Traditional Indian Antidiabetic Medicinal Plants as Inhibitors of Pancreatic $\alpha$-Amylase and $\alpha$-Glucosidase

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1 Introduction

Diabetes mellitus is a carbohydrate metabolism disorder resulting in abnormally high blood sugar level (hyperglycemia). It can be caused by hereditary, increasing age, poor diet, imperfect digestion, obesity, sedentary lifestyle, stress, drug-mediated, infection in pancreas, hypertension, high serum lipid and lipoproteins, less glucose utilization and other factors (Alberti & Zimmet, 1998). The World Health Organization (WHO) estimates that worldwide, 346 million people have diabetes with more than 80% of diabetics living in low- and middle-income countries. The number is expected to grow to double by 2030 (WHO, 2012). Of the two main forms of diabetes, type 2 accounts for over 90% of the cases globally. It is characterized by insulin resistance or abnormal insulin secretion, either of which may predominate (WHO, 1999; Zimmet et al., 2001). The primary aim of managing type 2 diabetes is to delay, or even prevent, the complications of the disease by achieving good glycemic control. In addition to drug therapy, this often involves changes in lifestyle, such as diet and exercise. The main groups of oral synthetic drugs available along with their mechanism of action and side effects are listed in Table 1. Insulin is increasingly considered part of a treatment regimen in type 2 diabetics, particularly the use of long-acting preparations to provide a constant basal insulin release (Hall & Nicholson, 2009). Increased side effects, lack of curative treatment for several chronic diseases, high cost of new drugs, are some reasons for renewed public interest in complementary and alternative medicines (Humber, 2002).

1.1 Complementary and Alternative Medicine in Diabetes Mellitus

Ayurveda, the traditional Indian medicine (TIM) as well as the traditional Chinese medicine (TCM) remain the most ancient yet living traditions in complementary and alternative therapy (Humber, 2002). Use of indigenous drugs of natural origin forms a major part of such therapies with more than 1500 herbs sold as dietary supplements or ethnic traditional medicines (WHO, 2001). Pharmaceutical companies have renewed their strategies in favor of natural product drug development and discovery (Seidl, 2002). Ethnobotanical studies of traditional herbal remedies used for diabetes around the world have identified more than 1200 species of plants with hypoglycemic activity. These plants are broadly distributed throughout 725 different genera. The pharmacopoeia of India is especially rich in herbal treatments for diabetes (Shekelle et al., 2005). While plant derivatives with purported hypoglycemic properties have been used in folk medicine and traditional healing systems, very few of these traditional anti-diabetic plants have received proper scientific or medical scrutiny despite recommendations by (WHO). Ayurveda and other Indian traditional approaches have described more than 800 plants in the Indian subcontinent, known to possess antidiabetic potential. These require to be effectively studied and in fact only few of them have been characterized for their mechanistic actions (Grover et al., 2002; Mukherjee et al., 2006; John et al., 2009).

1.2 Targets of Action: Pancreatic α-amylase and α-glucosidase

Type 2 diabetes is a multifactorial disease caused by oligo- and polygenic genetic factors as well as non-genetic factors that result from a lack of balance between the energy intake and output and other lifestyle related factors. Understanding of diabetes pathogenesis is essential to the development of new methods for treatment and strategies of this disease (Hansen, 2002). Of the many targets for antidiabetic agents, pancreatic α-amylase and α-glucosidase are discussed in this chapter.
<table>
<thead>
<tr>
<th>Oral Hypoglycemic Agent</th>
<th>Mechanism of Action</th>
<th>Side Effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sulphonylurea</td>
<td>Stimulates release of Insulin from β-cell of pancreas</td>
<td>Increase in appetite and weight gain (Kelle, 1995). Increase in occurrence of cardiovascular risk (Feinglos &amp; Bethel, 1999; Sheehan, 2003).</td>
</tr>
<tr>
<td>Meglitinides</td>
<td>Stimulate release of insulin from β-cell of pancreas by binding to ATP-sensitive potassium ion channels. Short acting and meal adjusted dosing.</td>
<td>High cost, gastrointestinal disturbances, hypersensitivity reactions including pruritus, rashes and urticaria (Bastaki, 2005).</td>
</tr>
<tr>
<td>Biguanides Metformin</td>
<td>Improves insulin sensitivity in skeletal muscles, decrease hepatic gluconeogenesis, inhibits glycogenolysis, and reduces plasma triglyceride of low density lipoprotein. In liver, stimulates AMP-protein kinase to enhance fatty acid oxidation with inhibition of lipogenesis and glucose production (Stumvoll et al., 2007).</td>
<td>No effect on insulin secretion, hypoglycemia occurs in monotherapy so always should be used in combinatorial therapy with other hypoglycemic agents. Gastrointestinal disturbances, metallic taste, nausea, abdominal pain and diarrhea, severe lactic acidosis (Stumvoll et al., 1995; Bolen et al., 2007).</td>
</tr>
<tr>
<td>Thiazolidinediones (TZD)</td>
<td>TZDs are selective agonists for nuclear peroxisome proliferator-activated receptor-gamma. The TZDs bind to PPAR-γ, which activates insulin-responsive genes that regulate carbohydrate and lipid metabolism. TZDs lower insulin resistance in peripheral tissue, but an effect to lower glucose production by the liver. Enhances the insulin sensitivity in adipocytes and muscles by increasing efficiency of glucose transporters. TZDs activate genes that regulate free fatty-acid (FFA) metabolism in peripheral tissue, thus lowering triglycerides and non-esterified fattyacid levels and inducing differentiation of adipocytes (Bastaki, 2005).</td>
<td>Weight gain, edema, and increase risk of cardiovascular death. Increases risk of mortality when used in intensive therapy (ACCORD et al., 2007).</td>
</tr>
<tr>
<td>α-Glucosidase Inhibitors</td>
<td>α-Glucosidase inhibitors competitively block small intestine brush border enzymes that are necessary to hydrolyze oligo and polysaccharides to monosaccharide. Inhibition of this enzyme slows the absorption of carbohydrates; the postprandial rise in plasma glucose is blunted in both normal and diabetic subjects (Bischoff, 1995).</td>
<td>Dose-related flatulence, diarrhea, and abdominal bloating, high cost. Hypoglycemia (Reabasa-Lhoret &amp; Chiasson, 1998).</td>
</tr>
</tbody>
</table>

