6, 7, 8 and 9 respectively. The \( R_f \) value of the different compounds of flavonoids present in the EEBI was found to be 0.19, 0.27, 0.36, 0.63, 0.72 and 0.74 of peak 1, 2, 3, 4, 5 and 6 respectively. Among them, peak 5 (\( R_f \) value 0.42) was identified as flavonoid glycoside in EEBS and peak 5 (\( R_f \) value 0.72) was identified as flavonoid glycoside in EEBI as per literatures.

The EEBS and EEBI were subjected to column chromatography for the separation of phytoconstituents. All the fractions were subjected to TLC studies by using Toluene : Methanol : Glacial acetic acid, 20:2:1, (v/v) as mobile phase. Fractions showing similar \( R_f \) value, melting point and identification test, were pooled together and solvents evaporated to get residues. The residues were named as BSC 1 and BIC 2. BSC 1 was collected from the fractions of ethyl acetate : ethanol (20:80, 40:60, 50:50). The fractions of ethyl acetate : ethanol (40:60, 50:50, 60:40) yielded BIC 2.

**Description of the isolated compound BSC 1 by column chromatography**

- **Nature**: Crystalline powder
- **Colour**: Yellow
- **Taste**: Tasteless
- **Solubility**: Soluble in ethanol
- **Melting point**: 202\(^0\)C - 207\(^0\)C
- **Mobile phase for TLC**: Toluene : Methanol : Glacial acetic acid, 20:2:1, (v/v)
- **\( R_f \) value**: 0.40
- **Identification test**: Answered positive for Shinoda’s test indicating the presence of flavonoids.

**Description of the isolated compound BIC 2 by column chromatography**

- **Nature**: Crystalline powder
- **Colour**: Yellow
- **Taste**: Tasteless
Solubility: Soluble in ethanol
Melting point: 225°C - 231°C
Mobile phase for TLC: Toluene : Methanol : Glacial acetic acid, 20:2:1, (v/v)
R_f value: 0.71
Identification test: Answered positive for Shinoda’s test indicating the presence of flavonoids.

Spectral characterization of isolated compounds BSC 1 and BIC 2

Fourier Transformed Infra Red spectroscopy (FT-IR spectroscopy)

The flavonoid glycosides present in both isolated compounds BSC 1 and BIC 2 were elucidated by Fourier Transformed Infra Red (FT-IR) spectrophotometer in KBr pellet method (Perkin Elmer spectrometer FT-IR SPECTRUMONE). IR values were measured in cm⁻¹.

Nuclear Magnetic Resonance spectroscopy (NMR spectroscopy)

The structures of flavonoid glycosides in isolated compounds BSC1 and BIC 2 were elucidated by ¹H-NMR and ¹³C-NMR (BRUKER Avance 111 300MHz) analysis. The samples were dissolved in D₂O and values were measured in δppm. From the ¹H-NMR and ¹³C-NMR spectrum, flavonoid glycosides were identified in both isolated compounds BSC 1 and BIC 2.

Liquid Chromatography-Mass Spectroscopy (LCMS)

The molecular weight of flavonoid glycosides present in both isolated compounds BSC 1 and BIC 2 were detected by LC-MS and LC-MS/MS. It relies of production of ions from a parent compound and the subsequent characterization of the pattern that are produced.

In pharmacological studies,

The effect of ethanolic extract of whole plants of Biophytum sensitivum DC and Biophytum intermedium Wight on acute oral toxicity test in male Sprague Dawley rats
The acute toxicity assay of ethanolic extracts of whole plants of *Biophytum sensitivum* DC. and *Biophytum intermedium* Wight were evaluated as per OECD guideline no.423. A single doses of EEBS and EEBI did not produce any signs of toxicity or mortality to the animals even at the dose of 2000 mg/kg. No animals showed any change in general behavior or other physiological activities. This revealed the non-toxic nature of the plants. So the extracts are safe for long term administration. The LD$_{50}$ was found to be 2000 mg/kg; so ED$_{50}$ was 200 mg/kg. Based on this evaluation, 200 and 400 mg/kg were taken as the lowest and highest dose respectively for remaining studies.

