Chapter III

Antidiabetic Activity of Cuminum cyminum L.: Effect of Agonist and Antagonist Bioactive Components on Insulin Secretion
1. Introduction

Hyperglycemia in patients with diabetes mellitus (DM) is always the result of a mismatch between the quantity of insulin necessary to regulate the person's metabolic processes and the amount of insulin being secreted by the person's β-cells. There is decreased or unregulated insulin secretion in type II diabetic patients (Ashcroft and Rorsman 2004). Type 2 diabetes is characterized by a progressive loss of β-cell function throughout the course of the disease. The reduced or absence of first phase of glucose induced insulin secretion is a hallmark of type 2 diabetes. Other insulin secretory abnormalities in type 2 diabetes mellitus are decreased glucose sensing, impaired ability to respond to elevations and reductions in glucose during an oscillatory glucose infusion, reduced or absent first-phase insulin secretion in response to intravenous glucose administration, reduced or absent early insulin secretory response to oral glucose, alterations in the rapid oscillations of insulin secretion, reduced effect of gastrointestinal hormones in potentiating glucose-mediated insulin secretion, inadequate insulin secretion for the magnitude of hyperglycemia etc. (Porte and Kahn 1995; Kahn et al. 1997).

The people who are in the type 2 prediabetic state already manifest some abnormalities in β-cell function or decrease β-cell mass (Clark et al. 2001). Glucose plays a central role among all the factors contributing to β-cell burnout or dysfunction. In addition to its effect on β-cell turnover, hyperglycemia also impairs β-cell secretory function (Robertson 1989). This is most striking in vitro, where a 4-day exposure of human islets to elevated glucose concentrations leads to almost complete ablation of β-cell secretory function, although <1% of β-cells are apoptotic (Maedler et al. 2002). However, it is unlikely that glucotoxicity acts alone, and the negative contribution of saturated fatty acids, lipoproteins, leptin, and circulating and locally produced cytokines will further burn out the β-cells. These factors will induce apoptosis and/or necrosis, which in the presence of proinflammatory cytokines may activate specific immunological phenomena, which ultimately result in autoimmunity (Mathis et al. 2001).

In diabetes mellitus, due to the state of continuous hyperglycemia, there is increased demand of insulin secretion which may lead to the overstimulation of the β-cells (Grill and Bjorklund 2001). One of the negative effects of overstimulation on β-cells is desensitization. In 1986, it was reported that 48 h of marked hyperglycemia in normal rats, achieved by massive glucose infusions, produced almost total insensitivity...
to glucose when insulin release was subsequently measured from the perfused pancreas. Although, the desensitization was reversible within 24 h (Leahy et al. 1986). It was later showed that inhibition of glucose-induced insulin secretion by simultaneous infusion of diazoxide prevents the further desensitization. Diazoxide is a K+-ATP channel opener which abolishes the glucose stimulated insulin secretion. It was concluded that the desensitizing effect was due to overstimulation of the β-cells rather than to effects of hyperglycemia (Sako and Grill 1990).

The mechanisms behind the effects of overstimulation are only partly clarified. In cultured human pancreatic islets, overstimulation by high glucose leads to a rise in cytoplasmic Ca\(^{2+}\) levels, which persists after normalization of the glucose levels. Persistent elevation of cytoplasmic Ca\(^{2+}\) may trigger apoptosis, thus participating in long-term irreversible deterioration of β-cell function (Bjorklund et al. 2000). Other studies in human islets have shown that culture in high glucose for several days leads to down regulation of insulin biosynthesis, possibly secondary to decreased expression of the relevant transcription factors (Marshak et al. 1999). Still other studies have reported decreased glucose metabolism (Eizirik et al. 1992). These data provide sufficient rationale for clinical studies to test the beneficial effects of relative β-cell rest in type 2 diabetic patients. The short-term treatment with diazoxide improves insulin secretion in type 2 diabetic subjects (Greenwood et al. 1976). These results support the importance of β-cell rest in type 2 diabetic patients.

Obesity is the main risk factor for the development of diabetes. It is often part of the metabolic syndrome and is accompanied by dyslipidemia and increased circulating leptin and cytokine levels. All of these factors have been shown to modulate β-cell function and survival. The influence of dyslipidemia on the β-cells of an individual will depend on his or her specific lipid profile. Whereas some free fatty acids and lipoproteins have been shown to be pro-apoptotic for the β-cell, others are protective. Thus, long-term exposure to saturated fatty acids such as palmitate appears highly toxic, whereas monounsaturated fatty acids such as olate protect against both palmitate- and glucose-induced β-cell apoptosis (Maedler et al. 2003).

Insulin secretagogues are extensively used in the treatment of diabetes mellitus. Sulfonlureas, a major class of insulin secretagogues, have been used in the treatment of type 2 diabetes mellitus for over 50 years. Though these synthetic remedies are extensively used due to their easy production and cost effectiveness, they do not appear
to correct the defect in early insulin secretion characteristic of type 2 diabetes (Shapiro et al. 1989) and also are associated with number of undesirable side effects (Harris 1971). The major clinical side effects concerning the use of insulin secretagogues are apoptosis of β-cells and reduction in their mass (Donath et al. 2005). The closure of the K\(^+\)-ATP channels by the sulfonylureas such as tolbutamide and glibenclamide may induce Ca\(^{2+}\)-dependent β-cell apoptosis in rodent and human islets (Efano et al. 1998, Maedler et al 2005). In contrast to sulfonylureas, K\(^+\)-ATP channels’ channel openers may exert protective effects on β-cells (Maedler et al. 2004, Ritzel et al. 2004). In 1976, Greenwood et al. were the first to report an improvement in insulin secretion after administration of diazoxide to diabetic subjects for 7 days (Greenwood et al. 1976). Similar protective effects were observed more recently in patients classified with type 1 and type 2 diabetes (Guldstrand et al. 2002).

The other major side effects of sulfonylureas are desensitization after a prolonged use (Rustenbeck et al. 2004), chance of hypoglycemic shock as sulfonylurea elicits insulin release regardless of plasma glucose concentrations (Henquin 2004; Lheureux et al. 2005), problem of weight gain and coronary artery disease (Kimmel and Inzucchi 2005). Also long-term treatment with sulfonylureas could overstimulate β-cells, resulting in negative consequences. Given the possible deleterious effect of some sulfonylureas, alternatives to these as well as alternative insulin secretagogues may have to be considered.