Table 1: Current synthetic oral hypoglycemic drugs for Type 2 diabetes with their side effects
Pancreatic $\alpha$-amylase (E.C. 3.2.1.1), and $\alpha$-glucosidase (E.C, 3.2.1.20) are the enzymes in the digestive system responsible for the break down of the carbohydrates from diet. Digestion of carbohydrates, about 5% is initiated by salivary amylases, which gets destroyed in the gut due to the high acid environment. When the food enters the intestine, the acidic pH from the gut is neutralized by bicarbonate from pancreas and mucous that lines the walls of the intestine. Pancreatic $\alpha$-amylase is secreted into the small intestines by the pancreas and $\alpha$-glucosidase enzymes are located in the brush border of the small intestines. Pancreatic $\alpha$-amylase breaks down the carbohydrates into oligosaccharides, which are subsequently broken down to monosaccharide by $\alpha$-glucosidase. Further, glucose and other monosaccharide are transported via the hepatic portal vein to the liver. Monosaccharides not immediately utilized for energy are stored as glycogen in the liver or as fat (triglycerides) in adipose tissue, liver and plasma. Carbohydrates that are resistant to digestion in the intestine enter the colon, where they are fermented by colonic bacteria to produce short-chain fatty acids, carbon dioxide and methane (Barrett & Udani, 2011).

In Type 2 diabetic patients, degradation of this dietary starch proceeds rapidly and leads to elevated post prandial hyperglycemia (PPHG). Hence retardation of starch digestion by inhibition of these enzymes viz., pancreatic $\alpha$-amylase and $\alpha$-glucosidase would play a key role in the control of diabetes. However, the discovery of safe, specific high-affinity inhibitors of these digestive enzymes for the development of therapeutics has remained elusive (Bhat et al., 2008; Sudha et al., 2010; Sudha et al., 2011) (Figure 1).

**Figure 1:** Carbohydrate from diet is partially broken down to oligosaccharides by salivary $\alpha$-amylase, which further catalyzed by pancreatic $\alpha$-amylase in gut to maltose, maltotriose and oligodextran. $\alpha$-glucosidase acts on maltose and maltotriose to form monosaccharide (glucose), which enters the blood vessels. In case of Type 2 diabetics, carbohydrate digestion after meals leads to a condition called as post prandial hyperglycemia (PPHG). Double bar marks (/\) indicate inhibition of pancreatic $\alpha$-amylase and $\alpha$-glucosidase activity leading to a reduction of maltose, oligosaccharide and glucose concentration and finally lowers the blood glucose level.
2 Pancreatic α-amylase and α-glucosidase Inhibitors from Indian Medicinal Plants

Indian medicinal plants have been an exemplary source of medicine. Research conducted in last few decades on plants mentioned in ancient literature or used traditionally for diabetes has shown antidiabetic property (Grover et al., 2002). Very few of these plants have been characterized for their mechanistic action on varying antidiabetic targets. Plants are known to posses enzyme inhibitors as a defensive mechanism against the predating insects. The enzyme inhibitors act on key insect gut digestive hydrolases, the α-amylases and proteinases. Several kinds of α-amylase and proteinase inhibitors, present in seeds and vegetative organs, act to regulate numbers of phytophagous insects. This defensive mechanism has been acquired by plants over a period of time due to evolution. Pancreatic α-amylases and α-glucosidases are potent targets for antidiabetic agents (amylase and glucosidase inhibitors). These inhibitors would retard the starch digestion (Figure 1) in type 2 diabetic patients subsequently lowering post prandial hyperglycemia (PPHG). The presence of amylase inhibitors naturally in plants for their defenses has thus therapeutic implication as antidiabetic agents (Franco et al., 2002; Bhat et al., 2008).

2.1 Objective

The major work discussed in this chapter is screening and identification of lead pancreatic α-amylase and α-glucosidase inhibitors form known hypoglycemic Indian plants. The plants screened are listed in Table 2.