**Selection of doses:**

In this study, dose of 2000 mg/kg was found to be safe, so dose $1/10^{th}$ and $1/5^{th}$ i.e. 200 and 400 mg/kg were chosen for the experimentation.

**Effect of ethanolic extracts of whole plants of *Biophytum sensitivum* DC and *Biophytum intermedium* Wight on oral glucose tolerance test (OGTT) in male Sprague Dawley rats**

EEBS and EEBI supplementation at both 200 mg/kg and 400 mg/kg showed a significant decrease in glucose levels at 120 minutes to 240 minutes after oral glucose administration when compared to the control group. In Glibenclamide (0.5 mg/kg. b.w., p.o) group also, a decrease in glucose levels were detected at 120 minutes to 240 minutes after oral glucose administration. Thus the plant extracts EEBS (200 and 400 mg/kg) and EEBI (200 and 400 mg/kg) exhibited significant antihyperglycemic activity at 120 minutes to 240 minutes after glucose load when compared to control.

**Effect of ethanolic extracts of whole plants of *Biophytum sensitivum* DC and *Biophytum intermedium* Wight on body weight against high fat diet and low dose Streptozotocin induced diabetes mellitus in male Sprague Dawley rats**

In our study, after 28 days of diabetes induction with HFD and low dose STZ (35 mg/kg b.w/i.p), diabetic rats showed very significant increase in body weight as compared to control animals. Diabetic rats treated with Glibenclamide (0.5 mg/kg b.w/p.o.), EEBS 200 and 400 mg/kg and EEBI 200 and 400 mg/kg showed very significant
decrease in body weight as compared to diabetic control animals. As treatment with EEBS and EEBI showed significant reduction in body weight, the drug exhibited marked effect in controlling obesity during diabetes mellitus.

**Effect of ethanolic extracts of whole plants of *Biophytum sensitivum* DC and *Biophytum intermedium* Wight on blood glucose level against high fat diet and low dose Streptozotocin induced diabetes mellitus in male Sprague Dawley rats**

In our study, after 28 days of HFD and low dose STZ induction, the HFD and STZ (35 mg/kg b.w/i.p) control group showed very significant increase in blood glucose level. The blood glucose levels of Glibenclamide (0.5 mg/kg b.w/p.o.) treated, EEBS 200 and 400 mg/kg treated and EEBI 200 and 400 mg/kg treated groups showed very significant decrease in final fasting blood glucose level. This indicates that the plant extracts improved glucose tolerance, as indicated by a reduction in peak glucose level possess antihyperglycemic activity. Our results were consistent with the findings of previous studies that show decrease in elevated blood glucose level in HFD and STZ diabetic rats treated with plant extracts may be due to the stimulation of the β-cell of the pancreatic islets.

**Effect of ethanolic extracts of whole plants of *Biophytum sensitivum* DC and *Biophytum intermedium* Wight on serum lipid profile against high fat diet and low dose Streptozotocin induced diabetes mellitus in male Sprague Dawley rats**

In our study, after 28 days of HFD and low dose STZ induction, the HFD and STZ (35 mg/kg b.w/i.p) treated rats showed very significant hypercholesterolemia. But Glibenclamide (0.5 mg/kg b.w/p.o.) treated, EEBS 200 and 400 mg/kg treated and EEBI 200 and 400 mg/kg treated groups showed very significant decrease in total cholesterol levels.

Hypercholesterolemia was associated with hypertriglyceridemia in HFD and low dose STZ induced diabetic rats after the study period. But glibenclamide treated, EEBS 200 and 400 mg/kg treated and EEBI 200 and 400 mg/kg treated groups showed very significant decrease in triglyceride levels.
When compared to control group, the LDL levels of HFD and low dose STZ (35 mg/kg b.w/i.p) control group showed very significant increase. While Glibenclamide (0.5 mg/kg b.w/p.o.) treated, EEBS 200 and 400 mg/kg treated and EEBI 200 and 400 mg/kg treated groups showed very significant decrease in serum LDL.