There are number of insulin secretagogues other than sulfonylureas are being used such as meglitinides, glucagon like peptide-1 (GLP-1) analogs, dipeptidyl peptidase IV (DPP IV) inhibitors (Maedler et al. 2005). Meglitinide are the non-sulfonylurea insulin secretagogues. The best known are compounds from meglitinide class are repaglinide, nateglinide and mitiglinide. Repaglinide, in contrast to sulfonylureas, did not stimulate insulin secretion in islets in the absence of glucose (Fuhlendorff et al. 1998) and hence, are without a side effect of hypoglycemia. Also when applied for their respective circulating half-lives in vitro, repaglinide and nateglinide do not appear to have an apoptotic effect on human islets (Maedler et al 2005). However, these compounds also show some side effects similar to that of sulfonyureas.

Thus, there is a need for better insulin secretagogue which with no or lesser side effects. The ideal insulin secretagogue should have the following characteristics: it acts rapidly, so that insulin secretion is stimulated soon after meal ingestion; its effect is
graded to increase as the plasma glucose increases from 60 to 180 mg/dl; it has little or no effect at plasma glucose levels of less than 60 mg/dl; and its duration of action is short so that it does not continue to stimulate insulin secretion beyond the postprandial period. Understanding that decreased β-cell mass is an important factor in the pathogenesis of type 2 diabetes raises a concern regarding the application of drugs potentially harmful to the remaining β-cells. Conversely, protection of β-cells from death presents itself as a new therapeutic target. Hence, insulin stimulating compounds possessing β-cell protective action are beneficial. The therapy to correct hyperglycemia in type 2 diabetic patients must be directed at decreasing insulin requirements in those who are insulin resistant, thereby bringing endogenous insulin secretion more closely in alignment with insulin need, or at increasing the insulin available adequately to meet the insulin requirements, whether normal or increased.

The treatment of diabetes mellitus is rapidly undergoing significant changes. The trend is now towards use of combinatorial therapy rather than single therapy. As mentioned before, diabetes is a complex disorder that involves multiple pathophysiological defects e.g. the level of alpha-glucosidase and GLUT-2 transporters in the intestine known to be upregulated. Likewise there is increased output from the liver through enhanced gluconeogenesis and glycogenolysis. A higher expression of SGLT2 in the kidney also prevents excessive loss of glucose from the urine. All these factors add to the existing hyperglycemic burden leads to insulin resistance. The overall scenario manifests as chronic hyperglycemia which can induce glucotoxicity to the pancreatic islets causing there destruction as well as initiating micro and macro vascular changes initiating secondary complications such as neuropathy, nephropathy etc. Almost ~ 50% loss of β-cell function was already present in newly diagnosed type 2 diabetic patients (Turner et al. 1999). As the disease progresses, further functional decline in β-cell output is apparent. As a result, patients can adequately control on monotherapy. Thus, combinational therapy involving agents with complementary mechanisms of action is not only logical but frequently necessary to achieve control. The use of a combination of alpha-glucosidase inhibitor, insulin sensitizers like metformin along with insulin secretagogue is currently considered as a better option. Published trials confirmed the additive beneficial effects on glucose control of agents from different therapeutic classes (Kimmel and Inzucchi 2005). Few studies, however, suggest an actual “synergistic” effect. Over the past several years, the availability of several combination
products incorporating sulfonylureas with metformin or metformin with a TZD has been marketed. These convenient formulations may enhance compliance. Their availability raises the potential for starting patients at the outset with two drugs. Such an approach is logical and will likely result in quicker achievement of target glucose levels, particularly in those with the greatest degree of baseline glycemia. However, precisely how various regimens function together metabolically remains incompletely understood but are an area of great interest that warrants further evaluation.

Ayurveda, the traditional Indian medicinal system is known to possess a holistic approach to treatment of diseases. Several plants used in Ayurvedic treatment are known to attack the root cause of the disease rather that just treatment of effects of the disease. In view of the earlier discussion on the current available secretagogues and their side effects as well as deleterious effects on the β-cells itself propelling the use of insulin as the only option to control glycemic; it is essential to devise secretagogue with the ability to protect the β-cell degradation.

One such plant used in Ayurvedic formulation to treat diabetes is *Cuminum cyminum*. In the present chapter this plant is evaluated for its holistic use in treatment of diabetes mellitus and to study the insulin secretagogue action as well as its protective action in details.

1.1 *Cuminum cyminum*

*Cuminum cyminum* is a flowering plant in the family Apiaceae. *Cuminum cyminum* Linn. is native from the east Mediterranean to India. Primary cultivation of cumin is in Europe, Asia, the Middle East, and North Africa with India and Iran as the largest cumin exporters. It is now mostly grown in Iran, Uzbekistan, Tajikistan, Turkey, Morocco, Egypt, India, Syria, Mexico, and Chile.

*Cuminum cyminum* is an herbaceous annual plant with a slender branched stem 20–30 cm tall. The leaves are 5–10 cm long, pinnate or bipinnate, thread-like leaflets. The flowers are small, white or pink, and borne in umbels. The fruit is a lateral fusiform or ovoid achene 4–5 mm long, containing a single seed.
The dried seeds of the herb are called as cumin seeds and locally known as “jeera”. Cumin seeds are oblong in shape, longitudinally ridged, and grey-brown in color. Cumin seeds, in both whole and ground form, are used in the cuisines of many different cultures from ancient time. The seeds of *Cuminum cyminum* have gained their place as main spice in Indian, African, Chinese, Cuban and Mexican cuisines, due to their distinctive popular aroma. It has a spicy-sweet aroma with pungent, powerful, sharp and slightly bitter flavour. It is mainly used to spice and season variety of dishes like curries, chutneys, masalas etc. It is extensively used in India to season dishes.

