Chapter II

Insulin Secretagogue, Alpha-glucosidase Inhibitory and Antioxidant Activity of Some Selected Spices
1. **Introduction**

Diabetes mellitus is a metabolic disorder characterized by hyperglycemia with impaired metabolism of carbohydrate, fat and proteins as a result of defects in insulin secretion, insulin action or both (WHO 1999). Type 2 diabetes mellitus has become a worldwide health problem and an important cause of morbidity and mortality. Through lifelong vascular complications, diabetes leads to enhanced rates of myocardial infarction, stroke, renal failure, blindness, and amputations.

The current thinking in diabetes treatment suggests use of combinatorial treatment at multiple targets as more desirable (DeFronzo 2010). Hence use of sulfonylurea along with alpha-glucosidase inhibitors or metformin is preferable. Use of alpha-glucosidase inhibitors is advocated to control post prandial glucose spikes. Accumulating evidence shows that excessive fluctuations in post-meal blood glucose levels (postprandial hyperglycemia) have adverse consequences for diabetes-related morbidity and mortality. It is now apparent that postprandial hyperglycemia plays a central role in the decline from impaired glucose tolerance (IGT) to overt diabetes and in the development and progression of diabetes complications (Diabetes control and complications trial research group 1995). Alpha-glucosidase inhibitors inhibit alpha-glucosidase enzymes such as maltase and sucrase in intestine consequently reducing postprandial hyperglycemia by delaying the absorption of carbohydrate from the small intestine. These agents reduce blood glucose without increasing insulin secretion and do not cause hypoglycemia or weight gain. In addition, treatment with an alpha-glucosidase inhibitor can improve lipid metabolism, reduce fasting plasma glucose levels, and improve insulin sensitivity in a non-invasive manner.

Among the plant remedies, there is growing interest in use of edible food components or spices in treatment of diabetes. The benefits of using food components, edible herbs and spices is that they are used for their medicinal properties from ancient time, possess nutrient value with no/less toxicity. From ancient years, spices are being extensively used to the increase taste and flavor of foods. These spice ingredients impart characteristic flavor, aroma, or piquancy and color to foods. Some spices, like fenugreek, can also modify the texture of food. It is a common experience that their distinct aroma stimulates the appetite. Not only are spices used as flavorings and seasonings, but many are also used in perfumery and cosmetics. In addition, several spices have long been
recognized to possess medicinal properties. Several spices or their isolated components were shown with the antidiabetic activity. Diet has been recognized as one of the most important factor in management of diabetes mellitus. As part of the dietary treatment of diabetes, there has been a continuous search for novel antidiabetic drugs from plant sources.

Spices, the natural and common food adjuncts, have also been examined in this direction. Various studies in experimentally induced diabetic animal models and human experiments shown that the spices significant antilipidemic and antidiabetic action. Many researchers showed antihyperglycemic activity of spices but mechanism of action was not evaluated (Srinivasan 2005; Kaushik et al. 2010). Coriander seeds fed to rats consuming a high-fat diet led to decreased LDL, VLDL, and total cholesterol and increased HDL cholesterol (Chithra and Leelamma 1997). In rats, curry leaf and mustard seeds decreased total serum cholesterol, LDL cholesterol, and VLDL cholesterol and increased HDL cholesterol levels (Khan et al. 1996). Fenugreek, turmeric, or its active principle curcumin, onion or its active principle allyl propyl disulfide, garlic, and cumin were observed to improve glycemic status in diabetic animals and non-insulin dependent diabetes mellitus (NIDDM) patients. Addition of fenugreek seeds to the diets of diabetic patients or animals results in a fall in blood glucose and improvement in glucose tolerance (Sharma et al. 1996; Khosla et al. 1995).

Cinnamon is a commonly used spice and possesses significant antihyperglycemic effect. Aqueous extracts from cinnamon have been shown to increase in vitro glucose uptake and glycogen synthesis and to increase phosphorylation of the insulin receptor (Imparl-Radosevich et al. 1998). In addition, these cinnamon extracts are likely to aid in triggering the insulin cascade system (Jarvill-Taylor et al. 2001). Intake of 1, 3 or 6 g of cinnamon per day reduces serum glucose, triglyceride, LDL cholesterol, and total cholesterol in people with type 2 diabetes (Khan et al. 2003; Hlebowicz et al. 2007). Cinnamaldehyde, a major component of cinnamon, was reported to possess hypoglycemic and hypolipidemic effects in STZ-induced diabetic rats (Babu et al. 2007).

The blind use of herbal remedies with unknown mechanism for blood glucose lowering effect is progressively decreasing and there is growing interest in use of remedies with known mechanism of action. The antihyperglycemic mechanism for several spices was reported and few were shown to possess insulin secretagogue action. *Coriandrum sativum* was reported for its insulin secretagogue action. Aqueous extract of
coriander at a concentration of 0.25–10 mg/ml evokes a stepwise i.e. 1.3 to 5.7-fold stimulation of insulin secretion from a clonal β-cell line (Gray and Flatt 1999). The spice probably delays gastric emptying by direct interference with glucose absorption. The spices with alpha-glucosidase inhibitory activities were also reported. The cinnamon extract inhibits β-glucosidase activity and hence beneficial in diabetes mellitus (Shihabudeen et al. 2011). The ellagitannins, casuarictin and eugeniin isolated from methanol extracts of clove (Syzgium aromaticum) inhibits the rat intestinal maltase activity (Toda et al. 2000). Cuminaldehyde isolated from Cuminum cyminum L. seeds shows aldose reductase and α-glucosidase inhibitory activity (Lee 2005).

Oxidative damage at the cellular or subcellular level is now considered to be an important event in disease processes like cardio vascular disease (CVD), inflammatory disease, carcinogenesis, and aging. Also the oxidative stress in diabetes is considered as responsible factor in development of secondary complications such as neuropathy, nephropathy and retinopathy. Tanaka et al. demonstrated that glucose itself is capable of generating reactive oxygen species (ROS) in β-cells is essential to the hypothesis that glucose-induced oxidative stress is a mechanism for glucose toxicity (Tanaka et al 1999).