<table>
<thead>
<tr>
<th>Plant Name</th>
<th>Family Name</th>
<th>Parts Used</th>
<th>Hypoglycemic and Medicinal Property</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Adansonia digitata</em> L.</td>
<td>Bombacaceae</td>
<td>Leaves</td>
<td>Lowers blood glucose level due to insulin like effect on peripheral tissues; by promoting glucose uptake and metabolism or by inhibiting hepatic Gluconeogenesis (Tanko et al., 2008).</td>
</tr>
<tr>
<td><em>Allium sativum</em> L.</td>
<td>Alliaceae</td>
<td>Rhizomes</td>
<td>Lowers blood pressure and improves lipid profile, decreases serum glucose, triglycerides, cholesterol, urea, uric acid, increases serum insulin levels (Eidia et al., 2006).</td>
</tr>
<tr>
<td><em>Aloe vera</em> (L.) Burm.F.</td>
<td>Liliaceae</td>
<td>Leaf Gel</td>
<td>Hypoglycemic activity, decreases fasting glucose levels, hepatic transaminases, plasma and liver cholesterol, triglycerides, free fatty acids and phospholipids. Improves plasma insulin level. Restores normal levels of LDL and HDL and cholesterol Reduces levels of hepatic phosphatidylcholine hydroperoxide and have hypocholesterimic efficacy, diminishes degenerative changes in kidney tissues (Yagi et al., 2009)</td>
</tr>
<tr>
<td><em>Azadirachta indica</em> A. Juss.</td>
<td>Meliaceae</td>
<td>Leaves</td>
<td>Antihyperglycemic activity, increase in glucose uptake and glycogen deposition, inhibits activity of epinephrine on glucose metabolism resulting in utilization of peripheral glucose. Does not alter cortisol concentration (Modak et al., 2007; Chattopadhyay, 1999)</td>
</tr>
<tr>
<td><em>Bixa orellana</em> L.</td>
<td>Bixaceae</td>
<td>Leaves</td>
<td>Hypoglycemic activity by lowering blood glucose by</td>
</tr>
<tr>
<td>Plant Name</td>
<td>Family</td>
<td>Part Used</td>
<td>Effect Description</td>
</tr>
<tr>
<td>------------------------------------------------</td>
<td>-----------------</td>
<td>----------</td>
<td>---------------------------------------------------------------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td><strong>Bougainvillea spectabilis Willd.</strong></td>
<td>Nyctaginaceae</td>
<td>Leaves</td>
<td>Hypoglycemic effect is by D-pinitol which exerts insulin-like effect and inhibits $\alpha$-glucosidase (Narayanan et al., 1987).</td>
</tr>
<tr>
<td><strong>Casia fistula L.</strong></td>
<td>Caesalpiniaceae</td>
<td>Leaves</td>
<td>Hypoglycemic activity decreases blood glucose level (Rizvi et al., 2009).</td>
</tr>
<tr>
<td><strong>Catharanthus roseus (L.) G. Don</strong></td>
<td>Apocynaceae</td>
<td>Leaves</td>
<td>Reduces blood glucose by enhancing secretion of insulin from b-cells of Langerhans or through extra pancreatic mechanism (Nammi et al., 2003).</td>
</tr>
<tr>
<td><strong>Cinnamomum verum J.S. Presl</strong></td>
<td>Lauraceae</td>
<td>Leaves, Bark</td>
<td>Hypoglycemic activity is by enhancing insulin activity, increasing lipid metabolism and antioxidant status, capillary function. Reduces the blood glucose and elevates the plasma insulin level (Khan et al., 2003; Verspohl et al., 2005).</td>
</tr>
<tr>
<td><strong>Coccinia grandis (L.) Voigt.</strong></td>
<td>Cucurbitaceae</td>
<td>Fruit</td>
<td>Reduces blood glucose and glycosylated hemoglobin content. C. indica extracts lowers blood glucose by depressing its synthesis, depression of glucose 6-phosphatase and fructose1,6, bisphosphatase and enhancing glucose oxidation by shunt pathway through activation of glucose 6-phosphate dehydrogenase (Shibib et al., 1993)</td>
</tr>
<tr>
<td><strong>Curcuma longa L.</strong></td>
<td>Zingiberaceae</td>
<td>Rhizome</td>
<td>Hypoglycemic, hypolipidemic, and antioxidant property. Decreased influx of glucose in polyol pathway, increasing NADPH/NADP ratio and increased activity of glucose peroxidase (Arun &amp; Nalini, 2002).</td>
</tr>
<tr>
<td><strong>Ficus benghalensis L.</strong></td>
<td>Moraceae</td>
<td>Leaves, Bark</td>
<td>Stimulates insulin secretion from beta cells inhibits insulin degradative process (Achrekar et al., 1991).</td>
</tr>
<tr>
<td><strong>Ficus racemosa L.</strong></td>
<td>Moraceae</td>
<td>Fruit pulp</td>
<td>Hypoglycemic activity by $\beta$-sitosterol isolated was found to lower the blood glucose level (Paarakh, 2009).</td>
</tr>
<tr>
<td><strong>Linum usitatissimum L.</strong></td>
<td>Linaceae</td>
<td>Seeds</td>
<td>Reduces fasting blood sugar levels, total cholesterol; reduces carbohydrate absorption from gut and clinical symptoms of diabetes associated with dyslipidamia (Thakur et al., 2009).</td>
</tr>
<tr>
<td><strong>Mangifera indica L.</strong></td>
<td>Anacardiaceae</td>
<td>Fruit, Leaves</td>
<td>Reduces glucose absorption in type 2 diabetes. Stimulates glycogenesis in liver causing reduction in blood glucose level (Bhowmik et al., 2009).</td>
</tr>
<tr>
<td><strong>Momordica charantia L.</strong></td>
<td>Cucurbitaceae</td>
<td>Leaves, fruit, seeds</td>
<td>Hypoglycemic effect by inhibition of glucose-6-phosphatase and fructose-1-6-biphosphatase in liver and stimulation of hepatic glucose-6-phosphate dehydrogenase (Shibib et al., 1993).</td>
</tr>
<tr>
<td><strong>Murraya koenigii L. Spreng</strong></td>
<td>Rutaceae</td>
<td>Leaves</td>
<td>Increases glucogenesis and decreases glycogenolysis and gluconeogenesis (Khan et al., 1995).</td>
</tr>
<tr>
<td><strong>Morus alba L.</strong></td>
<td>Moraceae</td>
<td>Leaves</td>
<td>Antiphlogistic, diuretic, expectorant and antidiabetic. Increases b-cell number in diabetic islets. Reduces levels of glycosylated hemoglobin. Decreases triglycerides, cholesterol and VLDL to normal levels in type II DM. Restores elevated levels of blood urea (Mohammadi&amp;Naik, 2008).</td>
</tr>
<tr>
<td><strong>Nerium oleander L.</strong></td>
<td>Apocynaceae</td>
<td>Leaves</td>
<td>Chlorogenic acid, quercetin and cathechin induce post prandial hyperglycemia by acting as $\alpha$-glucosidase inhibitors (Ishikawa et al., 2007).</td>
</tr>
<tr>
<td><strong>Ocimumtenuiflorum L.</strong></td>
<td>Laminaceae</td>
<td>Leaves</td>
<td>Lowers blood glucose level, modulates cellular antioxidant defense system. Improves b cell function and enhances insulin secretion. Inhibits absorption of glucose from the intestine (Sethi et al., 2004)</td>
</tr>
<tr>
<td><strong>Piper nigrum L.</strong></td>
<td>Piperaceae</td>
<td>Seeds</td>
<td>Reduces glucose and serum lipid levels (Kaleem et al., 2005).</td>
</tr>
<tr>
<td><strong>Syzygiumcumini L. Skeels (Jamun)</strong></td>
<td>Myrtaceae</td>
<td>Seeds</td>
<td>Reduces blood glucose level, increase in serum insulin level, exhibits insulinase activity. Hypoglycemic activity mediated through insulin release mechanism, glycogen content and hepatic glucokinase, hexokinase, glucose-6-phosphate, and phosphofructokinase levels in diabetic mice (Modak et al., 2007)</td>
</tr>
<tr>
<td><strong>TerminaliachebulaRetz.</strong></td>
<td>Combretaceae</td>
<td>Fruit</td>
<td>Decreases blood glucose levels by enhancing secretion of insulin from b cells of Langerhans or through extra pancreatic mechanism. Inhibits advanced glycosylation end products, which contribute to renal damage (Rao &amp; Nammi, 2006).</td>
</tr>
<tr>
<td><strong>Tinosporacordifolia (Willd.) Miers</strong></td>
<td>Menispermaceae</td>
<td>Stem</td>
<td>Decreases blood glucose level through glucose metabolism. It exhibits inhibitory effect on adrenaline-induced hyperglycemia (Singh et al., 2003).</td>
</tr>
<tr>
<td><strong>Trigonellafoenum-graceum L.</strong></td>
<td>Fabaceae</td>
<td>Seeds</td>
<td>Decreases post prandial blood glucose level (Ismail, 2009).</td>
</tr>
<tr>
<td><strong>TribulusterrestrisL.</strong></td>
<td>Zygophyllaceae</td>
<td>Seeds</td>
<td>Hypoglycemic activity by inhibiting oxidative stress (Kostova &amp; Dinchev, 2005).</td>
</tr>
<tr>
<td><strong>ZingiberofficinaleRosc.</strong></td>
<td>Zingiberaceae</td>
<td>Rhizome</td>
<td>Lowers plasma glucose level, (Agoreyo et al., 2006)</td>
</tr>
</tbody>
</table>