Due to its numerous medicinal properties, jeera is used as an ingredient in many home remedies and ayurvedic preparations. The strong aroma of jeera or cumin seeds is due to the presence of compound cuminaldehyde. Cumin is widely used in Ayurvedic medicine as a stimulant, carminative, and astringent and for the treatment of dyspepsia,
diarrhea and jaundice. This spice has been praised as jeeraka, jarana and ajaaji for its medicinal qualities in ayurvedic texts. These names refer to its carminative and digestive properties. According ayurvedic principles these seeds balance vata and kapha. It also has stomachic, diuretic, emmenagogic and antispasmodic properties.

_Cuminum cyminum_ shows several pharmacological actions. The anticarcinogenic property of cumin was investigated. The cumin seeds (_Cuminum cyminum_ Linn) were reported to decrease the incidence of both neoplasia and hepatomas. The results suggest that cumin seeds may prove to be valuable anticarcinogenic agent (Aruna and Sivaramakrishnan 1992). Cumin showed immunomodulatory properties in normal and Cyclosporine-A induced immune-suppressed animals. In both the groups _C. cyminum_ significantly increases T cells (CD4 and CD8) count and Th1 predominant immune response in a dose dependent manner. Hence, the immunomodulatory activity of _C. cyminum_ was supposed through modulation of T lymphocytes expression (Chauhan 2010). Methanolic extract of _Cuminum cyminum_ was reported to inhibit the ovariectomy-induced bone loss i.e. antosteoporotic activity in rats (Shirke _et al._ 2008).

Extracellular application of the fruit essential oil of _Cuminum cyminum_ Linn. reduces the epileptiform activity induced by pentylenetetrazol (PTZ) in a dose dependent manner (Janahmadi _et al._ 2006). The antibacterial and antifungal activity of _Cuminum cyminum_ was greatly exploited in various studies. The activity was particularly high against the genera Clavibacter, Curtobacterium, Rhodococcus, Erwinia, Xanthomonas, Ralstonia, and Agrobacterium, which are responsible for plant or cultivated mushroom diseases worldwide (Iacobellis _et al._ 2005). A biologically active compound 1-(2-Ethyl, 6-Heptyl) Phenol (EHP), extracted by benzene from _Cuminum cyminum_ shows inhibitory activity against a number of fungal pathogens. It also exhibited antitumor activity against six types of tumor cell lines viz. HEPG2, HELA, HCT116, MCF7, HEP2 and CACO2 (Mekawey _et al._ 2009).

Besides the whole extract, the essential oil of _Cuminum cyminum_ was greatly used in various studies and demonstrated good antibacterial, insecticidal or antifungal activity. The essential oil shows good insecticidal activity against larvae of _L. ingenua_. The components from cumin oil were isolated and individual compound was assessed for its insecticidal activities against larvae of _L. ingenua_. Among the isolated compounds cuminaldehyde shows effective insecticidal activity (Park _et al._ 2007). The essential oil of Cuminum shows antifungal activity against _B. cinerea_, _F. oxysporum_, _P. ultimum_ and
R. solani (Lee et al. 2007) and antibacterial effect against several food-borne pathogens, namely Staphylococcus aureus, Bacillus cereus, Escherichia coli O157:H7, Salmonella enteritidis, and Listeria monocytogenes (Oroojalian et al. 2010). The essential oil of Cuminum cyminum decreased biofilm formation ability and plasmid integrity of Klebsiella pneumonia. Hence the essential oil of cumin seed may be useful to treat bacterial infections. The antibacterial property of cumin essential oil was postulated due to the cuminaldehyde (Derakhshan 2010). Moreover, C. cyminum oil exhibited higher antibacterial and antifungal activities with a high effectiveness against Vibrio spp. strains (Hajlaoui et al. 2010) and also bactericidal effects against Bacillus cereus (Pajohi et al. 2011). The essential oil of Cuminum shows antioxidant activity and antimicrobial activity against E. coli, S. aureus, and S. faecalis (Allahghadri et al. 2010). Cumin oil and its isolated compound cuminic aldehyde exhibited a significant antimicrobial activity (Wanner et al. 2010).

The free radical scavenging and antioxidant activity of Cuminum cyminum was also exploited in various in vitro studies and shows good antioxidant activity (Thippeswamy and Naidu 2005; Gachkar et al. 2007; Topal et al. 2008; Bettaieb et al. 2010; Kim 2011). Cuminum cyminum also shows good in vivo antioxidant activity in prolonged treated albino rats (Surya et al. 2005).

The antihyperglycemic activity of cumin and its use in secondary complications associated with diabetes mellitus was evaluated in various studies. The Cuminum cyminum reported to significantly decrease the area under the glucose tolerance curve and the hyperglycemic peak in subcutaneous glucose tolerance tests performed in healthy rabbits (Roman-Ramos 1995). The prolonged effect of cumin seeds (1.25%) in a dietary regimen was studied by Willatgamuwa et al. in streptozotocin induced diabetic rats. During an eight week dietary regimen containing cumin powder (1.25%) was found to be remarkably beneficial, as indicated by reduction in hyperglycaemia and glucosuria. There was also improvement in body weights and other metabolic alterations as revealed by lowered blood urea level and reduced excretions of urea and creatinine by diabetic animals on the cumin diet. Since spices such as cumin which are the normal constituents of our daily diet possessing no known harmful side effects, the authors suggest that their antidiabetic potency can be exploited to the maximum extent by their liberal inclusion in the diets (Willatgamuwa et al. 1998).
Aqueous suspension of *Cuminum cyminum* L. fruits and its extracts were tested orally for hypoglycemic effects in normal healthy rats and in streptozotocin induced diabetic rats (Kalia *et al.* 2004). Aqueous suspension and petroleum ether extract of the *Cuminum cyminum* fruits are reported to cause significant reduction in blood glucose level in fasting normal healthy rats and improved glucose tolerance. The petroleum ether extracts observed to increase serum insulin after oral glucose infusion in the drug treated group of rats as compared to control group. The hypoglycemic response of the petroleum ether extract used in this study was observed better in comparison to tolbutamide both in the terms of duration and extent of hypoglycemia. Although, the authors reported no significant reduction in fasting blood glucose concentration by single dose of petroleum ether fraction in streptozotocin induced diabetic rats and postulated that this may be due to the destruction of pancreatic islets (Kalia *et al.* 2004).