There are multiple sources of oxidative stress in diabetes including non-enzymatic, enzymatic and mitochondrial pathways. Hyperglycemia can directly cause increased ROS generation. Glucose can undergo autoxidation and generate •OH radicals. In addition, glucose reacts with proteins in a nonenzymatic manner leading to the development of Amadori products followed by formation of AGEs. ROS is generated at multiple steps during this process. In hyperglycemia, there is enhanced metabolism of glucose through the polyol (sorbitol) pathway, which also results in enhanced production of •O²⁻ (Johansen et al. 2005). Reactive oxygen radicals are detrimental to cells at both membrane and genetic levels. They induce lipid peroxidation in cellular membranes, generating lipid peroxides that cause extensive damage to membranes in terms of cross-linking of membrane components, leaks and lysis, and membrane-mediated chromosomal damage.

Numerous studies demonstrated that oxidative stress, mediated mainly by hyperglycemia-induced generation of free radicals, contributes to the development and progression of diabetes and related contributions. Hence, ameliorating oxidative stress through treatment with antioxidants might be an effective strategy for reducing diabetic complications. Reactive species can be eliminated by a number of enzymatic and
Spices have been investigated for their antioxidant potency in food systems for at least 55 years. The commonly used spices such as cinnamon, cloves, fennel, ginger, lavender, parsley, rose, rosemary, sage and thyme were reported for their antioxidant capacities (Chohan et al. 2008). Cumin (Cuminum cyminum), black cumin (Nigella sativa) and bitter cumin (C. nigrum) shows good antioxidant activity (Thippeswamy and Naidu 2005; Surya et al 2010).

Spices having antihyperglycemic activity along with antioxidant potential will therefore play a vital role in prevention of progression of secondary complications. Hence, the objective of this study was to evaluate insulin secretagogue, alpha-glucosidase inhibitory and antioxidant activity of five spices which have not been studied extensively i.e. Myristica fragrans, Parmelia perlata, Illicium verum, Trachyspermum copticum, Myristica malabarica in streptozotocin induced diabetic rats.

2. **Introduction of spices used in study**

2.1 *Myristica fragrans* (mace)

*Myristica fragrans* Houtt (Family- Myristicaceae) is an aromatic evergreen tree, usually growing to 5 to 13 metres high, occasionally 20 metres. Indigenous to the Moluccas and Banda Islands in the South Pacific, it is seldom found truly wild. It is now cultivated in tropical regions, especially in Indonesia, Grenada in the West Indies, India and Sri Lanka.
**Parts used** - Two part of *Myristica fragrans* are greatly used as spices viz. nutmeg and mace. Nutmeg is the seed kernel inside the fruit and mace is the fleshy red, net like skin covering (aril) on the kernel. The seeds of *M. fragrans* (nutmeg) were imported into Europe during the 12th century and they have long been used indigenously as a spice in many kinds of Western food. When fresh, the aril (mace) is bright scarlet becoming horny, brittle and with a yellowish-brown color when dried.

**Medicinal uses** - Extracts of nutmeg show a good antidiarrhoeal effect, with a significant sedative property (Grover et al. 2002). Nutmeg has shown antioxidant (Olaleye 2006; Calliste 2010), anti-inflammatory (Jin et al. 2005), protein tyrosine phosphatase 1B inhibitory (Yang et al. 2006) and acetylcholine esterase inhibitory activities (Mukherjee et al. 2007). The AMP-activated protein kinase (AMPK) activators were isolated from nutmeg which can be beneficial in treatment of obesity and may be useful in treatment of type-2 diabetes or other metabolic disorders (Nguyen 2010). The nutmeg shows hypolipidaemic effect (Ram et al. 1996) and also used in prevention of hypercholesterolemia and atherosclerosis (Sharma et al. 1995). Myristicin (Morita et al. 2003) and macelignan (Sohn et al. 2008) isolated from nutmeg are reported to possess hepatoprotective action. Mace is widely used as a flavouring agent, a hair dye and a folk medicine. It also possesses antipapillomagenic, anticarcinogenic (Hussain and Rao 1991) and anti-inflammatory activities (Ozaki et al. 1989).

** Constituents** - It has been reported to contain 25–30% fixed oils and 5–15% volatile oils, such as camphene, elemicin, eugenol, isoelemicin, isoeugenol, methoxyeugenol, pinene, sabinene, and safrol; and chemical substances, such as dihydroguaiaretic acid, myristicin, and lignans.

### 2.2 Myristica malabarica

*Myristica malabarica* is commonly known as Bombay nutmeg tree. It is an endemic Indian plant in the Myristicaceae family. The fruit rind of the plant is commonly known as rampatri, Bombay mace or false nutmeg.
Chapter II

Spices

2.1 *Myristica malabarica*

It is used as an exotic spice in various Indian cuisines. It is also used for medical purposes such as hepatoprotective, anticarcinogenic, and antithrombotic activities (Morita et al. 2003). The antioxidant (Patro et al. 2005) and endopeptidase inhibitory activities of the methanol extract of *M. malabarica* were reported (Khanom et al. 2000). The *M. malabarica* also reported for nematicidal activity of (Choi et al. 2008) and antifungal activity (Choi et al. 2006).

2.3 *Illicium verum* Hook. f. (Star Anise)

*Ilicium verum* is a small evergreen tree. The characteristically shaped fruits (pods) in dried state commonly called star anise, star aniseed, or Chinese star anise. The star anise is used as spice worldwide. It is native of Egypt, Greece, Crete and Asia Minor and was transported by the ancient Egyptians to other parts of world.

**Culinary uses-** It is widely used in Chinese, Indian, Malay and Indonesian cuisine as a major component of garam masala. In India, it is also used as an ingredient of masala chai (tea). Star anise contains anethole, the same ingredient which gives the unrelated anise (*Pimpinella anisum*) its flavor. It is also used for chewing after meals in order to sweeten the breath. Star anise has found only limited use in the West. Its main application is as a substitute for anise seed in mulled wine and special desserts. The essential oil is used to flavour soft drinks, bakery products and, most importantly, liqueurs. It is also used as a flavouring agent in confectionery, candy and chewing gum. The oil finds application in a small way in perfumery and in the pharmaceutical industry.
Star anise oil, and (E)-anethol isolated from it, is used in anise liqueur (Anisette, Sambuca) and anise brandy (Pernod, Ouzo, Raki, Arak), liquorice sweets, tooth-paste etc. It has almost completely replaced the original anise seed oil, obtained from the umbellifer *Pimpinella anisum*.