After 28 days of HFD and low dose STZ (35 mg/kg b.w/i.p) induction, the VLDL levels of HFD and low dose STZ control group showed very significant increase. The Glibenclamide (0.5 mg/kg b.w/p.o.) treated, EEBS 200 and 400 mg/kg treated and EEBI 200 and 400 mg/kg treated groups showed very significant decrease in serum VLDL levels.

The HDL levels of HFD and low dose STZ (35 mg/kg b.w/i.p) control group showed very significant decrease after the study period. But Glibenclamide (0.5 mg/kg b.w/p.o.) treated, EEBS 200 and 400 mg/kg treated and EEBI 200 and 400 mg/kg treated groups showed very significant increase in serum HDL levels.

In our study, the HFD and low dose STZ (35 mg/kg b.w/i.p) control animals exhibited abnormalities in lipid metabolism as evidenced from the significant elevation of serum TC, TG, LDL, VLDL and reduction of HDL levels. Treatment with EEBS and EEBI for 28 days significantly reduced the serum TC, TG, LDL, VLDL levels and significantly increased HDL levels, which is considered to be good cholesterol that is anti-atherogenic in nature. Ethanolic extracts of *Biophytum sensitivum* and *Biophytum intermedium* thus have the potential to prevent the formation of atherosclerosis and CHD, which are secondary complications of diabetes mellitus. In all the cases the highest dose of EEBS and EEBI 400 mg/kg showed profound effect than the lowest dose of EEBS and EEBI 200 mg/kg which reveals that the drug acts on dose dependent manner.

**Effect of ethanolic extracts of whole plants of* Biophytum sensitivum* DC and *Biophytum intermedium* Wight on total protein, creatinine, urea and liver marker enzyme levels in serum against high fat diet and low dose Streptozotocin induced diabetes mellitus in male Sprague Dawley rats**

After 28 days of HFD and low dose STZ (35 mg/kg b.w/i.p) induction, the serum protein levels of HFD and low dose STZ control group showed very significant decrease.
But Glibenclamide (0.5 mg/kg b.w/p.o.) treated, EEBS 200 and 400 mg/kg treated and EEBI 200 and 400 mg/kg treated groups showed very significant increase in serum total protein levels.

After the treatment period of 28 days, the serum creatinine levels of HFD and low dose STZ (35 mg/kg b.w/i.p) control group showed very significant increase. But Glibenclamide (0.5 mg/kg b.w/p.o.) treated, EEBS 200 and 400 mg/kg treated and EEBI 200 and 400 mg/kg treated groups showed very significant decrease in serum creatinine levels.

At the end of treatment period, the serum urea levels of HFD and low dose STZ (35 mg/kg b.w/i.p) control group showed very significant increase. But Glibenclamide (0.5 mg/kg b.w/p.o.) treated, EEBS 200 and 400 mg/kg treated and EEBI 200 and 400 mg/kg treated groups showed very significant decrease in serum urea levels.

After 28 days of HFD and low dose STZ (35 mg/kg b.w/i.p) treatment, the SGOT levels of HFD and low dose STZ control group showed very significant increase. But Glibenclamide (0.5 mg/kg b.w/p.o.) treated, EEBS 200 and 400 mg/kg treated and EEBI 200 and 400 mg/kg treated groups showed very significant decrease in SGOT levels.

The SGPT levels of HFD and low dose STZ (35 mg/kg b.w/i.p) control group showed very significant increase after the study period. But Glibenclamide (0.5 mg/kg b.w/p.o.) treated, EEBS 200 and 400 mg/kg treated and EEBI 200 and 400 mg/kg treated groups showed very significant decrease in SGPT levels.

The treatment with EEBS and EEBI prevented such alterations and protected the histological aspects of kidney. Our work clearly showed elevated levels of kidney functional markers in serum creatinine and urea and significant reduction of total protein levels of HFD and low dose STZ control group. The serum GOT and GPT were used as markers to assess the extent of liver damage in HFD and low dose STZ-induced diabetic rats. In contrast, EEBS and EEBI treated rats showed significant changes in the level of these markers, bringing the values to near normalcy; thus showing its ability to protect against diabetes-induced kidney and liver damage. In all the evaluated kidney and liver
parameters, EEBI 400 mg/kg produced more effect than EEBI (200 mg/kg) and EEBS (200 and 400 mg/kg) that showed dose dependent action of the plant extracts.

