Int J Pharm Bio Sci 2015 July.; 6(3): (B) 732 - 741

Review Article **Pharmacology**

International Journal of Pharma and Bio Sciences **ISSN 0975-6299**

MODELS TO STUDY *IN VITRO* ANTIDIABETIC ACTIVITY OF PLANTS: A REVIEW

DORIN DSOUZA AND N. LAKSHMIDEVI*

Department of Studies in Microbiology, Manasagangotri, Mysore 570 006, Karnataka, India.

ABSTRACT

Antidiabetic effect of plants and their active principles can be assessed *in vitro* using a variety of biological test systems. They play a major role in evaluation of antidiabetic properties as an initial screening tool prior to *in vivo* studies. The present review focuses on *in vitro* assays that are available to study potential antidiabetic activity of plant extracts and their active constituents. It is evident that these plant extract and compounds derived from them are capable of lowering blood glucose level through different mechanism of action. This has attracted a great deal of research interest in exploring natural sources. *In vitro* assay provides a basic platform for accusing these plant extracts and help us understand various mechanisms that would alleviate hyperglycaemia in diabetes.

KEYWORDS: ***In vitro*, α-amylase, α-glucosidase, Glucose uptake, Cell lines, Glut 4, PPAR- γ**

***Corresponding author**

N. LAKSHMIDEVI
Department of Studies in Microbiology, Manasagangotri, Mysore 570 006, Karnataka, India.

INTRODUCTION

Diabetes Milletus (DM) is a major metabolic disorder causing morbidity and mortality worldwide[1], characterized by elevated plasma glucose concentrations resulting from insufficient insulin and insulin resistance, or both, along with disturbances in metabolism of lipids, carbohydrates and proteins[2, 3]. As the disease advances, it leads to complications like retinopathy, neuropathy, nephropathy, stroke, ischemic heart disease, peripheral vascular disease and a wide array of heterogeneous disease[4]. The incidence of diabetes in developing countries have reached to epidemic proportions and International Diabetes Federation (IDF) projects a rise from 382 million people with diabetes to 592 million between 2013 and 2035[5]. The current treatment to combat type 2 diabetes is the usage of oral hypoglycemic drugs such as α-glucosidase inhibitors, sulphonylureas,biguanides, thiazolidenediones and meglitinide analogues besides injectable insulin, which become important when blood glucose levels cannot be controlled by diet, exercise, weight loss and oral medications. Whereas, insulin replacement therapy is the mainstay for patients with type 1 DM[6, 7]. However, prominent side-effects of such drugs are the main reason for an increasing number of people seeking alternative therapies that may have no side-effects[8]. This has led to the growing interest in phytomedicine and the extracts are studied to know about their efficacy, safety and mechanism of action. The same has been recommended by World Health Organization (WHO), as 80% of the people rely on plant based medication[9]. The effect of these plant extracts can be studied *in vitro* that includes perfused whole organs, tissues, cells in primary or immortal culture, sub cellular membranes, purified receptor and enzymes that can establish mechanisms and definite toxicities. Further, biological systems can be studied using live animals which is necessary to study how such mechanisms behave under clinical or pathophysiological conditions[10]. *In vitro* models are fairly based on a specific process, wherein activity of an enzyme on a metabolic reaction or binding to a receptor within a given cell type can be studied. *In vitro* studies are of considerable value in identifying the mechanism of action of a test material and are more economical. It provides an alternative to animal testing in many aspects which includes investigation of organs or tissues derived from animals that could be used to test many replicates or samples. Immortalized cell lines, although originally derived from animals or humans, do away completely with the repeated need for animal tissue in assays. There is reduced variability in cells from a single cell line and have the advantage of genetic homogeneity. *In vitro* assay is an ideal way of obtaining active components with defined biological activity during fractionation process and later can be tested *in vivo* to confirm their effects[11].

IN VITRO MODELS USED IN DIABETIC RESEARCH

Inhibition of carbohydrate digesting enzyme

Pancreatic α-amylase

Pancreatic α-amylase, an important enzyme of digestive system hydrolyzes starch into mixture of smaller oligosaccharides comprising of maltose, maltotriose and oligoglucans which are further degraded by glucosidase into glucose that enters the blood stream upon absorption. This leads to elevated Post-prandial hypergylcemia (PPHG). Hence, it is important to control these two aspects in the treatment of type 2 diabetes[12]. The inhibition assay is carried out as illustrated by Miller[13] using the chromogenic 3,5- dinitrosalicylic acid (DNSA) method. The assay mixture is prepared using 500 µL of 0.02 M sodium phosphate buffer (pH 6.9) containing 6 mM sodium chloride, 0.04 units of Porcine Pancreatic α-amylase (PPA) solution and test samples with different concentrations are pre-incubated at 37°C for 10 minutes. To the same, 500 µL of 1% (w/v) starch solution (prepared using above buffer) is added and incubated at 37°C for 15 minutes. DNSA reagent (1.0 mL) is added to stop the reaction and placed in boiling water bath for 5 minutes, cooled, diluted and measured at 540 nm. The control without test sample represents 100% enzyme activity. The

absorbance produced by test sample is eliminated by including appropriate sample control without enzyme and starch. Acarbose, a known PPA inhibitor can be used as a positive control.