![Illicium verum](image)

**Medicinal values** - Pharmacology studies demonstrated that its crude extracts and active compounds possess wide pharmacological actions, especially as antimicrobial (De *et al.* 2002), antibacterial (Yang *et al.* 2010), antioxidant activity (Yingming 2004; Padmashree 2007; Yadav 2010), insecticidal (Ho *et al.* 1995) and other activities like analgesic, sedative and convulsive activities (Wang 2011). It is taken internally in the treatment of abdominal pain and digestive disturbances. It is often included in remedies for indigestion and also in cough mixtures. For children it is effective for digestive upsets, including colic pain. Some people chew the fruit after meals for better digestion. It is the major source of shikimic acid, a primary ingredient in the antiflu drug (Tamiflu) which is an oral antiviral treatment for Influenza virus A within the family of Orthomyxoviridae (Ohira 2009).

**Main constituents of Star Anise** - The dried fruits may contain 5 to 8% of essential oil, which dominated by anethole (85 to 90%). The other components are phellandrene, safrole and terpineol.
2.4 *Trachyspermum copticum* L.

*Trachyspermum copticum* L. belongs to the family Apiaceae. It is known as bishop’s weed, carum seed, carum, ajowan or ajwain. The other common synonyms are *Trachyspermum ammi* Linn, *Carum copticum* Benth and Hook, *Ammi copticum* Linn., *Ptychotis coptica* DC and *Lingusticum ajowain* Roxb. The correct generic position of this spice is very uncertain. Ajwain is an annual, aromatic and herbaceous plant. It is profusely branched with a height of 60–90 cm small, erect with soft fine hair. Ajwain is indigenous to India and Egypt. It is cultivated in the Mediterranean region and South-West Asian countries: Iran, Iraq, Afghanistan, Egypt and predominantly in India.

**Trachyspermum copticum**

**Uses**- The ajwain seed has been popular from ancient times for its use in folk medicines. In addition it has many uses for flavouring, culinary, household and cosmetic purposes. The entire plant has its herbal value in medicinal industry but commercially it is valued for its seed. Ajwain seeds have an aromatic smell and a warm pungent taste. They are used both as spices and condiment in many countries. They are used in India as a traditional spice in many foods, including curries. Several pharmacological activities of seed extract and oil from ajwain were reported such as antifungal (Bansod and Rai 2008), antibacterial (Kaur and Arora 2009; Oroojalian *et al.*, 2010), antioxidant (Singh *et al.*, 2004; Nickavar and Abolhasani 2009), antiinflammatory (Thangam and Dhananjayan 2003) and insecticidal activity (Chaubey 2007).
The seed extract shows antihypertensive, antispasmodic, bronchodilator and hepatoprotective activities (Gilani et al. 2005). The seeds are also used in the treatment of intestinal dysbiosis (Hawrelak et al. 2009). Intestinal dysbiosis is a qualitative and quantitative change in the gastrointestinal flora, their metabolic activities, and/or their local distribution that produces harmful effects on the host.

**Main constituents of *Trachyspermum copticum*** - The major components present in ajwain essential oil are thymol (39.1%) along with p-cymene (30.8%), γ-terpinene (23.2%), β-pinene (1.7%), terpinene-4-ol (0.8%) (Singh et al 2004)

### 2.5 *Parmelia perlata*

*Parmelia perlata* is lichen commonly known as stone flower. Botanical synonym is *Parmotrema chinense*. *Parmelia perlata* is used as a spice in Indian cuisine. It is also used in preparation of soup and in salads. In India and Chiana, it is used in folk medicines. It is kapha and pitta suppressant. It is a good pain reliever and also promotes early healing of wounds. It helps in treating the skin related problems. It is helpful in relieving from headache. It helps in reducing any kind of inflammation in the body. It shows antibacterial activity (Momoh and Adikwu 2008) and is fairly effective against Protozoans.

![Parmelia perlata](image)

The crude polysaccharide fraction from *Parmelia perlata* possesses specific antiviral activity against yellow fever virus (Esimone et al., 2007). It is a diuretic and
used in treatment of urinary stones or calculi (Chitme et al. 2010). *P. perlata* with other two *Parmelia* sp. viz. *P. sancti-angeli* and *P. peforatum*, is used in the formulation of Indian drug chharila, which is used as aphrodisiac (Malhotra et al. 2008). In Europe, *Parmelia perlata* has been used as light brown dye for wool as well as bio-indicator of air pollution of heavy metals such as zinc, lead, cadmium copper and mercury (Pilegaard, 1978). *P. perlata* contain acidic substance that has been used as an antibiotic in several countries as a topical antibacterial agent for human skin diseases (Ketchum, 1984).

**Chemical composition**- It contains no fat with carbohydrates and protein to the extent of 1-5% is present. It contains phenols and phenol acids, mainly lecanoric acid, beta-orsellinic acid, erythrinic and roccellic acids, usnic acid, evernic acid and lecanoric acid etc.

3. **Material and methods**

3.1 **Plant materials**

All the spices used in present study were purchased from local spice market Kolhapur, Maharashtra, India. Aril of the fruit from *Myristica fragrans* (i.e. mace) and *Myristica malabarica*, *Parmelia perlata*, pericarp of *Illicium verum*, fruits pods of *Trachyspermum copticum* was used. All the spices were identified by the Department of Botany, Shivaji University, Kolhapur, India.