Effect of ethanolic extracts of whole plants of *Biophytum sensitivum* DC and *Biophytum intermedium* Wight on kidney, pancreas and liver weights against high fat diet and low dose Streptozotocin induced diabetes mellitus in male Sprague Dawley rats

In HFD and low dose STZ (35 mg/kg b.w/i.p) control group, there was very significant increase in kidney weight when compared to control group. As compared to HFD and low dose STZ control group, Glibenclamide (0.5 mg/kg b.w/p.o.) treated, EEBS 200 and 400 mg/kg treated and EEBI 200 and 400 mg/kg treated groups showed very significant reduction in kidney weights.

The pancreas weight of HFD and low dose STZ (35 mg/kg b.w/i.p) control group, Glibenclamide (0.5 mg/kg b.w/p.o.) treated, EEBS 200 and 400 mg/kg treated and EEBI 200 and 400 mg/kg treated groups showed no significant changes when compared to control group. Diabetic rats treated with Glibenclamide (0.5 mg/kg b.w/p.o.), EEBS (200 & 400 mg/kg) and EEBI (200 & 400 mg/kg) groups also showed no significant changes when compared to HFD and low dose STZ control group.

In HFD and low dose STZ (35 mg/kg b.w/i.p) control group, there was very significant increase in liver weight when compared to control group. As compared to HFD and low dose STZ control group, Glibenclamide (0.5 mg/kg b.w/p.o.) treated, EEBS 200 and 400 mg/kg treated and EEBI 200 and 400 mg/kg treated groups showed very significant reduction in liver weights.

In HFD and low dose STZ (35 mg/kg b.w/i.p) control group, there was very significant increase in mesenteric fat pad weights when compared to control group. As compared to HFD and low dose STZ control group, Glibenclamide (0.5 mg/kg b.w/p.o.) treated, EEBS 200 and 400 mg/kg treated and EEBI 200 and 400 mg/kg treated groups showed very significant reduction in mesenteric fat pad weights.

In HFD and low dose STZ (35 mg/kg b.w/i.p) control group, there was very significant increase in perirenal fat weights when compared to control group. As compared
to HFD and low dose STZ control group, Glibenclamide (0.5 mg/kg b.w/p.o.) treated, EEBS 200 and 400 mg/kg treated and EEBI 200 and EEBI 400 mg/kg treated groups showed very significant reduction in perirenal fat weights. After the treatment with EEBS and EEBI showed significant decrease in weights of mesenteric and peri-renal fat pads compared with high fat diet group.

**Effect of ethanolic extracts of whole plants of *Biophytum sensitivum* DC and *Biophytum intermedium* Wight on serum antioxidant enzyme levels against high fat diet and low dose Streptozotocin induced diabetes mellitus in male Sprague Dawley rats**

After 28 days of HFD and low dose STZ (35 mg/kg b.w/i.p) induction the serum SOD level of HFD and low dose STZ treated group, showed very significant decrease. But after drug administration, Glibenclamide (0.5 mg/kg b.w/p.o.) treated, EEBS 200 and 400 mg/kg treated and EEBI 200 and 400 mg/kg treated groups showed very significant increase in serum SOD levels.

The serum CAT levels of HFD and low dose STZ (35 mg/kg b.w/i.p) control group showed very significant decrease after the study period. But Glibenclamide (0.5 mg/kg b.w/p.o.) treated, EEBS 200 and 400 mg/kg treated and EEBI 200 and 400 mg/kg treated groups showed very significant increase in serum CAT levels.