α-Glucosidase

α-Glucosidase and Sucrase are confined to the mucosal brush border of the small intestine and catalyze the final step of digestion of starch and sucrose[14]. The lowering of postprandial blood glucose and insulin levels in diabetic patients is due to the delayed breakdown of carbohydrates in small intestine by the α-glucosidase and sucrase inhibitors[15, 16]. The inhibition assay is carried out as illustrated by Andrade-Cetto[17]. The assay mixture is prepared using an incubated solution of starch substrate (2 % w/v maltose); 1 mL of 0.2 M Tris buffer (pH 8.0) and different concentrations of test sample for 5 minutes at 37°C. The reaction is initiated by adding 1 mL of α-glucosidase enzyme (1U/mL) and then incubating it for 10 minutes at 37°C. The reaction is terminated by heating for 2 minutes in boiling-water bath. The quantity of glucose liberated is measured. The standard control without test sample represents 100% enzyme activity. The absorbance produced by test sample is eliminated by including appropriate sample control without enzyme and substrate. The α-glucosidase inhibitor, acarbose can be used as a positive control.

The % of inhibition for α- amylase and α-glucosidase is calculated as follows

$$\% \text{ Inhibition} = EC - (ET - TC) / EC \times 100$$

Where, EC is enzyme activity of control, ET is enzyme activity of test and TC is test control.

Sucrase

The inhibition assay is carried out as illustrated by Honda and Hara[18]. Ten µL of the enzyme along with varying concentrations of the sample is incubated for 10 minutes at 37°C. Malate buffer (pH 6.0) is used to make up the volume up to 200 µL. The enzyme reaction is initiated by adding 100 µL sucrose solution (60 mM) and incubated for 30 minutes. The reaction is terminated by the addition of 200 µL of 3, 5-dinitrosalysilic acid reagent and treating the mixture in a boiling water bath for 5 minutes. The absorbance of the solution is read at 540 nm.

Sucrase inhibition rate is calculated as follows

$$\text{Inhibition rate (\%)} = A_C \text{ (Control)} - A_S \text{ (sample)} / A_C \text{ (Control)} \times 100$$

Where, A_C control is the absorbance of the control reaction (all reagents to be added except for the test sample), and the A_S sample is the absorbance of the test sample. Models to target Specific/Particular enzyme

Glucose uptake assays using dialysis bag

The effect of Glucose movement can be assessed using a dialysis tube as described by Edwards [19]. Fifteen mL of glucose solution and 0.15 M sodium chloride (NaCl) is introduced into a sealed dialysis tube and measured for the manifestation of glucose in the external solution. Two mL of 0.15M NaCl and 15mL of 0.22mM D-glucose solution is introduced into a 6cm×15mm dialysis tube (MWCO:2000) and sealed on both ends. The sealed dialysis tube is placed in a centrifugal tube containing 45 mL of 0.15M NaCl. The tubes are set on an orbital shaker and maintained at room temperature. Later the movement of glucose into the external solution is measured by Glucose oxidase method.

Dipeptidyl peptidase IV (DPP IV)

Dipeptidyl peptidase IV sequentially removes N-terminal dipeptides from polypeptides having unsubstituted N-terminals. The ultimate residue being proline and alanine[20]. Dipeptidyl peptidase IV is a multifunctional protein, which is said to be articulated on the surface of epithelial, endothelial and lymphoid cells [21, 22]. The soluble form of the enzyme gets released in plasma and contains a wide variety of proline-containing peptides such as growth factors, chemokines,

neuropeptides and vasoactive peptides. One major therapeutic approach for type 2 diabetes is inhibition of DPPIV as it cleaves N-terminal dipeptides from polypeptides and regulates glucose homeostasis. Apart from this, it is implicated in immune regulation, signal transduction, apoptosis and has a major role in tumour progression [23, 24]. The inhibition assay is carried out as illustrated by Kojima [25]. Test samples of various concentrations are prepared using 100mM Tris buffer (pH 8.0). Twenty five µL of the same is added with 25 µL of 1.59 mM Gly-Pro-*p*-nitroanilide prepared using 100mM Tris buffer (pH 8.0) the mixture is incubated for 20 minutes at 37°C. Fifty µL of DPP IV (0.01 units/mL) diluted with the same tris buffer is added to the reaction mixture and incubated at 37°C for 60 minutes. The reaction is seized by the addition of 100 µL of 1 M sodium acetate buffer (pH 4.0). The absorbance of the resulting solution is measured at 385 nm with an ELISA Reader.