3.2 **Preparation of extract**

All the plant parts purchased were cleaned properly and dried in shed protected from direct sunlight exposure. Each plant material was grounded to fine powder and extracted in methanol by using Soxhlet extraction. The extraction was allowed to continuou for 24 hours. Methanol extract was filtered through filter paper and concentrated in vacuum rotary evaporator. The yield of methanolic extracts from all plants is tabulated in Table 1. All dried extracts were stored at −4 °C until further use. The desired concentrations were prepared by dissolving the methanolic extract of the respective plant material in DMSO.
Table 1- Common name, part used and % yield of methanolic extract of each plant

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Botanical Name</th>
<th>Common Name</th>
<th>Plant part used</th>
<th>Yield (% w/w)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>Myristica fragrans</em></td>
<td>Mace</td>
<td>Aril of the fruit</td>
<td>23.61</td>
</tr>
<tr>
<td>2</td>
<td><em>Myristica malabarica</em></td>
<td>Bombay mace</td>
<td>Aril of the fruit</td>
<td>18.33</td>
</tr>
<tr>
<td>3</td>
<td><em>Parmelia perlata</em></td>
<td>Stone flower</td>
<td>Whole</td>
<td>17.89</td>
</tr>
<tr>
<td>4</td>
<td><em>Illicium verum</em></td>
<td>Star anise</td>
<td>Fruits pods</td>
<td>13.83</td>
</tr>
<tr>
<td>5</td>
<td><em>Trachyspermum copticum</em></td>
<td>Ajwain</td>
<td>Fruits pods</td>
<td>36.44</td>
</tr>
</tbody>
</table>

3.2 Chemicals and reagents

Streptozotocin, gliclazide, collagenase type IX, soyabean trypsin inhibitor (STI), HEPES, BSA were purchased from Sigma chemicals, USA. RPMI 1640, fetal calf serum (FCS), dithiozone, trypan blue dye etc. were purchased from Hi-media, India. All the other chemicals used were of analytical grade.

3.3 Experimental animals

Thirty nine male Wistar rats weighing about 200 ± 10 g were used in the experimental study. All the animals were maintained under standard laboratory conditions. All the animals were allowed free access to standard rat feed (Amruth, Pune, India) and water *ad libitum*. All the experiments were carried out as per the guidelines of the Institutional Animal Ethical Committee after due submission and approval of the protocols.

3.4 Induction of diabetes

Normal male rats weighing between 200 ± 10 g were fasted overnight and diabetes was induced by intraperitonal administration of freshly prepared streptozotocin (65 mg/kg) in 0.1 M citrate buffer (pH 4.5). After 14 days, animals showing stable
glycemic values above 250 mg/dl were considered diabetic and selected for further experimentation.

3.5 Oral Glucose Tolerance Test (OGTT)

For conducting the OGTT, rats were fasted overnight with free access to water. Initial blood glucose of each rat was measured. All rats were fed orally with glucose load of 3 mg/g body weight. They were divided into 13 groups (n=3) as normal control, diabetic control, positive control (gliclazide 1.6 mg/kg body weight) and remaining 10 groups were administrated 100 or 200 mg/kg body weight of methanolic extract. Blood samples were withdrawn from tail vein at intervals of 30, 60 and 120 min of glucose administration. Glucose levels were estimated with Glucose Oxidase-Peroxidase (GOD-POD) method with commercial kit (BIOLAB Diagnostics).

3.6 In vitro isolation of rat pancreatic islets

The isolation of islets was carried out using collagenase digestion method (Shewade et al., 1999; Smelt et al., 2008) with some modifications (Patil et al., 2011). The overnight fasted rats were sacrifical by cervical dislocation, and pancreata were removed aseptically. The pancreata was washed properly with ice cold HBSS pH 7.4 (mM 1.26 CaCl₂, 0.81 MgSO₄, 5.37 KCl, 0.44 KH₂PO₄, 4.17 NaHCO₃, 137 NaCl, 0.34 Na₂PO₄) to remove any traces of blood. The attached fat bodies were removed and pancreata were cut into small pieces of approximate 1 mm² and washed thrice with HBSS to remove proteases enzymes that might have leaked out of the exocrine part of the pancreas. It was then subjected to collagenase digestion in dissociation medium for 20 minutes with continuous shaking. The total volume of tissue and buffer was always 10 mL. The dissociation medium consist of collagenase type IX (1 mg/ml) in HBSS (without Ca²⁺ and Mg²⁺), supplemented with 10 mM HEPES, 2% BSA and 2 mg/ml soyabean trypsin inhibitor (STI). Digestion was stopped by addition of chilled HBSS and tissue digest was centrifuged twice at 1000 rpm for 2 min at 4°C to remove collagenase and exocrine-derived proteases. Digested pellet was again washed twice in HBSS and islets were handpicked under inverted microscope (LABOMED TC 400). The isolated islets were washed in RPMI 1640 containing 1% BSA at least 3 times and seeded in culture flasks (25 cm² Falcon).
Islets were cultured overnight in RPMI 1640 supplemented with 2.8 mM D-glucose, 100 units/ml penicillin, 100 µg/ml streptomycin, gentamycin (25 mg/ml) and 10% fetal calf serum (FCS) at 37 °C in a CO₂ incubator (Thermo Scientific, USA) gassed with 5% CO₂ in air. The antibiotics used in the present study do not affect the viability and the functioning of the islets at their pharmacological concentrations (Shewade et al. 2001). The next morning, islets were collected and washed in phosphate-buffered saline twice before being dispersed into single cells by mechanical shaking at 37 °C for 3 minutes. On average, 100 islets per pancreas were obtained. Viability of isolated islets was assessed by trypan blue exclusion test while specificity of islet was determined with Dithiozone dye. Exclusion of trypan blue positive cells indicated that more than 95% of the cells were viable after the procedure.

3.7 DTZ assay for islet specificity

DTZ assay was performed by previously reported method (Shiroi et al. 2002; Jin Lu et al. 2005). This stain can be used to differentiate between pancreatic islets and acinar tissue because of the high zinc content of islet beta cells.

**Stock dithizone solution**- Dithizone (20 mg) was added to 0.6ml of 95% ethanol. Mixed thoroughly by vortexing. 1-2 drops of ammonium hydroxide were added and mixed thoroughly. After complete dissolution of dithizone, solution turns into bright orange.