After the treatment period of 28 days, the serum LPO level of HFD and low dose STZ (35 mg/kg b.w/i.p) control group, showed very significant increase. But after drug administration, Glibenclamide (0.5 mg/kg b.w/p.o.) treated, EEBS 200 and 400 mg/kg treated and EEBI 200 and 400 mg/kg treated groups showed very significant decrease in serum LPO levels.

The serum GPx levels of HFD and low dose STZ (35 mg/kg b.w/i.p) control group showed very significant decrease after the study period. But Glibenclamide (0.5 mg/kg b.w/p.o.) treated, EEBS 200 and 400 mg/kg treated and EEBI 200 and 400 mg/kg treated groups showed very significant increase in serum GPx levels.

Administartion of EEBS and EEBI and Glibenclamide decreased the TBARS significantly in diabetic rats. Administration of EEBS and EEBI to the diabetic rats
brought back the antioxidant enzyme levels to near normal values. This profound changes in the activities of the antioxidant enzymes in EEBS and EEBI treated rats unravels the efficacy of the drug in resisting oxidative insult due to diabetes. In all the above mentioned parameters, the highest dose EEBI 400mg/kg showed profound effect than the EEBI 200mg/kg and EEBS 200 & 400mg/kg which reveals that the drug acts on dose dependent manner.

**Effect of ethanolic extracts of whole plants of *Biophytum sensitivum* DC and *Biophytum intermedium* Wight on liver triglyceride and cholesterol against high fat diet and low dose Streptozotocin induced diabetes mellitus in male Sprague Dawley rats**

After the treatment period of 28 days, the liver triglyceride level of HFD and low dose STZ (35 mg/kg b.w/i.p) control group, showed very significant increase. But after drug administration, Glibenclamide (0.5 mg/kg b.w/p.o.) treated, EEBS 200 and 400 mg/kg treated and EEBI 200 and 400 mg/kg treated groups showed very significant decrease in liver triglyceride levels.

At the end of 28 days, the liver cholesterol level of HFD and low dose STZ (35 mg/kg b.w/i.p) control group showed very significant increase. But after drug administration, Glibenclamide (0.5 mg/kg b.w/p.o.) treated, EEBS 200 and 400 mg/kg treated and EEBI 200 and 400 mg/kg treated groups showed very significant decrease in liver cholesterol levels.

From the above investigation, EEBS and EEBI can be useful in lowering the liver content of TG and cholesterol and treating fatty liver in hyperlipidemic regime. Therefore, consumption of EEBS and EEBI can be useful to reduce the risk of cardiovascular diseases, fatty liver, and atherosclerosis.

**Effect of ethanolic extracts of whole plants of *Biophytum sensitivum* DC and *Biophytum intermedium* Wight on histopathological analysis of toxicity studies in male Sprague Dawley rats**

The toxic effect of EEBS and EEBI on histopathological analysis of kidney, heart, liver and brain showed normal nephrotic bundles in kidney, no signs of infarct and normal
fiber appears in heart and showed marginal hepatocytes at regular interval and normal lumen of hepatic veins appears in liver and showed no signs of edema or degeneration and neuron appears normal with prominent nucleus in control and EEBS and EEBI treated groups.

**Effect of ethanolic extracts of whole plants of *Biophytum sensitivum* DC and *Biophytum intermedium* Wight on histopathological sections of kidney against high fat diet and low dose Streptozotocin induced diabetes mellitus in male Sprague Dawley rats**

In the histopathological studies, kidney sections in normal control group showed glomeruli and proximal convoluted tubules. The kidney sections of diabetic rats showed congestion, proteinuria, haemorrhage and tubular degeneration. In diabetic rats treated with EEBS and EEBI (200 & 400mg/kg) showed glomeruli and tubules without proteinuria and haemorrhage. The diabetic rats treated with Glibenclamide showed improved features like somewhat normal glomerulus and tubules.