The DPP IV inhibition rate is calculated as follows

$$\text{Inhibition rate (\%)} = [(A_C - A_B) - (A_T - A_B)] / (A_C - A_B)] \times 100$$

Where A_C is the absorbance of the control solution; A_B is the absorbance of the blank solution; A_T is the absorbance of the reaction solution.

Protein tyrosine phosphatase 1B (PTP 1B)

Tyrosine phosphorylation of proteins is a basic mechanism to control cell growth and differentiation. Protein tyrosine phosphatase 1B has been implicated in modulating signalling pathways initiated by the activation of the tyrosine kinase receptor super family. It regulatesinsulinsignallingby dephosphorylating and inactivating insulin receptor. Protein tyrosine phosphatase 1B inhibition could provide a new therapeutic optionin the treatment of type 2 diabetes. It is directed by the opposing activities of protein tyrosine kinases (PTKs), which catalyse phosphorylation and protein tyrosine phosphatases (PTPs) that are responsible for dephosphorylation. An inappropriate operation leads to aberrant tyrosine phosphorylation, contributing to the development of many diseases *viz*., cancer and diabetes [26]. The assay is carried out as illustrated by Zhang and Lee[27]. The reaction mixture contains 22.5 µL of Bovine Serum Albumin (BSA) solution (100 µg/mL), 19µL of Para-Nitrophenyl phosphate Tyrosine (pNPP Tyr) assay buffer (pH 7.2)/ positive control/ test solution of various concentration and 3.5 µL of enzyme (46.72 U/100µL). After pre incubation, 5µL of 50mM substrate is added, mixed and incubated at 37°C for 60 minutes. The absorbance is measured at 405 nm. Controls are run devoid of test samples.

Cell lines as Model to study different in vitro antidiabetic activity

Cell lines facilitate to examine cell types for their physiological and pathophysiological processes without the use of animal model. It is used to study the physiology of cells and to test the effects of a variety of compounds on specific cell type. The main advantage of using cell lines is to obtain more reliable and reproducible results from a batch of clonal cells. In contrast to this, cell line characteristics keep changing due to the continuous growth of cells and also cell cultures grow without limits being one of the disadvantages. This can be associated to their tumour origin and these cells may have modified metabolism, abnormal chromosomal content or abnormal protein expression with other genetic mutations [28]. Bioengineered technologies have provided better opportunities to establish a more appropriate cultured cell line which helps in studying the mechanism involved in insulin secretion and glucose uptake by target organs; it also helps in studying transcriptional factors such as Peroxisome Proliferator-Activated Receptor-*γ* (PPAR-*γ*), the targets of modern therapy[29]. Defect in insulin secretion leads to a number of diseases which includes diabetes, constant hyperinsulinemichypoglycaemiaand insulinoma. New therapies can be developed by knowing the underlying mechanisms involved in insulin secretion that will contribute to engineer insulin producing cells which can be used as a

replacement therapy to treat diabetes. Glucose phosphorylation in beta-cells is a key regulatory event in pairing insulin secretion to extracellular glucose concentrations[28]. Glucose is the major physiological insulin secretagogue and a potent regulator of beta-cell activity which in turn produces insulin in response to an elevated blood glucose concentration and with an increase in cytoplasmic calcium concentration (Ca^{2+}). In the absence of glucose, the ATP-sensitive K-channels (K^{ATP}) remain close due to low cytoplasmic ATP concentration. Elevated extracellular glucose concentration facilitates glucose entry into the cells through GLUT2 transporters and glycolysis increases production of ATP thereby closing K^{ATP} in the plasma membrane, depolarization takes place resulting in opening of voltage-gated Ca^{2+} channels and increased influx of extracellular Ca^{2+}. Electrical activity together with Ca^{2+} influx occurs in bursts and results in insulin release [30].