The inhibitory activity of *Cuminum cyminum* seed oil isolated component was reported against lens aldose reductase and alpha-glucosidase isolated from Sprague-Dawley male rats. Among the 11 components derived from *C. cyminum* seed oil, the cuminaldehyde was reported to possess significant biologically activity with the IC\textsubscript{50} value of 0.00085 mg/ml against aldose reductase and 0.5 mg/mL against alpha-glucosidase, respectively. Hence, cuminaldehyde was postulated to be useful as a lead compound and a new agent for antidiabetic therapeutics (Lee 2005).

The cumin was reported with good hypolipidemic action. Oral administration of 0.25 g kg\textsuperscript{-1} body weight of *C. cyminum* for 6 weeks to alloxan induced diabetic rats resulted in significant reduction in blood glucose. There was a significant reduction in plasma and tissue cholesterol, phospholipids, free fatty acids and triglycerides. It also prevented a decrease in body weight. Moreover, *C. cyminum* supplementation was found to be more effective than glibenclamide in the treatment of diabetes mellitus (Dhandapani 2002). The antihyperglycemic and hypolipidemic effect of cumin seeds was also studied in type 2 diabetic patients. Significant glycemic control was observed in patients with cumin seed therapy also there was a significant decrease in levels of cholesterol (47%), triglycerides (26%), plasma free fatty acids (4%), phospholipids (9%), LDL-cholesterol (5%), VLDL-cholesterol (26%) and atherogenic index (21%) while significantly increase in HDL-cholesterol (10%) (Andallu and Ramya 2007).
The delay of diabetic cataract in rats by antiglycating potential of cumin was also investigated by feeding streptozotocin (STZ)-induced diabetic rats with diet containing 0.5% cumin powder. The supplementation of cumin shows delayed progression and maturation of STZ-induced cataract in rats (Kumar et al. 2009). Antihyperglycemic activity and inhibition of advanced glycation end product formation by methanolic extract of seeds *Cuminum cyminum* was reported in streptozotocin induced diabetic rats. *In vitro* studies indicated that *Cuminum cyminum* inhibited free radicals and AGE formation. Treatment of streptozotocin-diabetic rats with *Cuminum cyminum* and glibenclamide for 28 days showed a reduction in blood glucose, glycosylated hemoglobin, creatinine, blood urea nitrogen and improved serum insulin and glycogen (liver and skeletal muscle) content when compared to diabetic control rats. Significant reduction in renal oxidative stress and AGE was observed with *Cuminum cyminum* when compared to diabetic control and glibenclamide. *Cuminum cyminum* and glibenclamide improved antioxidant status in kidney and pancreas of diabetic rats. Diabetic rats are reported to show an increase in rat tail tendon collagen, glycated collagen, collagen linked fluorescence and reduction in pepsin digestion. Treatment with *Cuminum cyminum* significantly improved these parameters when compared to diabetic control and glibenclamide group. The cumin shows better effect in controlling oxidative stress and inhibiting the AGE formation, which are implicated in the pathogenesis of diabetic microvascular complications (Jagtap and Patil 2010).

### 1.2 Chemical composition

Cumin oil is a pale yellow to brownish yellow liquid; it occasionally displays a greenish tint. Many phytochemical studies have been conducted thus far to investigate the chemical composition of the essential oil of cumin seeds. The major components of cumin are aldehydes, where the most prominent one is cuminaldehyde 36.31%. The other major components are cuminic alcohol (16.92%), γ-terpinene (11.14%), safranal (10.87%), p-cymene (9.85%) and β-pinene (7.75%) are the major components (Li and Jiang 2004; Hashemi et al. 2009).
2. **Material and methods**

2.1. **Plant material**

   The cumin seeds were used in the present study and purchased from local spice market Kolhapur, Maharashtra, India. The spice was identified from Department of Botany, Shivaji University, Kolhapur, India.

2.2 **Extraction of fractions from steam distillate**

   The seeds of cumin were ground to powder and subjected to steam distillation. About 100 gm of cumin seed powder was placed in round bottomed flask and final volume was adjusted to 1000 ml with distilled water and subjected to steam distillation at 60 °C. The distillate was collected and subjected to sequential extraction with organic solvents as petroleum ether (pet ether), chloroform followed by dichloromethane (DCM). Each fraction extracted was then evaporated in vacuum rotary evaporator and dried over anhydrous Sodium sulphate. The distillate remaining after the organic extraction was considered as aqueous fraction. The % yield of pet ether, chloroform and DCM fractions with respect to cumin powder was 1.01%, 0.05% and 0.03% w/w respectively. All the dried fractions were stored in airtight screw cap glass vials at 4 °C until further use. Each fraction was diluted with dimethyl sulfoxide (DMSO) just before the use. The final concentration of DMSO did not exceed more than 0.1% in each dilution. Vehicle control is used in each *in vivo* and *in vitro* experiment.

2.3 **Induction of diabetes**

   Diabetes was induced in normal male Wistar rats as per method mentioned in previous chapter.

2.4 **Oral Glucose Tolerance Test (OGTT)**

   Total 42 rats (6 normal and 36 diabetic) were fasted overnight with free access to water. Initial blood glucose of each rat was measured. They were divided into 7 groups (in each group n=6) as normal control, diabetic control, positive control (glibenclamide 2.5 mg/kg body weight) and remaining four groups were as pet ether fraction (10 mg/kg body weight), chloroform fraction (10 mg/kg body weight), DCM fraction (10 mg/kg body weight) and aqueous fraction (10 ml/kg body weight). All rats were fed orally with glucose load of 3 mg/gm body weight. The normal and control group rats were
administrated orally with vehicle while others with respective test component ten min prior to glucose administration. Blood glucose was measured with ACCU-CHECK at 0, 30, 60 and 120 min.

2.5 Experimental design

All the experiments were carried out as per the guidelines of the Institutional Animal Ethical Committee after due submission and approval of the protocols. The biological active pet ether fraction was carried out for further prolonged and in vitro studies.

2.5.1 In vivo prolonged treatment

For prolonged experiment, total 30 male rats (6 normal and 24 diabetic) were used and divided into 5 groups as below.