**Effect of ethanolic extracts of whole plants of *Biophytum sensitivum* DC and *Biophytum intermedium* Wight on histopathologic sections of pancreas against high fat diet and low dose Streptozotocin induced diabetes mellitus in male Sprague Dawley rats**

However compared to the HFD and low dose STZ (35 mg/kg b.w/i.p) control rats, histopathological examination of the EEBS and EEBI treated diabetic rats revealed an increase in the number of pancreatic islets and the number of $\beta$-cells i.e. EEBS and EEBI treated diabetic samples histopathologically approach the corresponding healthy pancreatic samples. The regeneration of the $\beta$-cells of the HFD and low dose destructed islets is probably due to the fact that pancreas contains stable (Quiescent) cells which have the capacity of regeneration. Therefore, the surviving cells can proliferate to replace the lost cells.
Effect of ethanolic extracts of whole plants of *Biophytum sensitivum* DC and *Biophytum intermedium* Wight on histopathologic sections of liver against high fat diet and low dose Streptozotocin induced diabetes mellitus in male Sprague Dawley rats

The histological findings of the liver sections normal control rats showed shows intact parenchyma with marginal arrangement of hepatocyte and no signs of edema and inflammation. On the contrary, in the diabetic rats exhibited signs of degeneration with infiltration of polymorpho nuclear cells, glycogen deposition, fatty changes. In diabetes, degradation of liver glycogen and gluconeogenesis are increased while glucose utilization is inhibited. The pathological alteration of diabetic liver was due to glycosylation of proteins leading to abnormalities in hepatic ultrastructures. Treatment with the EEBS and EEBI and Glibenclamide showed improvement in histological structure of the liver sections of the diabetic rats and showed decreased intra hepatocellular space.
SUMMARY AND CONCLUSION

Diabetes mellitus is the most common endocrine disorder that affects more than 285 million people worldwide. The number is expected to grow to 438 million by 2030, corresponding to 7.8% of the adult population. In addition to the primary effects of diabetes, this disease is accompanied by increased risk factors such as hyperglycemia, hypertension, dyslipidemia, severe atherosclerosis etc. Though there are wide developments in the treatment of diabetes mellitus by synthetic drugs, side effects such as hypoglycemia at higher dose administration, low oral bioavailability due to degradation in stomach makes it necessary to find other alternatives. Therefore, there is an owing interest in discovering natural treatments without negative side effects that can reduce these risk factors in diabetic patients.

The plant *Biophytum sensitivum* have a long history of use as a natural medicine by the native in the tropics. But *Biophytum intermedium* have not been scientifically validated. The flavonoids present in *Biophytum sensitivum* have shown in clinical research to possess active cancer-killing properties against various cancers. This may be due to its action as antioxidant. The *Biophytum* genus plants belonging to Oxalidaceae, had proven its effect as antidiabetic. Based on these literature reviews, ethanolic extract of whole plants of *Biophytum sensitivum* DC. and *Biophytum intermedium* Wight were selected for the study.

The macroscopical, microscopical studies of leaflet, stem and root to ascertain the arrangement of tissues of both selected medicinal plants were evaluated. The T.S. of leaflet through midrib, crystal distribution around the vascular bundle of the midrib, leaf lamina, leaf margin, stomata, T.S. of stem, T.S. of root and the powder analysis of both plants were evaluated. Quantitative microscopy, physico-chemical evaluation, extractive values and fluorescence analysis for both plants were determined.

The shade-dried coarse powdered material (500 gm) separately extracted in Soxhlet apparatus using various solvents according to their polarity. As per literatures, we have selected ethanolic extracts of both plants for further study. The ethanolic extracts of both plants showed the presence of plant constituents like flavonoids, alkaloids, carbohydrates,
glycosides, phytosterol, saponins, tannins and phenolic compounds, proteins and free amino acids, lignins, Volatile oils by qualitative chemical tests.

The various extracts of whole plant powders of *Biophytum sensitivum* and *Biophytum intermedium* were subjected to thin layer chromatography using mobile phase, Toluene : Methanol : Acetic acid 20:2:1, (v/v) was found to be effective. showed six spots with R_f values were calculated as 0.25, 0.36, 0.40, 0.51, 0.65 and 0.77 in EEBS and five spots with R_f values were calculated as 0.29, 0.38, 0.64, 0.71 and 0.76 in EEBI detected in UV 365nm.