Insulin secretion assay using pancreatic RIN m5F cells

RIN m5F (Rat insulinoma) cells are one of the most extensively used insulin-secreting cell lines and contain insulin, small amount of somatostatin and glucagon. Abnormal properties of glucose transport have been reported for RINm5F and RINr cells, and both cells exhibit inappropriate sensitivity to glucose [31]. A solid phase two-site enzyme immunoassay is used in Mercodia Rat insulin ELISA (Enzyme Linked Immuno Sorbent Assay) which is purely based on direct sandwich technique as described by Persaud[32]. Two monoclonal antibodies are fixed against separate antigenic determinants on a molecule of insulin. During incubation, insulin in the sample reacts with anti-insulin antibodies and peroxidise-conjugated anti-insulin antibodies which are bound to microtitre well. The unbound enzyme labelled antibodies are removed by washing and the bound conjugate is detected using 3,3',5,5'- tetramethyl benzidine with the addition of acid to give a colorimetric endpoint which is read spectrophotometrically.

Cytotoxicity assay (MTT reagent)

The MTT cytotoxicity assay is carried out as illustrated by Mossman[33]. The cells are seeded at a density of 1×10^5 cells/well in 96 well microtitre plate along with various concentration of test samples and incubated for 24 hours. MTT working solution is prepared by mixing 1 mL of MTS solution with 50 µL of PMS (Phenazine methosulfate) solution. After incubation the media is removed from the wells and normal growth media is added. MTT working solution (20 µL/ well) is assessed and incubated for 3 hours and the absorbance is measured at 485 nm.

Insulin release assay

The assay is carried out as illustrated by Persaud[32] using RIN m5F cells. Cells are washed with KRB (Krebs-Ringer Bicarbonate) buffer (100 µL) to remove serum and then the cells ($1x10^5$ cells/well) are seeded into the 96 well microtitre plate and incubated for 24 hours at 37°C. Various concentrations of test samples are prepared using 100 µL KRB buffer and incubated for an hour at 37°C. After incubation, the insulin is measured by sandwich ELISA methods. *Sandwich ELISA Procedure (Mercodia Rat Insulin ELISA)* Twenty five µL of the cell lysates and 50 µL of the enzyme conjugate are added to anti-insulin coated 96 well microtitre plate. The whole mixture is incubated for 2 hours at room temperature; the incubated solution is then washed with KRB buffer. Two hundred µL of the substrate (tetramethylbenzidine) is added to each well and incubated for 15 minutes. After the incubation, 50 µL of the stop solution (0.5% H_2SO_4) is added and is placed on a shaker for 5 sec to ensure proper mixing. The absorbance is measured at 450 nm.

Insulin secretion assay using pancreatic β-cell line

The release of Insulin from the pancreatic β-cell line depends on glucose load in dose dependent manner. Insulin secretion released into the medium in response to increasing concentrations of glucose is used in detecting the changes in cell secretory properties like GSIS, basal secretion and a shift in glucose sensitivity. Cells are cultured at a density of 2.5×10^5 cells/well on a 12 well microtitre plate. The growth medium is supplemented with 1 mg/mL tetracycline upon reaching 60-80% confluence for 48 hours. The

cells which ceased growth are then incubated for 18 hours with or without test sample in the growth medium. Later, the cells are rinsed and pre-incubated without glucose for an hour at 37°C in a Krebs-Ringer buffer (25mM $NaHCO_3$, 10mM HEPES, 118mM NaCl, 2mM NaH_2PO_4, 2.5mM $CaCl_2$, 5mM KCl, 0.1% fatty-acid-free BSA, 1mM $MgSO_4$, pH 7.4) which does not contain glucose in presence or absence of test sample. Insulin secretion in presence or absence of test sample in buffer containing 0.5 mM IBMX and either 2 mM glucose (basal secretion), 16 mM glucose (GSIS) or 2 mM glucose + 50 mM K^+ (non-fuel secretagogue) (buffer adjusted to 50mM KCl and 73mM NaCl) is measured for a period of 2 hours. By the end of the secretion period, the insulin released is estimated using radioimmunoassay [34].

β-Cell proliferation assay

β- Cells located in the islet of langerhans, produce, store and secrete insulin. Type 1 and 2 diabetes involve loss of beta-cell mass and function. These cells are destroyed by the immune system (type 1) or do not produce sufficient amount of insulin to maintain normal blood glucose concentration (type 2). Generally, beta-cell mass is regulated by various processes, including apoptosis, neogenesis, proliferation and differentiation. The compounds which can restore the β-cells or promote their proliferation are of at most importance [35]. The *β* TC-tet cells are seeded at a density of 1.0×10^5 cells/well in 24 microtitre well plate and incubated for 24 hours in growth medium. The incubation is prolonged for 48 hours in growth medium while one group is treated with tetracycline (1 µg/mL) to cease growth in the medium. The replicating cells are incubated with or without extracts for 24 hours. Around 1 µCi/mL of methyl ^{3}H-thymidine is added by the last 6 hours of treatment. The cells are rinsed thrice in PBS (Phosphate-buffered saline), lysed using 0.1M NaOH (Sodium hydroxide) for 30 minutes and scraped. Lysate is added to 1ml of liquid scintillation cocktail and measured for the radioactivity incorporated [36]. Four replicates are maintained for each experimental condition. The average count form the tetracycline treated i.e., the growth ceased wells are considered as nonspecific incorporation and are subtracted from all other measures.