Group I - Normal control
Group II - Diabetic control
Group III - Diabetic + glibenclamide (2.5 mg/kg body weight)
Group IV - Diabetic + 5 mg pet ether fraction/kg body weight
Group V - Diabetic + 10 mg pet ether fraction/kg body weight

The glibenclamide or pet ether fraction was fed orally to the animals twice a day to respective group while the normal and diabetic control group rats were administrated vehicle (0.1% DMSO).

2.6 Estimation of biochemical parameters

Fasting blood glucose level of all the groups were estimated by ACCU-CHECK on day 0, 8, 16, 24, 32, 40 and 45. Blood was collected from tail tip. At the end of 45 days, all the rats were fasted overnight and sacrificed by cervical dislocation. Blood was withdrawn immediately and serum collected was used for biochemical assays. Whole blood was used for estimation of glycated hemoglobin. For lipid profile of all the groups; Serum total cholesterol and HDL-Cholesterol was estimated by biochemical kit from Crest Biosystems while total triglycerides were estimated by liquid gold kit. Serum LDL and VLDL was calculated by using Friedewald’s formula as:
VLDL= Triglycerides/5
LDL= Total cholesterol – HDL – VLDL

Serum insulin was measured by rat insulin ELISA kit (CalBiotech). The amount of insulin released was quantified by using ELISA microplate reader (Multiskan EX, Thermo Scientific) at 450 nm.

2.7  \textit{In vitro} studies
2.7.1  Isolation of rat pancreatic islets

The isolation of islets was carried out using collagenase digestion method (Shewade \textit{et al.}, 1999) with some modifications (Patil \textit{et al.}, 2011). The detailed procedure was mentioned in previous chapter.

2.7.2  Insulin release assay

Groups of 10 islets were placed in wells 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 with 11.8 mM glucose in presence of test components. Glibenclamide (10 µg/ml of reaction mixture) which is a commercially used sulfonylurea, was used as positive control. In primary studies, pet ether fraction found to enhance the insulin secretion and was used for further \textit{in vitro} studies. To illustrate probable mechanism of insulin secretion by bioactive isolated compounds, isolated islets were incubated presence or absence of isolated compound under following conditions as, in presence of 2.8 mM, 5.8 mM and 11.8 mM glucose, 11.8 mM glucose and 300 µM diazoxide (an established opener of K$^+$-ATP channel) or 20 µM nifedipine (an established the L-type Ca$^{2+}$ channel blocker) or 30 mM KCl (artificial depolarizing agent), 5.6 mM glucose and 1 mM IBMX, 2.8 mM glucose and 10 mM alanine. After 1 hour incubation of islets in above mentioned conditions, supernatant from each well was collected and stored at -20 °C until further use. The insulin concentration in all the stored samples was determined by ELISA kit (CalBiotech) and quantified by using ELISA microplate reader (Multiskan EX, Thermo Scientific) at 450 nm.

2.7.3  Viability assessment by MTT conversion

The viability of isolated islets after experimental treatment was assessed by MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay (Mosman 1983;
Latha et al., 2009). MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) is a yellow water soluble tetrazolium salt. The dye is converted to water-insoluble purple formazan by the active living cells. The viability of cells was expressed in percent viability (% viability) relative to vehicle control group islets which were considered as 100% viable.

3. Isolation and identification of bioactive compound form pet ether fraction

3.1 Isolation of compounds by silica gel column chromatography

The petroleum ether fraction was subjected to silica gel column chromatography. The petroleum ether fraction was loaded on a silica gel column (60-120 mesh size, 1.2 internal diameter x 50 cm) and successively eluted with stepwise gradient of Hexane: Ethyl acetate system (100:0, 80:20, 60:40, 40:60, 20:80 and 0:100).

Among the six fractions, the third fraction i.e. 60:40 was found to possess bioactivity and was further separated by repeated silica gel column chromatography. The fraction was loaded on a column and successively eluted with stepwise gradient of Hexane: ethyl acetate system (60:40, 55:45, 50:50, 45:55 and 40:60). Three subfractions were collected for each gradient. Hence, total 12 fractions were collected for this step. The bioactive fractions were subjected to further structural elucidation by GC-MS analysis followed by IR, H¹ NMR analysis.

3.2 GC-MS analysis

GC-MS analysis of active fraction was carried out Common Facility Center (CFC), Shivaji University, Kolhapur using a GCD-1800 A model (Shimadzu) of GC-MS. 0.2 μl of sample was injected on a HP-5 column with a starting temperature of 100°C and a hold time of 2 min at 150 -160 °C.

3.3 FTIR studies

Infrared (IR) analysis was carried out done in Common Facility Center (CFC), Shivaji University, Kolhapur using a FTIR spectrometer in the range 450–4000 cm⁻¹ by KBr pellet technique.
3.4 NMR analysis

$^1H$ NMR was performed at Shivaji University, Kolhapur using deuterated chloroform (CDCl$_3$) at 300 MHz on a Bruker spectrophotometer.

4. Statistical analysis

All the data obtained was expressed as mean ± SD. Statistical analysis was performed using ANOVA and Unpaired Student $t$-test. A value with $p < 0.05$ was considered as statistically significant while $p < 0.005$ as extremely significant.

5. Results and discussion

Cumin seeds are widely used as spice worldwide. Beside just a spice, cumin is widely used in Ayurvedic medicine as a stimulant, carminative, astringent and also for the treatment of dyspepsia, diarrhea and jaundice. Various pharmacological actions of cumin were reported including the antidiabetic activity. However, its mechanism of action is not specified. Hence, the antihyperglycemic activities of the extracts were evaluated through oral glucose tolerance test (OGTT) and other in vitro and in vivo studies.