Table 2: Indian Medicinal plant sources and their traditional uses.

Screening is initiated by amylase and glucosidase inhibition assays of plant extracts prepared by solvent extraction protocol. The positive extracts are further subjected to bioactivity guided isolation of lead inhibitor components by varying chromatographic techniques. Enzyme inhibition kinetics performed would reveal the mode of inhibition of the lead purified inhibitor.
2.2 Extraction Protocols

Extraction, as the term is used pharmaceutically, involves the separation of medicinally active portions of plant or animal tissues from the inactive or inert components by using selective solvents in standard extraction procedures. The extract thus obtained can be further fractionated to isolate individual lead bioactive entities. Thus, standardization of extraction procedures contributes significantly to the final quality of the herbal drug (Reichardt, 2003).

Sequential solvent extraction is one such extraction protocol used to extract the desired components from plants based on their solubility in solvents varying in polarity. In Ayurveda, the herbal formulations prepared are generally aqueous in nature. Moreover, aqueous extracts contain peptides, proteins, or glycans, which would otherwise be denatured by organic solvents and high-temperature extraction. The recommended sequential solvent extraction is from a higher polar solvent (water) sequentially to a nonpolar solvent based on their relative polarity (Sudha et al., 2010; Sudha et al., 2011). The values for relative polarity are normalized from measurements of solvent shifts of absorption spectra (Reichardt, 2003). The extraction scheme is represented in Figure 2. Twenty-seven hypoglycemic plants listed in Table 2 yielded 217 extracts.

![Sequential Solvent Extraction of Plant Samples](image)

**Figure 2:** Plant samples are crushed with liquid nitrogen and sequentially extracted with solvents of decreasing polarity, initiating with water (Relative Polarity RP) of 1.0 and ending with cyclohexane (RP:0.006). CWE: Cold water extract; HWE: Hot water extract; ME: Methanol extract; IPE: Isopropanol extract; AE: Acetone extract; MTBE: Methyl-tertiary Butyl-ether extract; CHE: Cyclohexane extract.