Glucose uptake assay Using 3T3 L1 cells

Insulin promotes glucose uptake, metabolism and storage in adipose tissue and skeletal muscle. Insulin stimulates phosphorylation of insulin receptor substrates (IRS) by kinase, which leads to activation of PI3 kinase, PKB and protein kinase C isoforms. Activated PKB translocate Glut4 to the cell surface and stimulate glucose transport in muscle and fat cells, whereas it phosphorylates and inhibits GSK-3. Inhibitor of GSK-3 enhances insulin signalling thereby favouring glucose entry into the muscle cells and adipose tissue. Inactivation of GSK-3 in 3T3 L1 differentiated cells [adipocytes] stimulates glucose uptake which can be measured by incorporation of 2-deoxy-^{14}C-glucose and quantified by using scintillation counter.

Cell culture

The cell line 3T3-L1 pre-adipocytes are cultured and made to differentiate as described by Ardevol[37]. The pre-adipocytes are then treated with 0.5 mM 3-isobutylmethylxanthine, 5 µg/mL insulin and 0.25 µM dexamethasone in 10% FBS containing DMEM for 2 days. Later, the cells are transferred to 10% FBS/DMEM containing only insulin and then to 10% FBS/DMEM without insulin for next 2 days. After ten days of differentiation, the cells are used for the experiments. Chinese hamster ovaric cells over expressing the human insulin receptor (CHO-IR) cells are grown in Ham's F12 supplemented with 100 U/mL penicillin; 10% fetal bovine serum; 2.5 µg/mL fungizone; 0.5% G-418 and 100 mg/ml streptomycin in 5% CO_2/humidified atmosphere at 37°C. Cells are passed two to three times a week. Confluent cells are used for the experiments.

Glucose uptake assay using 3T3-L1adipocytes

2-deoxy-D-[3H] glucose uptake of 3T3-L1 adipocyte is used to measure the glucose transport system[38]. 3T3-L1adipocyte cells cultured on 12 well microtitre plate are incubated in a transport solution containing 1 µCi 2-deoxy-

D-[3H] glucose (10 mCi/ mmol) and 0.1 mM 2-deoxy-Dglucose for 7 minutes. Uptake of glucose is terminated by the addition of 50 mM glucose and 0.1MNaOH/ 0.1% PBS for disruption of cells. Radioactivity incorporated in the cells is determined using scintillation counter. Protein is used to standardize the glucose transport values[39].

Immunoblotting

CHO-IR cells are deprived of serum for 18 hours and stimulated using insulin (100 nM) or GSPE (100 mg/L). Cells are harvested using cell lysis buffer (150mM NaCl, 1% Brij, 80mM Tris-HCl pH 7.5, 200mM, 20mM EDTA, 10nM okadaic acid, 4mM sodium vanadate and a protease inhibitor cocktail). SDS/PAGE is used to separate proteins and then transferred to nitrocellulose membranes as described by Onnockx[40]. Specific antibodies are used to detect individual proteins and identified by horseradish peroxidase-linked to a secondary antibody using ECL (enhanced chemiluminescence) kit. The band's intensity is estimated using the Scion Image software.

Glucose transport assay

Differentiated 3T3-L1 adipocytes and differentiated C2C12 skeletal myotubes are incubated with test samples. Insulin-regulated glucose uptake is exhibited by both cell lines and has Glut-1 and Glut-4 glucose transporters[41, 42]. Prior to the assay, differentiated cells grown in 12-well plate are incubated with DMSO, test sample and positive control either for 1 or 18–21 hours. For an hour's treatment, differentiation medium is replaced with serum-free medium, 1hour prior to start of incubation period. For 18-21 hours treatment, the last 3 hours of incubation is carried out in serum-free medium. Cells are washed twice following incubation with Krebs-phosphate buffer (4.05mM Na_2HPO_4; 0.95mM NaH_2- PO_4; 20mM HEPES; 4.7mM KCl; 136mM NaCl; 1mM $MgSO_4$; 1mM $CaCl_2$; 0.5% BSA; 5mM glucose; pH 7.4) at 37°C. Later the cells are treated with 100nM insulin in the same buffer for 30 minutes at 37°C in presence or absence of test samples. Cells are washed twice with Krebs-phosphate buffer free of glucose and treated with 0.5 mCi/mL 2-deoxy-D-[1-3H]-glucose for 10 minutes at 37°C without test samples. Subsequently, the cells are placed on ice and washed immediately with ice-cold Krebs-phosphate buffer around 3 times. It is lysed using 0.1M NAOH for 30 minutes and scraped. The lysate is measured for incorporated radioactivity [43].