**Working dithizone solution**- From stock DTZ solution, 0.3 ml was diluted to 100 ml with phosphate buffered saline and mixed properly. The pH of the solution was adjusted to 7.4. This working solution was then filtered and was stored at –20 °C until use.

For staining, few ml of the working dithizone solution was added in a petri dish containing isolated islets. Islets were incubated with stain for 1-2 minutes. After the incubation, dishes were rinsed thrice with HBSS. After examining under the inverted microscope, islets will appear as crimson red colored and acinar tissue remains gray.

3.8 Assessment of islet viability using trypan blue dye exclusion test

The viability of the islets was checked by trypan blue dye exclusion test (Warburton and James 1995). In brief, 0.4% (w/v) trypan blue was prepared in HBSS (pH 7.4). Few drop of this dye was added to the petri-plate containing isolated islets and
observed under inverted microscope. The viable cells remain unstained while the dead or cell membrane damaged cells stains blue. Blue stained islets were scored as non-viable and the unstained were scored as viable islets.

3.9 Insulin release assay

Groups of 10 islets were placed in eppendorf tubes each containing 1 ml HBSS (pH 7.4) supplemented with 10 mmol/l HEPES and 2 mg/ml BSA. Cells were then incubated for 1 h in 11.8 mM glucose in presence of 1 or 2 mg/ml plant extract. The positive control is 11.8 mM glucose in presence of gliclazide (10 and 20 µg/ml). After incubation, supernatant was collected and stored at -20 ºC until further use. The insulin concentration in all the stored samples was determined by ELISA kit (CalBiotech). The amount of insulin released was quantified by using ELISA microplate reader (Multiskan EX, Thermo Scientific).

3.10 Isolation of rat intestinal alpha-glucosidase enzyme

Intestinal alpha glucosidase was isolated by previously described method (Chougle et al. 2009). For isolation of enzyme, normal healthy rats were sacrificed by cervical dislocation. The small intestine was removed and washed several times with ice-cold 0.01 M potassium phosphate buffer (pH 7.0). The intestine was cleaned from adipose tissue and cut longitudinally. The intestinal mucosa was scraped with a glass slide on an ice-cold glass surface. The collected mucosa was washed with chilled phosphate buffer saline (PBS). The mucosa was then homogenized in the Teflon homogenizer with 0.1% Triton-X 100 in PBS and centrifuged at 3000 rpm for 15 min at 4 ºC. The pellet was discarded and supernatant was collected which was used as a crude source of alpha-glucosidase enzyme. The enzyme was semi purified by addition of chilled n-butanol to supernatant in 1:1 proportion and centrifuged at 3000 rpm for 15 min at 4 ºC. The lipoproteins were precipitated by n-butanol. Aqueous layer containing alpha-glucosidase enzyme was withdrawn and dialyzed against 0.01 M potassium phosphate buffer (pH 7.0). The concentrated enzyme was stored at -20 ºC till further use. All procedure for isolation of rat intestinal alpha-glucosidase was carried out under cold condition.
3.11 Alpha-glucosidase inhibition assay

The alpha-glucosidase inhibitory activity was determined by using the method described previously (Jong-Anurakkun et al. 2007) with slight modifications. The percent inhibitory activity of each plant extract was determined by using variable concentrations (0.1, 0.2, 0.5 and 1.0 mg/ml). Briefly, 200 µl enzyme solution was mixed with various concentrations of test sample or vehicle. The volume was adjusted to 800 µl using 0.01 M potassium phosphate buffer (pH 7.0). After pre-incubated for 10 min, the reaction was started by adding 200 µl of 3.5 mM maltose prepared in buffer. Reaction was terminated after 30 min by heating at 90–100 °C. The amount of released glucose was estimated with a commercial glucose Oxidase-Peroxidase (GOD-POD) assay kit (BIOLAB Diagnostics). Inhibition rates were calculated as a percent with respect to blank controls, and the IC$_{50}$ value for each extract was calculated. The IC$_{50}$ value was defined as the concentration required to inhibit 50% of the alpha-glucosidase activity under the assay conditions specified. All the assays were performed in replicates of six (n=6).

3.12 Free radical scavenging activity

The antioxidant activity of the extracts was measured on the basis of scavenging activity of the stable DPPH (1, 1-diphenyl-2-picryl-hydrazyl) (Pari et al., 2008). The methanolic extract of each plant (0.05, 0.1, 0.2, 0.5 and 1 mg/ml) or standard ascorbic acid (5 to 100 µg/ml) was added to 1 ml of methanolic solution of DPPH (0.25 mM). After 20 min incubation at room temperature in dark, absorbance was measured at 517 nm. Methanol was used as blank while absorbance of DPPH without addition of respective extract was taken as control. Percent inhibition was calculated by using formula:

\[
\text{% Inhibition} = \frac{(\text{Abs}_{\text{control}} - \text{Abs}_{\text{test}})}{\text{Abs}_{\text{control}}} \times 100
\]

The concentration of extract required for 50% inhibition (IC$_{50}$) was calculated. Ascorbic acid was used as a positive control for comparison. All the assays were performed in replicates of six (n=6).
3.13 Determination of total phenolic content

The total phenolic content of each plant extract was determined by using Folin-Ciocalteu reagent method (Abdel-Hameed 2009). About 100 µl of plant extracts (1 mg/ml) were mixed with 500 µl of Folin-Ciocalteu reagent and 1.5 ml of 20% sodium carbonate. The mixture was shaken thoroughly and incubated for 5 min. at 37 °C in dark. The final volume was made up to 10 ml using distilled water. The mixture was allowed to stand for 2 h. Then the absorbance was determined at 765 nm against a blank which contain all reagents without the samples at the same conditions. For each extract, control blank was prepared with an equivalent volume of distilled water instead of Folin-Ciocalteu reagent. The total phenolic content is expressed as the number of equivalents of gallic acid (GAE) per gram of sample. All the assays were performed in replicates of six (n=6).