2.3 Biochemical Assay Protocols

Enzyme assays are experimental protocols that make enzyme-catalyzed chemical transformations visible. Such assays are very important for high-throughput screening in the context of drug discovery (Goddard & Reymond, 2004). One Unit (U) is defined as the amount of the enzyme that catalyses the conversion of 1 micromole of substrate or formation of 1 micromole of product per minute under the given assay conditions (Lehninger, 4th edition).
2.3.1 Pancreatic α-amylase Assay

There are mainly two types of assays that are used to determine the activity of α-amylase. One is based on measuring the amount of product formed i.e maltose a reducing sugar by the dinitrosalicylic acid (DNS) assay or the Nelson–Somogyi (Miller, 1959; Somogyi, 1952) method, whereas the other is based on the decreased staining value of blue starch–iodine complexes i.e measurement of substrate depletion (Fuwa, 1954). There are many chlorogenic substrates which are been approved by IFCC (International Federation for Clinical Chemistry) to determine the α-amylase activity.

**Starch-iodine assay:** The assay followed for screening of 126 extracts (Sudha *et al*., 2011) of the 217 extracts initially was microtitre plate based on the starch-iodine test (Xiao *et al*., 2006). Acarbose, was used as a positive control. Appropriate controls were kept. A dark-blue color indicates the presence of starch; a yellow color indicates the absence of starch while a brownish color indicates partially degraded starch in the reaction mixture. In the presence of inhibitors from the extracts the starch added to the enzyme assay mixture is not degraded and gives a dark blue color complex whereas no color complex is developed in the absence of the inhibitor, indicating that starch is completely hydrolyzed by α-amylase.

**3, 5-dinitrosalicylic acid assay:** The positively screened 21 extracts of the 126 by starch-iodine test along with the remaining 91 extracts (Sudha *et al*., 2010) were quantitatively assayed for pancreatic α-amylase inhibition by chromogenic DNSA method. In this method, in presence of the free carbonyl group (C=O), present in the reducing sugars, (maltose formed due to amylase activity on starch) 3,5-dinitrosalicylic acid (DNSA) is reduced to 3-amino,5-nitrosalicylic acid under alkaline conditions:

\[
\begin{align*}
\text{oxidation} & : & \text{aldehyde group} & \rightarrow \text{carboxyl group} \\
\text{reduction} & : & 3,5\text{-dinitrosalicylic acid} & \rightarrow 3\text{-amino,5-nitrosalicylic acid.}
\end{align*}
\]

Appropriate enzyme and extract controls were kept.

One unit of enzyme activity is defined as the amount of enzyme required to release one micromole of maltose from starch per min under the assay conditions.

\[
\text{% Relative enzyme activity} = \frac{\text{enzyme activity of test}}{\text{enzyme activity of control}} \times 100;
\]

\[
\text{% Inhibition in the } \alpha\text{-amylase activity} = (100 - \% \text{ Relative enzyme activity}).
\]

The extracts are evaluated in terms of their IC\text{50} value. The IC\text{50} values were defined as the concentration of the extract, containing the α-amylase inhibitor that inhibited 50% of the pancreatic-α-amylase activity.

Structurally as well as mechanistically, porcine pancreatic α-amylase is closely related to human pancreatic-α-amylase (Brayer *et al*., 1995). The enzyme used for screening is porcine pancreatic α-amylase. Further work is carried on human pancreatic α-amylase. Aqueous extracts (both hot and cold water) of *F. bengalensis* bark, *S. cumini*, methanol extracts of *B. orellana*, *C. longa*, *C. verum*, isopropanol extracts of *C. longa*, *C. verum*, *M. koenigi*, *L. usitatissimum*, *M. alba* and, *O. tenuiflorum*, and acetone extracts of *C. longa* and *T. terrestris* exhibited significant porcine pancreatic-α-amylase inhibition ≥ 50% at a concentration ranging from 9.4 and 1900 µgmL\text{−1}. On comparing the IC\text{50} values of extracts against PPA and HPA with that of the positive control Acarbose (IC\text{50} value both against PPA and HPA is 10.2 µgmL\text{−1}), and the stability of the extracts over a period of time, except methanol extracts of *C. longa* and *C. verum* the remaining 13 extracts listed in Table.3 were taken further to isolate the lead inhibitor com-
ponents. Of these, the 4 extracts, highlighted in red, exhibited a concentration independent inhibition whereas the other 8 extracts exhibited concentration dependent inhibition. *C. longa* isopropanol extract exhibited the least IC$_{50}$ value of 0.16 $\mu$g/mL$^{-1}$ on HPA.

### 2.3.2 $\alpha$-glucosidase Assay

$\alpha$-Glucosidase catalyzes the hydrolysis of terminal $\alpha$-1,4- and $\alpha$-1,6-glucosidic linkages of glycogen. There are a few enzyme assays. The fluorescence assay uses 4-methylumbelliferyl-$\alpha$-D-glucopyranoside (4MU-$\alpha$-glc) as the fluorogenic substrate. The product of this substrate, 4-methylumbelliferone (4MU), emits at a peak of 440 nm in the fluorescence spectra. Chromogenic assays that use p-nitrophenyl-$\alpha$-D-glucopyranoside and 2-naphthyl-$\alpha$-D-glucopyranoside as substrates are also available for $\alpha$-glucosidase assay. In addition, p-nitrophenyl-$\alpha$-D-maltoheptaoside has been used for compound screening, but the screen throughput was relatively low (Motabar *et al.*, 2009). The $\alpha$-glucosidase assay performed used the fluorescence assay with 4-MU-$\alpha$-Glc as the substrate. One unit of enzyme activity is defined as the amount of enzyme required to release one micromole of 4-MU from 4-MU-$\alpha$-Glc per min under the assay conditions (Sudha *et al.*, 2012).