PeroxisomeProliferator-Activated Receptor-γ luciferase assay

Peroxisome Proliferator-Activated Receptor-*γ* (PPAR-*γ*), a nuclear receptor has an important role in lipid and glucose metabolism. PPAR*γ* is articulated mainly in the white adipose tissue. Upon activation, it stimulates the expression of genes involved in insulin sensitivity and glucose homeostasis [44-46]. Conformational changes occur upon the binding of ligand and this stabilizes its interaction with Retinoid X Receptor (RXR). The heterodimer (PPAR-*γ*/RXR) then takes on a set of co-activators to the promoter regions of target genes and brings about transcription [47, 48]. A ligand-stimulated trans-repression of genes is mediated through a peroxisome proliferator response in which the PPAR*γ* ligand binds to the SMRT/NCoR co-repressor complex [46]. This interaction prevents proteasomal degradation of the co-repressor and maintains the gene in a repressed mode**.** Activation of PPAR*γ* is an important step in glucose uptake. To determine this, luciferase assay is performed according to Asha[49] using PPRE-tk-LUC reporter plasmid along with the expression vector for human PPAR-γ. At first, the CV1 cells are plated onto 24 well microtitre plate and transitory transfections are conducted and incubated over-night using lipofectamine. After 5 hours of transfection, the cells are treated with each test sample for next 24 hours.

CONCLUSION

These assays provide information of various *in vitro* studies used in antidiabetic assessment, which can establish a mechanism for the antidiabetic activity of drug. Besides, intensive studies of the mechanism of action of the known drugs have provided further validation of several new molecular drug targets.

ACKNOWLEDGEMENT

Dorin Dsouza is grateful to UGC New Delhi, for Maulana Azad National Fellowship.

DECLARATION OF INTEREST

The authors report no declaration of interest.

REFERENCES

1. Stenfen Ma., Jeffery Cutter., Chee Eng Tan., Suok Kai Chew., Shyong Tai. Associations of diabetes milletus andethinicity with mortality in a multiethnic Asaian population: data from the 1992 Singapore National Health Survey.American Journal of Epidemiology, 158: 543-552, (2003).
2. Patel DK., Kumar R., Prasad SK., Sairam K., Hemalatha S. Anti-diabetic and *in vitro* antioxidant potential of Hybanthus enneaspermus (Linn) F. Muell in streptozotocin-induced diabetic rats. Asian Pacific Journal of Tropical Biomedicine, 1(4): 316-322, (2011).
3. Warjeet Singh L. Traditional medicinal plants of Manipur as anti-diabetics. Journal of Medicinal Plants Research, 5: 677-687, (2011).
4. World Health Organization. Definition and Diagnosis of Diabetes Mellitus and Intermediate Hyperglycemia: Report of a WHO/IDF Consultation, WHO, Geneva, Switzerland, pp 5, (2006).
5. International Diabetes Federation. IDF Diabetes Atlas, 6th edn. Brussels, Belgium: Internationaldiabetes Federation, Accessed on 14-03-2014. http://www.idf.org/diabetesatlas.
6. Kumar PJ., Clark M. Textbook of Clinical Medicine. London: Saunders publishers, pp 1099-1121, (2002).
7. O'Dea K. Marked improvement in carbohydrate and lipid metabolism in diabetic Australian Aborigines after temporary reversion to traditional lifestyle. Diabetes, 33: 596-603, (1984).
8. Salim Bastaki. Review-diabetes milletus and its treatment. International Journal of Diabetes & Metabolism, 13: 111-134, (2005).
9. Mentreddy SR., Mohamed AI., Rimando AM. Medicinal plants with hypoglycemic/anti-hyperglycemic properties: a review. IMIDA, AAIC, 341-353, (2005).
10. Kruthika Thorat., LeenaPatil., Dnyanesh Limaye., Vilasrao Kadam. *In vitro* Models for antidiabetic assessment. International Journal of Research in Pharmaceutical and Biomedical Sciences, 3(2): 730-733, (2012).
11. Amalasoumyanath (nee Raman), Sairavee srijayant. Traditional Medicine for modern times: Antidiabetic Plants, Chapter 4: In vitro models for assessing Antidiabetic activity, CRC Press, Taylor and FrancisGroup, LLC: NW, pp. 99-116, (2006).
12. Eichler HG., Korn A., Gasic S., Prison W., Businger J. The effect of new specific α-amylase inhibitor on post-prandial glucose and insulin excursions innormal subjects and Type 2 (non-insulin dependent) diabetic patients. Diabetologia, 26(4): 278-281, (1984).
13. Miller GL. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Analytical chemistry, 31: 426-428, (1959).
14. Nichols BL., Avery SE., Karnsakul W., Jahoor F., Sen P., Swallow DM., Luginbuehl U., Hahn D., Sterchi EE. Congenitalmaltase-glucoamylase deficiency associated with lactase and sucrase deficiencies. Journal of Pediatric Gastroenterology and Nutrition, 35: 573-579, (2002).
15. Hillebrand I., Boehme K., Frank G., Fink H., Berchtold P. The effects of the alpha-glucosidase inhibitor BAY g 5421 (Acarbose) on meal-stimulated elevations of circulating glucose, insulin, and