3.14 Determination of total flavonoid content.

Total flavonoid content of all five extracts were also measured by colorimetric method (Chang _et al._ 2002). Diluted methanolic extract of respective plant (0.5 ml) was mixed with methanol (1.5 ml), to which 10% aluminium chloride (0.1 ml), 1 M potassium acetate (0.1 ml) and distilled water (2.8 ml) were added. The solution was mixed well, and allowed to stand for 30 min at room temperature. The absorbance of the reaction mixture was measured at 415 nm using a UV–Visible spectrophotometer (Hitachi U-2800; Hitachi, Tokyo, Japan). The total flavonoids content was quantified according to the standard curve prepared for quercetin (10 to 100 µg/ml) and the concentration of flavonoids was reported as mg of quercetin equivalents per gram of sample.

4. Statistical analysis

All the data obtained was expressed as mean ± SD. Statistical analysis was performed using one-way analysis of variance (ANOVA). _p_-values of less than 0.05 were considered to be statistical significant.
5. Results and discussion

5.1 Oral Glucose Tolerance Test

The antihyperglycemic effect of methanolic extracts of the five spices is shown in Table 2. Among the five plants, *M. fragrans*, *P. perlata* and *M. malabarica* showed significant reduction in blood glucose levels in a dose dependent manner.

Among the five plants, *Myristica fragrans* was previously reported to have antidiabetic activity. Somani *et al* demonstrated that petroleum ether extract of *Myristica fragrans* seeds at dose of 200 mg/kg shows significant decrease in blood glucose level and also improves body weight, lipid profiles and haemoglobin content as compared to diabetic control rats (Somani *et al.*, 2008).

Han *et al* isolated a bioactive component “macelignan” from *M. fragrans*. The authors found that 10 and 25 mg/kg macelignan improved blood glucose levels and glucose tolerance in *db/db*. Macelignan reduced serum glucose, insulin, triglycerides, free fatty acid levels, and triglycerides levels in the skeletal muscle and liver of *db/db* mice. Macelignan was reported to increase adiponectin expression in adipose tissue and serum, whereas the expression and serum levels of tumor necrosis factor-α and interleukin-6 decreased. Macelignan downregulated inflammatory gene expression in the liver and increased AMP-activated protein kinase activation in the skeletal muscle of *db/db* mice. Strikingly, macelignan reduced endoplasmic reticulum (ER) stress and c-Jun NH2-terminal kinase activation in the liver and adipose tissue of *db/db* mice and subsequently increased insulin signaling. Overall macelignan is reported to enhance the insulin sensitivity and improved lipid metabolic disorders by activating peroxisome proliferator receptor (PPAR, α/γ) and attenuating endoplasmic reticulum stress, suggesting that it can be used as an antidiabetic agent for the treatment of type 2 diabetes (Han *et al.*, 2008).

In the present study also *M. fragrans* showed significant reduction in blood glucose levels in a dose dependent manner and results were similar to previously reported data. At a dose of 200 mg extract/kg rat body weight, the reduction in blood glucose level was almost equally comparable to positive control gliclazide (1.6 mg/ kg rat body weight). Though the *Myristica fragrans* was previously reported to have antidiabetic activity, its effect on insulin secretion through isolated islets or alpha-glucosidase inhibitory activity was not assessed previously. Hence, work on *Myristica*
fragrans was continued further to evaluate its insulin secretagogue and alpha-glucosidase inhibitory activity.

Table 2 - Effect of plant methanolic extracts on blood glucose levels

<table>
<thead>
<tr>
<th>Groups</th>
<th>Blood Glucose (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 min</td>
</tr>
<tr>
<td>Normal</td>
<td>85±4</td>
</tr>
<tr>
<td>Diabetic Control</td>
<td>347.3±9.5</td>
</tr>
<tr>
<td>Diabetic + Gliclazide (1.6 mg/kg)</td>
<td>342.7±10.0</td>
</tr>
<tr>
<td>Diabetic + M. fragrans (100mg/kg)</td>
<td>326.7±8.6</td>
</tr>
<tr>
<td>Diabetic + M. fragrans (200mg/kg)</td>
<td>346.3±8.5</td>
</tr>
<tr>
<td>Diabetic + P. perlata (100mg/kg)</td>
<td>323.3±8.3</td>
</tr>
<tr>
<td>Diabetic + P. perlata (200mg/kg)</td>
<td>308.0±8.5</td>
</tr>
<tr>
<td>Diabetic + I. verum (100mg/kg)</td>
<td>311.7±6.5</td>
</tr>
<tr>
<td>Diabetic + I. verum (200mg/kg)</td>
<td>292.7±8.0</td>
</tr>
<tr>
<td>Diabetic + T. copticum (100mg/kg)</td>
<td>338.0±6.6</td>
</tr>
<tr>
<td>Diabetic + T. copticum (200mg/kg)</td>
<td>326.3±6.1</td>
</tr>
<tr>
<td>Diabetic + M. malabarica (100mg/kg)</td>
<td>317.7±8.6</td>
</tr>
<tr>
<td>Diabetic + M. malabarica (200mg/kg)</td>
<td>344.3±7.6</td>
</tr>
</tbody>
</table>

Values are given as mean ± S.D. from three rats in each group.

**p<0.001 significant from diabetic control.

*p<0.01 significant from diabetic control.

The lichen, P. perlata also showed very significant and dose dependent reduction in blood glucose levels. The antihyperglycemic effect of P. perlata at a dose 200 mg
extract/kg rat body weight was also almost equally comparable to gliclazide (1.6 mg/kg rat body weight). Among the five plants, *M. Malabarica* showed very significant blood glucose lowering activity. At a concentration of 200 mg extract/kg rat body weight, *M. Malabarica* showed better antihyperglycemic activity than the commercially used sulfonylurea, gliclazide. The remaining two plants, *I. verum* and *T. copticum* demonstrated lowering of blood glucose as compared to diabetic control however the activity was not very significant.

5.2 *In vitro* insulin secretion studies on isolated pancreatic islets

The effect of extracts on *in vitro* insulin secretion is shown in Figure 1. The insulin secretion in presence of 11.8 mM glucose is considered as control. The insulin release in presence of gliclazide or plant extract is expressed in terms of fold insulin increase with respect to this control.