$$\%\text{ Relative enzyme activity} = \frac{\text{enzyme activity of test}}{\text{enzyme activity of control}} \times 100;$$

$$\%\text{ Inhibition in the } \alpha\text{-amylase activity} = (100 - \%\text{ Relative enzyme activity})$$

### 2.4 Isolation of Lead Inhibitor Component

Natural products remain a prolific source of discovery of newer drugs and drug leads from vedic period. The conventional drug discovery process aims in identifying single pure active constituent from an active extract and a method to estimate it in the crude drug (Bhutani *et al.*, 2010). Bioassay-guided fractionation of medicinal plants is a routine feature in the attempt to isolate bioactive components from natural sources. Bioassay-guided fractionation linked to chromatographic separation techniques leads to the isolation of biologically active molecules whose chemical structures can readily be determined by modern spectroscopic methods (Phillipson *et al.*, 2001).

Bioactive guided purification of lead human pancreatic $\alpha$-amylase inhibitor from the 13 extracts listed in Table 3 initiated with a qualitative phytochemical analysis of the extract, revealing the possible class of phytoconstituents present in these crude extracts. Alkaloids, proteins, tannins, cardiac glycosides, flavonoids, saponins and steroids are the class of compounds qualitatively detected in the extracts. GCMS analysis of the extracts revealed the probable compounds present.