triglyceride levels in man. Research in Experimental Medicine (Berl), 175: 81-86, (1979).
16. Van de Laar FA. α-Glucosidase inhibitors in the early treatment of type 2 diabetes. Vascular Health and Risk Management, 4: 1189-1195, (2008).
17. Andrade-Cetto A., Becerra-Jimenez J., Cardenas-Vazquez R. Alfa-glucosidase inhibiting activity of some Mexican plants used in the treatment of type 2diabetes. Journal of Ethnopharmacology, 116: 27-32, (2008).
18. Honda M., and Hara Y. Inhibition of rat small intestinal sucrase and α- glucosidase activities by tea polyphenols. Bioscience, Biotechnology and Biochemistry, 57: 123-4, (1993).
19. Edwards CA., Johnson IT., Read NW. Do viscous polysaccharides slow absorption by inhibiting diffusion or convection? European Journal of Clinical Nutrition, 42: 307–12, (1988).
20. Hopsu-Havu VK., Sarimo SR. Purificatiaon and characterization of an aminopeptidase hydrolyzing glycyl- proline- naphthylamide. Hoppe-Seyler's Zeitschrift fur Physiologische Chemie, 348: 1540-50, (1967).
21. Villhauer EB., Brinkman JA., Naderi GB., Burkey BF., Dunning BE., Prasad K., Mangold BL., Russel ME., Hughes TE. 1-[(3-hydroxy-1 adamantyl) amino acetyl]-2-cyano-(S)-pyrrolidine: a potent, selective, and orally bioavailable dipeptidyl peptidase IV inhibitor with antihyperglycemic properties. Journal of Medicinal Chemistry, 46: 2774–2789, (2003).
22. Engel M., Hoffmann T., Wagner L., Wermann M., Heiser U., Kiefersauer R., Huber R., Bode W., Demuth HU., Brandstetter H. The crystal structure of dipeptidyl peptidase IV (CD26) reveals its functional regulation and enzymatic mechanism. Proceedings of the National Academy Sciences, 100: 5063–5068, (2003).
23. Didier Marguet., Laurie Baggio., Takashi Kobayashi., Anne-Marie Bernard., Michel Pierres., PerF.Nielsen., Ulla Ribel., Takeshi Watanabe., Daniel J. Drucker., Nicolai Wagtmann. Enhanced insulin secretion and improved glucose tolerance in mice lacking CD26. Proceedings of the National Academy Sciences USA, 97: 6874–6879, (2000).
24. Pro B Dang N. CD26/dipeptidyl peptidase IV and its role in cancer. Histology and Histopathology,19 (4):1345–1351, (2004).
25. Kojima K., Ham T., Kato T. Rapid chromatographic purification of Dipeptidyl peptidase IV in human submaxiallry gland. Journal of Chromatography, 189: 233-240, (1980).
26. HajFG., MarkovaB., Klaman LD., Bohmer FD., Neel BG. Regulation of receptor tyrosine kinase signaling by protein tyrosine phosphatase-1B. Journal of Biolological chemistry, 278: 739-744, (2003).
27. Zhang ZY., Lee SY. PTP1B inhibitors as potential therapeutics in the treatment of type2 diabetes and obesity. Expert Opinion of Investigational drugs, 12: 223-233, (2003).
28. Masa Skelin., Marjan Rupnik., Avrelija Cencic. Pancreatic Beta Cell Lines and their Applications in Diabetes Mellitus Research. Altex, 27: 105-113, (2010).
29. Hansotia T., Duckers DJ. GIP and GLP-1 as incretin hormones: lessons from single and double incretin receptor knockout mice. Regulatory Peptides, 128: 125-134, (2005).
30. AshcroftFM.,RorsmanP. Electrophysiology of the pancreatic β-cell. Progress in Biophysics and Molecular Biology, 54: 87-143, (1989).
31. Halban PA., Praz GA., Wollheim CB. Abnormalglucosemetabolism accompanies failure of glucose to stimuprotein late insulin release from a rat pancreaticcellline(RINm5F). Biochemical Journal, 212(2): 439-443, (1983).
32. Persaud SJ., Harris TE., Burns CJ., Jones PM. Tyrosine kinases play a permissive role in glucose-induced insulin secretion from adult rat islets. Journal of Molecular Endocrinology, 22: 19–28, (1999).
33. Mossman T. Rapid colorimetric assay for cellular growth and survival: application to