The EEBS and EEBI were subjected to HPTLC for the separation of phytoconstituents using toluene : methanol : Glacial acetic acid, 20:2:1, (v/v) as mobile phase with the standard flavonoid compound at 254nm and observed that the extracts showed the presence of flavonoids and confirmed from the chromatogram after derivatization. The R_f value of the different compounds of flavonoids present in the EEBS was found to be 0.02, 0.20, 0.26, 0.35, 0.42, 0.50, 0.63, 0.69 and 0.76 of peak 1, 2, 3, 4, 5, 6, 7, 8 and 9 respectively. The R_f value of the different compounds of flavonoid glycoside present in the EEBI was found to be 0.19, 0.27, 0.36, 0.63, 0.72 and 0.74 of peak 1, 2, 3, 4, 5 and 6 respectively. Among them, peak 5 (R_f value 0.42) was identified as flavonoid glycoside in EEBS and peak 5 (R_f value 0.72) was identified as flavonoid glycoside in EEBI as per literatures.

The EEBS and EEBI were subjected to column chromatography for the separation of phytoconstituents. All the fractions were subjected to TLC studies by using Toluene : Methanol : Glacial acetic acid, 20:2:1, (v/v) as mobile phase. Fractions showing similar R_f value, melting point and identification test, were pooled together and solvents evaporated to get residues. The residues were named as BSC 1 and BIC 2. BSC 1 was collected from the fractions of ethyl acetate : ethanol (20:80, 40:60, 50:50). The fractions of ethyl acetate : ethanol (40:60, 50:50, 60:40) yielded BIC 2.

From the FT-IR, ^1^H-NMR and ^13^C-NMR and LC-MS spectrum of both isolated compounds BSC 1 and BIC 2, provides details about presence of functional groups, structural elucidation and molecular weights for the flavonoid glycosides present in both
isolated compounds BSC 1 and BIC 2. Finally from these spectral studies, flavonoid glycosides were identified in both isolated compounds BSC 1 and BIC 2.

The acute toxicity assay of ethanolic extracts of whole plants of *Biophytum sensitivum* and *Biophytum intermedium* revealed the non-toxic nature of the plants.

Administration of ethanolic extracts of whole plants of *Biophytum sensitivum* and *Biophytum intermedium* (EEBS and EEBI) to diabetic rats significantly recovered the levels of blood glucose, body weight, serum profiles such as total cholesterol, HDL cholesterol, LDL cholesterol, VLDL cholesterol, triglycerides, total protein, serum creatinine, serum urea, SGOT, SGPT, antioxidant enzyme levels such as SOD liver tissue, CAT liver tissue, LPO, GPx liver tissue, organ weight such as kidney, liver, mesenteric fat pad, perirenal fat, liver cholesterol and triglyceride level compared to untreated diabetic rats in type 2 diabetes models. It was found that oral administration of extract shows nearly equal effectiveness in controlling diabetics when compared with diabetic rats treated with standard drug (glibenclamide). Histopathological studies of kidney, pancreas and liver showed better improvement after EEBS and EEBI treated rats compared to diabetic rats.

It was observed that, treatment of diabetic rats with ethanolic extracts of whole plants of *Biophytum sensitivum* and *Biophytum intermedium* had not only shown a significant antidiabetic activity but also possess an effective antioxidant activity which was evident from the observations. All the diabetic rats treated with ethanolic extracts elevated the levels of antioxidant enzymes. The extracts are thus expected to possess antioxidant activity.

In conclusion, ethanolic extracts of whole plants of *Biophytum sensitivum* DC. and *Biophytum intermedium* Wight showed significant antidiabetic and antioxidant effect in diabetic rats after oral administration. It could be speculated that the observed antihyperglycemic and antioxidant activity of *Biophytum sensitivum* DC. and *Biophytum intermedium* Wight due to the presence of flavonoid glycosides as active constituents. The present investigation has also opened an avenue for further research especially with reference to the development of potent formulation for diabetes mellitus from the whole plants of *Biophytum sensitivum* DC. and *Biophytum intermedium* Wight.
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