Of these, *C. longa* isopropanol extract which exhibited the most potent human pancreatic $\alpha$-amylase inhibition with the least IC$_{50}$ value was worked on initially for the lead inhibitor compound. Preliminary phytochemical and GCMS analysis of this crude *C. longa* isopropanol extract suggested the presence of curcuminoids, sesquiterpenes and cinnamic acid as the components present. Bioactivity guided isolation of the lead inhibitor component with solvent fractionation and chromatographic techniques with characterization with HPLC and NMR in comparison with the standards revealed bisdemethoxycurcumin as a lead small molecule inhibitor of porcine and human pancreatic $\alpha$-amylase with an IC$_{50}$ value of 0.026 and 0.025 mM, respectively. The crude extract from rat gut was also checked for inhibition of $\alpha$-amylase and $\alpha$-glucosidase activity with BDMC at the HPA IC$_{50}$ value (0.025 mM). The $\alpha$-glucosidase from Baker’s yeast and rat intestinal crude extract exhibited weaker inhibition of 16.4% and 21.0%, respectively, as compared to 53% for $\alpha$-amylase from rat intestine, suggesting that BDMC is a better pancreatic $\alpha$-amylase inhibitor as compared to $\alpha$-glucosidase (Sudha *et al.*, 2012).
<table>
<thead>
<tr>
<th>Plants</th>
<th>Extracts</th>
<th>Name of Compound</th>
<th>Molecular Formula</th>
<th>Molecular Wt</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; (µg/ml) value HPA</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. orellana</em> L.(Leaves)</td>
<td>Methanol</td>
<td>β-tocopherol Vitamin E</td>
<td>C&lt;sub&gt;28&lt;/sub&gt;H&lt;sub&gt;46&lt;/sub&gt;O&lt;sub&gt;2&lt;/sub&gt; C&lt;sub&gt;29&lt;/sub&gt;H&lt;sub&gt;50&lt;/sub&gt;O&lt;sub&gt;2&lt;/sub&gt;</td>
<td>416</td>
<td>49.0 ± 5.4E-3</td>
</tr>
<tr>
<td><em>C. longa</em> (Rhizomes)</td>
<td>Isopropanol</td>
<td>Podocarpic acid Curlone Cinnamic-acid</td>
<td>C&lt;sub&gt;17&lt;/sub&gt;H&lt;sub&gt;22&lt;/sub&gt;O&lt;sub&gt;3&lt;/sub&gt; C&lt;sub&gt;15&lt;/sub&gt;H&lt;sub&gt;28&lt;/sub&gt;O C&lt;sub&gt;8&lt;/sub&gt;H&lt;sub&gt;8&lt;/sub&gt;O&lt;sub&gt;2&lt;/sub&gt;</td>
<td>274</td>
<td>0.16 ± 4.0E-5</td>
</tr>
<tr>
<td><em>C. longa</em> (Rhizomes)</td>
<td>Acetone</td>
<td>3-Cyano-7-hydroxy-4-methylcoumarin Curlone 5-amino-2-hydroxybenzoic acid</td>
<td>C&lt;sub&gt;11&lt;/sub&gt;H&lt;sub&gt;22&lt;/sub&gt;O&lt;sub&gt;3&lt;/sub&gt; C&lt;sub&gt;15&lt;/sub&gt;H&lt;sub&gt;28&lt;/sub&gt;O C&lt;sub&gt;8&lt;/sub&gt;H&lt;sub&gt;8&lt;/sub&gt;O&lt;sub&gt;2&lt;/sub&gt;</td>
<td>201</td>
<td>7.4 ± 1.0E-4</td>
</tr>
<tr>
<td><em>C. verum</em> (Leaves)</td>
<td>Isopropanol</td>
<td>Naphthalene,1,2,3,4- tetrahydro-1,1,6-trimethyl Eugenol 4-acetoxyccinnamic acid</td>
<td>C&lt;sub&gt;13&lt;/sub&gt;H&lt;sub&gt;18&lt;/sub&gt; C&lt;sub&gt;10&lt;/sub&gt;H&lt;sub&gt;12&lt;/sub&gt;O&lt;sub&gt;2&lt;/sub&gt; C&lt;sub&gt;11&lt;/sub&gt;H&lt;sub&gt;10&lt;/sub&gt;O&lt;sub&gt;4&lt;/sub&gt;</td>
<td>174</td>
<td>1.0 ± 1.0E-4</td>
</tr>
<tr>
<td><em>F. bengalensis</em> (Bark)</td>
<td>Cold water</td>
<td>ND</td>
<td></td>
<td></td>
<td>4.4 ± 2.3E-4</td>
</tr>
<tr>
<td><em>F. bengalensis</em> (Bark)</td>
<td>Hot water</td>
<td>ND</td>
<td></td>
<td></td>
<td>125 ± 1.0E-3</td>
</tr>
<tr>
<td><em>L. usitatumum</em> (Seeds)</td>
<td>Isopropanol</td>
<td>2-cyclopentene-1-undecanoic acid cyclopentane undecanoic acid</td>
<td>C&lt;sub&gt;16&lt;/sub&gt;H&lt;sub&gt;28&lt;/sub&gt;O&lt;sub&gt;2&lt;/sub&gt; C&lt;sub&gt;16&lt;/sub&gt;H&lt;sub&gt;30&lt;/sub&gt;O&lt;sub&gt;2&lt;/sub&gt;</td>
<td>252</td>
<td>540 ± 2.0E-3*</td>
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<tr>
<td><em>M. alba</em> (Leaves)</td>
<td>Isopropanol</td>
<td>Hexadecanoic acid 9, 12-octadecadienic acid</td>
<td>C&lt;sub&gt;16&lt;/sub&gt;H&lt;sub&gt;32&lt;/sub&gt;O&lt;sub&gt;2&lt;/sub&gt; C&lt;sub&gt;17&lt;/sub&gt;H&lt;sub&gt;31&lt;/sub&gt;COOH</td>
<td>128.6 256.4</td>
<td>1440 ± 6.0E-3*</td>
</tr>
<tr>
<td><em>M. koenigii</em> L.(Leaves)</td>
<td>Isopropanol</td>
<td>Cyclohexanone, 2-methyl-5-(1-methylene) 2,3,5,6- tetrachlorohydroquinone Vitamin E</td>
<td>C&lt;sub&gt;10&lt;/sub&gt;H&lt;sub&gt;16&lt;/sub&gt; C&lt;sub&gt;4&lt;/sub&gt;H&lt;sub&gt;2&lt;/sub&gt;Cl&lt;sub&gt;4&lt;/sub&gt;O&lt;sub&gt;2&lt;/sub&gt; C&lt;sub&gt;29&lt;/sub&gt;H&lt;sub&gt;50&lt;/sub&gt;O&lt;sub&gt;2&lt;/sub&gt;</td>
<td>152 247 430</td>
<td>127 ± 4.0E-3</td>
</tr>
<tr>
<td><em>Ocimum tenuiflorum</em> L.(Leaves)</td>
<td>Isopropanol</td>
<td>Camphene Methyleneugenol 2-heptanol, 5-ethyl</td>
<td>C&lt;sub&gt;10&lt;/sub&gt;H&lt;sub&gt;16&lt;/sub&gt; C&lt;sub&gt;11&lt;/sub&gt;H&lt;sub&gt;14&lt;/sub&gt;O&lt;sub&gt;2&lt;/sub&gt; C&lt;sub&gt;3&lt;/sub&gt;H&lt;sub&gt;20&lt;/sub&gt;O</td>
<td>136.24 178.23 144.25</td>
<td>8.9 ± 3.5E-3*</td>
</tr>
<tr>
<td><em>S. cumini</em> (Seeds)</td>
<td>Cold water</td>
<td>ND</td>
<td></td>
<td></td>
<td>42.1 ± 2.6E-3</td>
</tr>
<tr>
<td><em>S. cumini</em> (Seeds)</td>
<td>Hot water</td>
<td>ND</td>
<td></td>
<td></td>
<td>4.1 ± 2.8E-4</td>
</tr>
<tr>
<td><em>T. terrestris</em> L. (Seeds)</td>
<td>Acetone</td>
<td>Sorbinose Ethyl crotonate</td>
<td>C&lt;sub&gt;6&lt;/sub&gt;H&lt;sub&gt;12&lt;/sub&gt;O&lt;sub&gt;6&lt;/sub&gt; C&lt;sub&gt;6&lt;/sub&gt;H&lt;sub&gt;10&lt;/sub&gt;O&lt;sub&gt;2&lt;/sub&gt;</td>
<td>180 114</td>
<td>511 ± 5.0E-3</td>
</tr>
</tbody>
</table>

Table 3: GCMS analysis of extracts exhibiting ≥ 50% inhibition on α-amylase activity. ND: Not determined. * IC<sub>50</sub> (µg /ml) for PPA.
2.5 Enzyme inhibition kinetics