5.1 Oral glucose tolerance test for solvent extracts

The antihyperglycemic activity of petroleum ether, chloroform, dichloromethane and aqueous fraction isolated from *Cuminum cyminum* distillate was evaluated through OGTT and shown in Table 1. The results were compared with diabetic rats as negative control and glibenclamide (2.5 mg/kg body weight) as a positive control. In diabetic control group, blood glucose levels dramatically increases at 60 min and remains high even after a period of 120 min as compared to normal control. Glibenclamide, a commercially used sulfonylurea at a concentration of 2.5 mg/kg rat body weight shows very good antihyperglycemic action. All the fractions viz. petroleum ether, chloroform, dichloromethane and aqueous fraction were used at a concentration of 10 mg/kg body weight.
Table 1- Effect of *Cuminum cyminum* solvent fractions on blood glucose levels

<table>
<thead>
<tr>
<th>Group</th>
<th>Blood glucose (mg/dl)</th>
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<tbody>
<tr>
<td></td>
<td>0 min</td>
</tr>
<tr>
<td>Normal control</td>
<td>76.7±3.1</td>
</tr>
<tr>
<td>Diabetic control</td>
<td>250.2±5.8</td>
</tr>
<tr>
<td>Diabetic + Glibenclamide (2.5 mg/kg)</td>
<td>242.2±6.2</td>
</tr>
<tr>
<td>Diabetic + Pet ether fraction (10 mg/kg)</td>
<td>238.7±6.0</td>
</tr>
<tr>
<td>Diabetic + Chloroform fraction (10 mg/kg)</td>
<td>243.3±5.9</td>
</tr>
<tr>
<td>Diabetic + DCM fraction (10 mg/kg)</td>
<td>235.3±5.6</td>
</tr>
<tr>
<td>Diabetic + Aqueous fraction (10 ml/kg)</td>
<td>251.5±5.6</td>
</tr>
</tbody>
</table>

Values are mean ± SD; n=6. *p<0.05 and $p<0.005 with respect to diabetic control.

Among the four fractions, pet ether fraction showed most prominent antihyperglycemic action. It reduces blood glucose levels after both 60 min and 120 min time interval as compared to diabetic control. The antihyperglycemic potency and glucose level lowering pattern of pet ether fraction (10 mg/kg body wt) resembles with that of glibenclamide control; however it is not as potent in action as glibenclamide. The remaining three fractions viz. chloroform, dichloromethane and aqueous fractions were not able to reduce blood glucose levels as compared to glibenclamide control and pet ether fraction group. Hence, the antihyperglycemic activity was supposed due to pet ether fraction of *Cuminum cyminum* seeds distillate. Thus, the bioactive pet ether fraction was used for further studies.

The antihyperglycemic activity of cumin by means of oral and subcutaneous glucose tolerance test was studied previously. The *Cuminum cyminum* was reported to significantly decrease the area under the glucose tolerance curve and the hyperglycemic peak in subcutaneous glucose tolerance tests performed in healthy rabbits (Roman-Ramos 1995). Kalia *et al.* reported that aqueous suspension and petroleum ether extract of the *Cuminum cyminum* fruits causes significant reduction in blood glucose level in fasting normal healthy rats and improves glucose tolerance (Kalia *et al.* 2004). The hypoglycemic response of the petroleum ether extract was reported to be better in
comparison to tolbutamide. Although, the authors observed no significant reduction in fasting blood glucose concentration by single dose of petroleum ether fraction in streptozotocin induced diabetic rats. However, in present study we observed potential antihyperglycemic effect of pet ether fraction even in a single dose in the oral glucose tolerance test. The antihyperglycemic action of pet ether fraction was not as potent as glibenclamide but showed good antihyperglycemic activity.

Though the antihyperglycemic activity of cumin seed was reported previously in which whole extract was used, in present study various fractions extracted from steam distillate of cumin seeds are used. The benefits of using steam distillate and further extraction is that the process greatly eliminates number of non-volatile compounds and reduced number of compounds eases the process of isolation and identification of bioactive compound.

5.2 *In vitro* insulin secretion studies of solvent extracts

Beside OGTT, the effect of pet ether, chloroform, dichloromethane and aqueous fractions extracted from *Cuminum cyminum* seeds distillate was assessed for insulin secretagogue action and is shown in Figure 1.

Figure 1. Effect of pet ether, chloroform, DCM and aqueous extracts on insulin secretion

Results are mean ± S.D.; n=6. *p*<0.05 and **p*<0.005 significant from 11.8 mM glucose control.
Pet ether, chloroform and DCM extracts at a concentration of (25 µg/ml reaction mixture) while aqueous fraction at concentration of 50 µl/ml were incubated with isolated islets in presence of 11.8 mM glucose.

Among all the four extracts, the pet ether extract shows maximum insulin secretion (100.70 µIU) as compared to other fractions. The insulin stimulation due to pet ether fraction was almost 2.51 times higher than that of 11.8 mM glucose. The chloroform fraction showed some stimulatory action but is not as pronounced as pet ether fraction. The DCM and aqueous fractions also not showed significant insulin secretagogue action. Hence, pet ether fraction was used for further analysis.

5.3 Prolonged treatment experiments

The pet ether fraction with blood glucose lowering activity was used for prolonged treatment at concentrations of 5 mg and 10 mg/kg body weight for 45 days. The effect of treatment on blood glucose, glycated hemoglobin, serum insulin levels and lipid profile was evaluated.

5.3.1 Changes in blood glucose, glycated hemoglobin, serum insulin

The effect of prolong treatment of glibenclamide (2.5 mg/kg body weight) or pet ether fraction (5 mg and 10 mg/kg body weight) on fasting blood glucose level, glycated Hb and serum insulin was shown in Table 2. The results were compared with diabetic control group. After prolonged treatment for 45 days, there was progressive increase in fasting blood glucose levels in diabetic control group. In glibenclamide treated rats, there was progressive lowering of blood glucose values and after the completion of treatment, the fasting values are even much controlled than the day of starting the treatment. Glibenclamide is a commercially used sulfonylurea which stimulates the insulin secretion and the enhanced insulin secretion which is responsible for the effective glycemic control. It was observed that pet ether fraction significantly reduced the fasting blood glucose levels at both the concentration 5 mg and 10 mg/kg body weight as compared to diabetic control. The glycemic control was more pronounced at dose of 10 mg/kg body weight and it was even better than glibenclamide treated group. Thus, the results show that the pet ether fraction of Cuminum cyminum showed antihyperglycemic action even in prolonged treatment.
Table 2: *In vivo* long term effect of *Cuminum cyminum* pet ether fraction on blood glucose, glycosylated hemoglobin and serum insulin.