![Figure 1 - Effect of methanolic extracts on insulin secretion from isolated pancreatic islets.](image)

The insulin release in presence of two concentrations of gliclazide viz. 10 μg/ml (square) and 20 μg/ml (triangle) or plant extract (1 mg and 2 mg/ml) is expressed in terms of fold increase with respect to 11.8 nM glucose control. Results are the mean ± S.D. of four replicates of each group. *p<0.001* significant from 2.8 mM and 11.8 mM glucose control.
Gliclazide, a second generation sulphonylurea, is widely used in the treatment of type 2 diabetes. The molecule reduces blood glucose levels by increasing insulin secretion from pancreatic beta-cells through interaction with the sulphonylurea receptor (SUR1) of the K\(^+\)-ATP channel (Scherntaner 2003). At concentrations of 10 μg and 20 g/ml, gliclazide showed a dose dependent insulin secretion i.e. 1.72 and 3.62 fold respectively. The *M. fragrans, T. copticum* and *M. malabarica* also showed dose dependent insulin secretion. Although *T. copticum* does not show significant antihyperglycemic activity in OGTT, it demonstrates *in vitro* insulin secretion in a dose dependent manner i.e. 1.41 and 2.95 fold increase at 1 and 2 mg/ml concentration respectively. This may be due to lack of proper absorption or biotransformation of the active constituent during *in vivo* studies.

The remaining two plants *P. perlata* and *I. verum* did not demonstrate any insulin secretion. The *P. perlata* surprisingly inhibited the 11.8 mM glucose stimulated insulin secretion. Several natural or synthetic components have shown to possess such insulin stimulation inhibitory action. Resveratrol is a naturally occurring phytoalexin and considerably found in plants such as *Polygonum cuspidatum*, grape vines, peanuts and groundnuts. Resveratrol inhibits insulin secretion from rat pancreatic islets (Szkudelski 2006). Other components like phenylcyanoguanidines (Tagmose *et al.* 2001), 3, 3-Diamino-sulfonylacrylonitriles (Tagmose *et al.* 2003), ghrelin (Reimer *et al.* 2003) and diphenyhydantoin (Kizer *et al.* 1970) also inhibits the *in vitro* insulin secretion. The insulin stimulation inhibitors are used as drug in patients with persistent hyperinsulinemic hypoglycemia of infancy (PHHI), congenital hyperinsulinism in infancy (CHI) and hyperinsulinism in infancy (HI). Diazoxide is the most commonly used drug in medical treatment for hyperinsulinism (Dunne *et al.* 2004). Though *P. perlata* inhibits the insulin secretion *in vitro*, it significantly reduces the blood glucose levels in OGTT. Hence, the glucose lowering effect of *P. perlata* could be due to mechanisms other than the insulin secretagogue activity. The viability of cells after each experiment was tested and it was not affected by either 1 or 2 mg/ml concentration of extracts. Hence, it was confirmed that the insulin secretion or inhibition was not due to cell damage.
5.3 Alpha-glucosidase inhibition assay

The in vitro alpha-glucosidase inhibitory activity of the plants was assessed. Acarbose was used as a positive control. Acarbose is a commercially used alpha-glucosidase inhibitor. Four concentrations of acarbose (0.05, 0.1, 0.2 and 0.5 mg/ml) are used to calculate the IC\textsubscript{50} value. The IC\textsubscript{50} value for acarbose was estimated to be 0.031 mg/ml.

Figure 2- The in vitro rat intestinal alpha-glucosidase inhibitory activity of acarbose

Results are the mean ± S.D. of six replicates of each group.

The in vitro rat intestinal alpha-glucosidase inhibitory activity of methanolic extracts was shown in Figure 3. At a concentration of 1 mg/ml, P. perlata showed significant alpha-glucosidase inhibitory activity i.e. 98.56%. The IC\textsubscript{50} value of P. perlata was estimated to be 0.14 mg/ml.
The in vitro rat intestinal alpha-glucosidase inhibitory activity of methanolic extracts with four concentrations (i.e. 0.1, 0.2, 0.5 and 1 mg/ml). Results are the mean ± S.D. of six replicates of each group.

The remaining plants demonstrated inhibitory activity in decreasing order as I. verum (71.75%) > M. fragrans (62.27%) > M. malabarica (62.02%) > T. copticum (55.67%). The IC50 values of the plants were as M. malabarica (0.64 mg/ml), I. verum (0.67 mg/ml), M. fragrans (0.85 mg/ml) and T. copticum (0.92 mg/ml). Hence, among the five plants studied, P. perlata showed significant alpha-glucosidase inhibitory activity.

5.4 Total phenolic content

The total phenolic content of five spices is shown in Table 3. The total phenolic contents of methanolic extracts were determined using Folin–Ciocalteu assay and expressed in terms of gallic acid equivalents. The P. perlata has highest phenolic content (118.5 mg/gm) followed by M. malabarica (84.13 mg/gm). The M. fragrans, T. copticum and I. verum showed 47.73, 40.01 and 24.81 mg/gm gallic acid equivalents respectively.
Table 3. Total phenolic content of methanolic extracts

<table>
<thead>
<tr>
<th>Plant</th>
<th>Total phenols (mg gallic acid equivalents/gm plant extract)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>M. fragrans</em></td>
<td>47.73±0.08</td>
</tr>
<tr>
<td><em>P. perlata</em></td>
<td>118.50±0.15</td>
</tr>
<tr>
<td><em>I. verum</em></td>
<td>24.81±0.04</td>
</tr>
<tr>
<td><em>T. copticum</em></td>
<td>40.01±0.04</td>
</tr>
<tr>
<td><em>M. malabarica</em></td>
<td>84.13±0.09</td>
</tr>
</tbody>
</table>

Values are given as mean ± S.D. All tests were performed in n=6.

5.5 Total flavonoid content

Total flavonoid contents of plant extracts was measured by colorimetric method and are expressed in terms of quercetin equivalents per gram of sample. The results are shown in Table 4.