Enzyme inhibition is defined as a reduction in enzyme activity through the binding of an inhibitor to a catalytic or regulatory site on the enzyme, or in the case of uncompetitive inhibition, to the enzyme–substrate complex. Inhibition can be reversible which involves non covalent bonding irreversible involving covalent binding. Inhibitors differ in the mechanism by which they decrease enzyme activity. There are three basic mechanisms of inhibition–competitive, noncompetitive, and uncompetitive inhibition. A competitive inhibitor is usually a close analogue of the substrate. It binds at the catalytic site but does not undergo catalysis. A noncompetitive inhibitor does not bind to the catalytic site but binds to a second site on the enzyme and acts by reducing the turnover rate of the reaction. An uncompetitive inhibitor does not bind to the enzyme but only the enzyme–substrate complex. Enzyme kinetics is principally concerned with the measurement and mathematical description of the enzyme reaction rate and its associated constants. The Michaelis–Menten equation, as presented by Michaelis and Menton and further developed by Briggs and Haldane (Briggs & Haldane, 1925; Michaelis & Menton, 1913) is fundamentally important to enzyme kinetics.

\[
\frac{v}{v_{\text{max}}} = \frac{[A]}{K_m + [A]}
\]

where \([A]\) is the substrate concentration; \(K_m\) is an inverse measure of the affinity or strength of binding between the enzyme and its substrate. The lower the \(K_m\), the greater the affinity (so the lower the concentration of substrate needed to achieve a given rate). The \(k_{\text{cat}}\), is the turnover number of the enzyme, is a measure of the maximum catalytic production of the product under saturating substrate conditions per unit time per unit enzyme. The larger the values of \(k_{\text{cat}}\), the more rapidly catalytic events occur. The \(v_{\text{max}}\) is the maximum velocity that an enzyme could achieve. The measurement is theoretical because at given time, it would require all enzyme molecules to be tightly bound to their substrates.

Evaluating enzyme kinetics using a nonlinear plot Michaelis and Menten is avoided by using one of the three common linearization methods, Lineweaver–Burke, Eadie–Hofstee plots; Hanes plots to obtain estimates for \(K_m\) and \(v_{\text{max}}\). In case of competitive inhibitor, an increase in the inhibitor concentration will increase the apparent \(K_m\) of the enzyme. However, since an infinite substrate concentration will exclude the competitive inhibitor, there is no effect on \(v_{\text{max}}\). Whereas, in case of a simple noncompetitive inhibitor \(K_m\) is not altered, but \(v_{\text{max}}\) reduces as inhibitor concentration increases. In case of an uncompetitive inhibitor, sequestration of the enzyme–substrate complex will reduce the apparent \(k_{\text{cat}}\) because the inhibited enzyme is less catalytically effective. Apparent \(v_{\text{max}}\) is reduced (and apparent \(K_m\) increased) because binding of the inhibitor cannot be prevented by increasing the substrate concentration.

Enzyme inhibition kinetics in other words reveals the mechanism of inhibition. The kinetics alters with respect to purity of the inhibitor and the substrate worked on. The effect of bisdemethoxycurcumin on the kinetics of HPA catalyzed hydrolysis of starch was studied at differing inhibitor concentrations. The double reciprocal LB plots revealed that the mode of bisdemethoxycurcumin inhibition is uncompetitive for starch as a substrate with an apparent \(K_i\) of 3.0 \(\mu\)M and decrease in both the apparent \(K_m\) and \(v_{\text{max}}\) values. Thus bisdemethoxycurcumin binds to the enzyme at a site other than the active site. Stoichiometry in enzyme inhibition kinetics accounts for the ratio in which the inhibitor molecules bind to an enzyme molecule to exhibit enzyme inhibition. The stoichiometry of bisdemethoxycurcumin:HPA when calculated at varying concentration of inhibitor to a fixed concentration of enzyme accounts for one molecule of bisdemethoxycurcumin binding to one molecule of HPA, thus exhibiting a stoichiometry1:1 (Sudha et al., 2012).
3 Conclusion

Many different plants have been used individually or in formulations for treatment of diabetes and its complications due to their advantages over current drug therapy. One of the major problems with this herbal formulation is that the active ingredients are not well defined. It is important to know the active component and their molecular interaction, which will help to analyze therapeutic efficacy of the product and also to standardize the product. In this limelight, target based drug discovery would reveal the mode of action of the drug on the antidiabetic targets. Human pancreatic α-amylase and α-glucosidase are the preliminary enzymes in carbohydrate metabolism, the retardation of their activities by inhibitors would play a key role in management of diabetes. Plants are a good source of glycosidase inhibitors as a key defensive mechanism against insects. This property has been exploited for its therapeutic implication against diabetes mellitus for retarding the activities of these glycosidases. With this rationale, in our research work carried out, 217 plant extracts from 27 different hypoglycemic plants have been screened for α-amylase and α-glucosidase inhibitors. The screening finally concluded with C.longa isopropanol extract exhibiting potent pancreatic α-amylase inhibition at an IC$_{50}$ value of 0.16µgm$^{-1}$. Bioactivity guided isolation and characterization of lead inhibitor compound from C.longa isopropanol extract resulted in bisdemethoxycurcumin as the potent lead inhibitor component with an IC$_{50}$ value of 0.025 mM. HPA inhibition kinetics revealed its mode of inhibition to be uncompetitive with a stoichiometry of 1:1(Sudha et al., 2012).Thus, an inactivator of HPA viz., BDMC isolated from C. longa functioning via uncompetitive inhibition could be developed as a lead anti-diabetic compound. Knowledge gained in this study would help future developments of functional foods for controlling starch digestion and postprandial hyperglycemia. As per the drug discovery module, in vivo preclinical studies would reveal the pharmacokinetic and pharmacodynamic parameters for the drug to be an eligible lead for clinical studies.

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References