proliferation and cytotoxicity assays. Journal of Immunology, 65: 55 –63, (1983).
34. Louis C., Martineaua., Audrey Couturea., Danielle Spoora., Ali Benhaddou-Andaloussia., Cory Harris C., Bouchra Meddaha., Charles Leduca., Andrew Burtc., Tri Vuonga., Phuong Mai Lea., Marc Prentkie., Steffany A. Bennettd., John T. Arnasonc., Pierre Haddada S. Anti-diabetic properties of the Canadian lowbush blueberry Vaccinium angustifolium Ait. Phytomedicine, 13: 612–623, (2006).
35. Butler AE., J Janson., S Bonner-Weir., R Ritzel., RA Rizza., Butler PC. "β-cell deficit and increased β-cell apoptosis in humans with type 2 diabetes". Diabetes, 52(1): 102–110, (2003).
36. Michelle M Hill., Sharon F., Clark., David F., Tucker., Morris J. Bimbaum., David E. James., Lance Macaulay S. A Role for Protein Kinase Bβ/Akt2 in Insulin-Stimulated GLUT4 Translocation in Adipocytes. Molecular and Cell Biology. 19(11): 7771- 7781, (1999).
37. Ardevol A., Blade C., Salvado MJ., Arola L. Changes in lipolysis and hormone-sensitive lipase expression caused by procyanidins in 3T3-L1 adipocytes. International Journal of Obesity and Related Metabolic Disorder, 24: 319–324, (2000).
38. Pinent M., Blay M., Blade MC., Salvado MJ., Arola L., Ardevol A. Grape seed-derived procyanidins have an antihyperglycemic effect in streptozotocin-induced diabetic rats and insulinomimetic activity in insulin-sensitive cell lines. Endocrinology, 145(11):4985-4990, (2004).
39. Bradford M. A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein dye binding. Analytical Biochemistry, 72: 248–254, (1976).
40. Onnockx S., De Schutter J., Blockmans M., Xie J., Jacobs C., Vanderwinden JM., Erneux C., Pirson I. The association between the SH2-containing inositol polyphosphate 5-phosphatase 2 (SHIP2) and the adaptor protein APS has an impact on biochemical properties of both partners. Journal of Cellular Physiology, 214: 260–272, (2008).
41. Klip A., Paquet MR. Glucose transport and glucose transporters in muscle and their metabolic regulation. Diabetes Care, 13: 228–243, (1990).
42. Sweeney MI., Kalt W., Mackinnon SL., Ashby J., Gottschall-Pass KT. Feeding rats diets enriched in low bush blueberries for six weeks decreases ischemiainduced brain damage. Nutritional Neuroscience, 5, 427–431, (2002).
43. Kumar N., Dey CS. Metformine enhances insulin signalling in insulin dependent and-independent pathways in insulin resistant muscle cells. BritishJournal of Pharmacology, 137: 329–336, (2002).
44. Auwerx J. PPAR- γ, the ultimate thrifty gene. Diabetologia, 42: 1033-1049, (1999).
45. Debril MB., Renaud JP., Fajas L., Auwerx J. The pleiotropic functions of peroxisome proliferator-activated receptor gamma. Journal of Molecular Medicine, 79: 30–47, (2001).
46. Pascual G., Fong AL., Ogawa S., Gamliel A., Li AC., Perissi V., Rose DW., Willson TM., Rosenfeld MG., Glass CK. A SUMOylation-dependentpathway mediates transrepression of inflammatory response genes by PPAR. Nature, 437: 759–763, (2005).
47. Berger J and Wagner JA. Physiological and therapeutic roles of peroxisome proliferator-activated receptors. Diabetes Technology and Therapeutics, 4:163–174, (2002).
48. Evans RM., Barish GD., Wang YX. PPARs and the complex journey to obesity. Nature Medicine, 10, 355–361, (2004).
49. Asha Kulkarni-Almeida A., Manoja BrahmaK., Prabhu Padmanabhan., Prabhu Mishra D., et al Fermentation, Isolation, Structure, and antidiabetic activity of NFAT-133 produced by Streptomyces strain PM0324667. AMB Express, 1: 42, (2011).