Table 4. Total flavonoid content of methanolic extracts

<table>
<thead>
<tr>
<th>Plant</th>
<th>Total flavonoids (mg quercetin equivalents/gm plant extract)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>M. fragrans</em></td>
<td>2.69±0.08</td>
</tr>
<tr>
<td><em>P. perlata</em></td>
<td>6.89±0.07</td>
</tr>
<tr>
<td><em>I. verum</em></td>
<td>1.87±0.06</td>
</tr>
<tr>
<td><em>T. copticum</em></td>
<td>0.55±0.02</td>
</tr>
<tr>
<td><em>M. malabarica</em></td>
<td>38.35±0.08</td>
</tr>
</tbody>
</table>

Values are given as mean ± S.D. All tests were performed in n=6.
Among five extracts, *M. malabarica* showed highest (38.35 mg/gm) while *T. copticum* showed lowest (0.55 mg/gm) flavonoid content. The *P. perlata, M. fragrans and I. verum* showed 6.89, 2.69 and 1.87 mg/gm quercetin equivalents respectively.

### 5.6 Free radical scavenging activity of spices

The antioxidant activity of the plant extracts was assessed on the basis of the radical scavenging effect on the stable DPPH and is shown in Figure 3. Antioxidants neutralizes the DPPH free radical and converts it to a product 2, 2-diphenyl-2-picrylhydrazine.

Figure 4 – DPPH free radical scavenging activity of plant methanolic extracts.

![Graph showing DPPH free radical scavenging activity](image)

Results are the mean ± S.D. of six replicates of each group.

The degree of discoloration indicates the free radical scavenging activity of extract. At a concentration of 1 mg/ml the methanolic extracts shows free radical scavenging activity in a decreasing order as *M. malabarica* (90.45%), *M. fragrans* (89.89%), *I. verum* (87.22%), *P. perlata* (76.70%) and *T. copticum* (38.14%). Though there is no significant difference in scavenging at concentration of 1 mg/ml of plant extract, the *M. malabarica* showed very potent activity with IC$_{50}$ value of 0.03 mg/ml as
compared to *M. fragrans* (0.25 mg/ml), *I. verum* (0.36 mg/ml) and *P. perlata* (0.54 mg/ml). Ascorbic acid, a standard antioxidant at a concentration range of 5 to 100µg/ml was used as a positive control. The IC_{50} value of ascorbic acid was estimated 8 g/ml. Antioxidant studies of *Myristica fragrans* (Olaleye MT 2006; Calliste CA 2010), *M. malabarica* (Patro et al. 2005; Khanom et al. 2000), *Illicium verum* (Yingming 2004; Padmashree 2007; Yadav 2010), *Trachyspermum copticum* (Singh et al. 2004; Nickavar and Abolhasani 2009) were previously reported and the results reported are similar to our findings.

There is controversy between the relation of antioxidant activity with phenolic and flavonoid content. Some authors have reported that there is no such correlation (Liangli et al. 2002) while some other postulated that phenolic and flavonoid compounds are mainly responsible for the antioxidant activity (Kumaran and Karunakaran 2007). However, in present study no significant correlation was found between the total phenolic and flavonoid content with antioxidant activity.

Oxidative damage plays a critical role in progression of various disease processes like cardiovascular disease (CVD), inflammatory disease, carcinogenesis, and aging. Also the oxidative stress in diabetes is considered as responsible factor in development of secondary complications such as neuropathy, nephropathy and retinopathy. Oxidative stress is also responsible for β-cell dysfunction. Antioxidants are hence beneficial in diabetes and it was shown that antioxidants may prevent or delay beta cell dysfunction by providing protection against glucose toxicity as well as prevents progression of diabetes into secondary complications (Kaneto et al. 1999). Hence, antioxidant activity of spices in addition to the antihyperglycemic activity will be beneficial in treatment in diabetes mellitus.

Combinatorial therapy is preferred over mono therapy so as to reduce the quantity of a single drug used for prolonged period in order to minimize the side effects of the drug. Although alpha-glucosidase inhibitors prevent sharp spikes in postprandial glucose levels and therefore have a beta cell protective effect by preventing glucose toxicity (Kaneto et al. 1999). In frank diabetic condition, blood glucose level still continues to remain high and necessitates use of a secretagogue or insulin in addition. However, the content of secretagogue or insulin used can be reduced through the use of alpha-glucosidase inhibitors in a non-invasive manner.
6. Conclusion

In conclusion, among five plants studied, *M. fragrans*, *P. perlata*, and *M. malabarica* showed significant blood glucose lowering activity in OGTT. The blood glucose lowering effect of *M. fragrans* and *M. malabarica* may be due to insulin secretagogue activity while *P. perlata* may reduce blood glucose due to mechanisms other than insulin secretagogue activity. All the five plants showed significant alpha-glucosidase inhibitory activity. Among them *P. perlata* showed maximum alpha-glucosidase inhibitory activity i.e. 98.56%. All five plants except *T. copticum*, showed significant free radical scavenging activity. This is the first report of antihyperglycemic activity of *P. perlata* and insulin secretagogue activity of *M. fragrans*, *T. copticum* and *M. malabarica*. Hence, the use of these spices in diet of diabetics may prevent increase in postprandial glucose levels. They may help to maintain serum insulin levels and thereby blood glucose levels and through antioxidant action may prevent progression of diabetes into secondary complications.

7. References


Chaubey MK (2007) Insecticidal activity of *Trachyspermum ammi* (Umbelliferae), *Anethum graveolens* (Umbelliferae) and *Nigella sativa* (Ranunculaceae) essential


Diabetes control and complications trial research group (1995) The relationship of glycemic exposure (HbA$_{1c}$) to the risk of development and progression of retinopathy in the diabetes control and complications trial. *Diabetes* 44: 968-983.


Chapter II

Spices

Ho SH, Ma Y, Goh PM, Sim KY (1995) Star anise, Illicium verum Hook f. as a potential grain protectant against Tribolium castaneum (Herbst) and Sitophilus zeamais Motsch. Post harvest Biology and Technology 6: 341-347.