<table>
<thead>
<tr>
<th>Group (Treatment and dose)</th>
<th>Blood glucose (mg/dl)</th>
<th>GHb (%)</th>
<th>Serum insulin (µIU)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 0</td>
<td>Day 45</td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>75.8±3.9</td>
<td>80.3±4.2</td>
<td>5.75±0.73</td>
</tr>
<tr>
<td>Diabetic Control</td>
<td>235.6±6.2</td>
<td>398.0±7.3</td>
<td>13.46±0.64</td>
</tr>
<tr>
<td>Diabetic + Glibenclamide (2.5 mg/kg body wt)</td>
<td>248.4±6.6</td>
<td>158.0±7.1</td>
<td>8.16±0.79</td>
</tr>
<tr>
<td>Diabetic + pet ether fraction (5 mg/kg body wt)</td>
<td>233.7±5.2</td>
<td>187.0±6.3</td>
<td>9.17±0.76</td>
</tr>
<tr>
<td>Diabetic + pet ether fraction (10 mg/kg body wt)</td>
<td>244.4±6.9</td>
<td>132.5±6.5</td>
<td>6.82±0.64</td>
</tr>
</tbody>
</table>

Values are mean ± SD; n=6. *p*<0.005 with respect to diabetic control for all the values.

In diabetic control group, there is a very high percentage of glycated hemoglobin (GHb) value. The uncontrolled and continuously high free glucose circulating in blood for a prolonged time leads to formation of Advanced Glycated End (AGE) products. The formation of AGE product leads to formation of glycated hemoglobin and other secondary complications of diabetes. There is a controlled glycated Hb level in glibenclamide treated rats. The glibenclamide effectively controls the blood glucose level which decreases the glycated hemoglobin. There is dose dependent decrease in extent of glycated hemoglobin in a pet ether treated rats as compared to diabetic control group. At 10 mg/kg dose, pet ether fraction showed decreased glycated hemoglobin (6.82% GHb) than 5 mg/kg dose (9.17% GHb). The control on glycated Hb at 10 mg/kg dose was even better than that of glibenclamide treated rats (8.16 % GHb). The blood glucose lowering effect of pet ether fraction is not as pronounced as glibenclamide; still it effectively controls the extent of hemoglobin glycation. The effective control on glycated hemoglobin in pet ether fraction treated rats may be due the antioxidant activity of the
fraction. The antioxidants play a vital role in prevention of formation of advanced glycation end products and further formation of secondary complications.

There are decreased serum insulin levels in diabetic control rats as compared to the normal control. The glucose toxicity towards the β-cells due to continuous hypoglycemic condition was reported in several studies. Chronic hyperglycemia may impair β-cell function at the level of insulin synthesis as well as insulin secretion (Robertson et al. 1992; Moran et al. 1997). In case of glibenclamide treated rats, improved serum insulin levels were observed as compared to diabetic control. This may be due to the antihyperglycemic activity and controlled glycemic status by glibenclamide. After treatment with pet ether fraction, there was dose dependent improvement in serum insulin levels. The increase in serum insulin levels at 5 mg and 10 mg/kg doses were 1.75 and 2.34 times higher than that of diabetic control group. At 10 mg/kg dose the insulin secretion improvement was better than glibenclamide treatment. The dose dependent improvement in serum insulin levels may be due to the antihyperglycemic activity which reduces the glucose toxicity and/or β-cell protective ability of the fraction. The petroleum ether extract extracted from the whole cumin seeds was reported to increase serum insulin as compared to control group and was even better in comparison to tolbutamide (1st generation sulfonylurea) (Kalia et al. 2004). We also observed that pet ether fraction increases the serum insulin levels.

5.3.2 Lipid profile

Obesity is the main risk factor for the development of diabetes. It is often part of the metabolic syndrome and is accompanied by dyslipidemia and increased circulating leptin and cytokine levels. All of these factors have been shown to modulate β-cell function and survival. The influence of dyslipidemia on the β-cells of an individual will depend on specific lipid profile. Whereas some free fatty acids and lipoproteins have been shown to be pro-apoptotic for the β-cell, others are protective. Thus, long-term exposure to saturated fatty acids such as palmitate appears highly toxic, whereas monounsaturated fatty acids such as oleate protect against both palmitate- and glucose-induced β-cell apoptosis (Maedler et al. 2003). In concert with these observations, the compounds showing good antilipidemic action are beneficial in treatment of diabetes. The effect of pet ether fraction on lipid profile evaluated and is summarized in Table 3.
Table 3 - *In vivo* long term effect of *Cuminum cyminum* pet ether fraction on lipid profile.

<table>
<thead>
<tr>
<th>Group (Treatment and doses)</th>
<th>TC (mg/dl)</th>
<th>TG (mg/dl)</th>
<th>HDL (mg/dl)</th>
<th>LDL (mg/dl)</th>
<th>VLDL (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control</td>
<td>70.6±3.5</td>
<td>73.1±3.9(^{§})</td>
<td>40.4±4.3(^{§})</td>
<td>15.4±3.5(^{§})</td>
<td>14.6±0.8(^{§})</td>
</tr>
<tr>
<td>Diabetic control</td>
<td>128.5±4.7</td>
<td>132.7±3.4</td>
<td>25.5±3.8</td>
<td>76.8±2.8</td>
<td>26.5±0.7</td>
</tr>
<tr>
<td>Glibenclamide (2.5 mg/kg body wt)</td>
<td>91.9±3.0</td>
<td>89.5±3.3(^{§})</td>
<td>32.9±3.4(^{*})</td>
<td>38.0±4.3(^{§})</td>
<td>17.9±0.7(^{§})</td>
</tr>
<tr>
<td>Diabetic + 5 mg pet ether fraction/kg body wt</td>
<td>108.1±4.6</td>
<td>111.3±3.7(^{§})</td>
<td>28.4±2.8</td>
<td>58.4±4.3(^{§})</td>
<td>22.3±0.7(^{§})</td>
</tr>
<tr>
<td>Diabetic + 10 mg pet ether fraction/kg body wt</td>
<td>87.1±4.3</td>
<td>98.8±4.3(^{§})</td>
<td>35.4±3.7(^{§})</td>
<td>32.4±1.2(^{§})</td>
<td>19.8±0.9(^{§})</td>
</tr>
</tbody>
</table>

Values are mean ± SD; n=6. \(^{*}\)p<0.01 and \(^{§}\)p<0.001 with respect to diabetic control.

In diabetic rats the lipid profile was greatly imbalanced as compared to normal control rats. Treatment with pet ether fraction of cumin also improves the lipid profile in dose dependent manner. There was lowering of total cholesterol (TC), triglyceride (TG), low density lipoprotein cholesterol (LDL), very low density lipoprotein cholesterol (VLDL) levels and improvement in high density lipoprotein (HDL) levels in pet ether treated groups. However, the improvement was better at a concentration of 10 mg/kg body weight than that of 5 mg dose and even better than glibenclamide group.

The hypolipidemic action of cumin was previously reported. Oral administration of 0.25 gm/kg body weight of *C. cyminum* for 6 weeks to alloxan induced diabetic rats was reported to significantly lower blood glucose level, and an increase in total hemoglobin and glycosylated hemoglobin. It was observed that cumin significantly reduces the plasma and tissue cholesterol, phospholipids, free fatty acids and triglycerides and also prevents a decrease in body weight. Moreover, *C. cyminum* supplementation was reported to be more effective than glibenclamide in the treatment of diabetes mellitus (Dhandapani 2002).

The antihyperglycemic and hypolipidemic effect of cumin seeds was also studied in type 2 diabetic patients. Significant glycemic control was reported in patients with
cumin seed therapy along with significant decrease in levels of cholesterol (47%), triglycerides (26%), plasma free fatty acids (4%), phospholipids (9%), LDL-cholesterol (5%), VLDL-cholesterol (26%) and atherogenic index (21%) while significantly increase in HDL-cholesterol (10%) (Andallu and Ramya 2007). In present study, results after prolonged treatment also confirmed the antihyperlipidemic effect of cumin in treated rats. Cuminaldehyde, the isolated component from cumin was reported to possess significant aldose reductase inhibitory and alpha-glucosidase inhibitory action thereby can prevent progression of secondary complications (Lee 2005). We have observed that after prolonged treatment with pet ether fraction the animals showed no evidence of cataract or nephrotoxicity.

The insulin stimulatory effect of pet ether extract and other solvent extracts from steam distillate of *Cuminum cyminum* were evaluated on isolated islets.

6. **Isolation and characterization of bioactive component/s from pet ether fraction**

6.1 **Isolation of compounds by silica gel column chromatography**

The petroleum ether fraction was separated on silica gel column chromatography using Hexane: Ethyl acetate gradient system as 100:0, 80:20, 60:40, 40:60, 20:80 and 0:100. The % yield (w/w of initially loaded sample) for each fraction is tabulated in Table 4.

**Table 4. Isolation and identification of bioactive compound form pet ether fraction.**

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Gradient (Hexane: Ethyl Acetate)</th>
<th>% wt of fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>100:0</td>
<td>2.19</td>
</tr>
<tr>
<td>2</td>
<td>80:20</td>
<td>2.99</td>
</tr>
<tr>
<td>3</td>
<td>60:40</td>
<td>53.17</td>
</tr>
<tr>
<td>4</td>
<td>40:60</td>
<td>7.37</td>
</tr>
<tr>
<td>5</td>
<td>20:80</td>
<td>14.31</td>
</tr>
<tr>
<td>6</td>
<td>0:100</td>
<td>19.94</td>
</tr>
</tbody>
</table>
Among these six fractions, the third fraction i.e. 60:40 was found to be most bioactive in stimulation of insulin secretion. The in vivo activity of fraction was further verified through oral glucose tolerance test (Figure 2).

### 6.2 Oral Glucose Tolerance Test for fraction 60:40

The antihyperglycemic activity of fraction 60:40 at concentration of 5 mg and 10 mg/kg body weight was verified (Figure 2). The results were compared with diabetic rats as negative control and glibenclamide (2.5 mg/kg body weight) as a positive control.

Figure 2. Oral Glucose Tolerance Test of fraction 60:40

The bioactive fraction 60:40 also showed dose dependent antihyperglycemic action in OGTT. At increased concentration of 10 mg/kg, the antihyperglycemic effect is more pronounced and is as effective as glibenclamide control.
For the further isolation of insulin stimulatory compounds, the 60:40 fraction was loaded on a column and successively eluted with stepwise gradient of Hexane: Ethyl acetate system (60:40, 55:45, 50:50, 45:55 and 40:60). Three sub-fractions were collected for each gradient. Hence, total 12 fractions were collected for this step. The % yield (w/w of initially loaded sample) for each fraction was tabulated in Table 5.

**Table 5.** Isolation of bioactive compound form 60:40 pet ether fraction.

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Gradient (Hexane: Ethyl Acetate)</th>
<th>% wt of fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>60:40 (Sub fraction 1)</td>
<td>1.35</td>
</tr>
<tr>
<td>2</td>
<td>60:40 (Sub fraction 2)</td>
<td>36.93</td>
</tr>
<tr>
<td>3</td>
<td>60:40 (Sub fraction 3)</td>
<td><strong>34.93</strong></td>
</tr>
<tr>
<td>4</td>
<td>55:45 (Sub fraction 1)</td>
<td>11.71</td>
</tr>
<tr>
<td>5</td>
<td>55:45 (Sub fraction 2)</td>
<td><strong>4.50</strong></td>
</tr>
<tr>
<td>6</td>
<td>55:45 (Sub fraction 3)</td>
<td>2.25</td>
</tr>
<tr>
<td>7</td>
<td>50:50 (Sub fraction 1)</td>
<td>0.90</td>
</tr>
<tr>
<td>8</td>
<td>50:50 (Sub fraction 2)</td>
<td>1.35</td>
</tr>
<tr>
<td>9</td>
<td>50:50 (Sub fraction 3)</td>
<td>2.70</td>
</tr>
<tr>
<td>10</td>
<td>45:55 (Sub fraction 1)</td>
<td>1.80</td>
</tr>
<tr>
<td>11</td>
<td>45:55 (Sub fraction 2)</td>
<td>1.35</td>
</tr>
<tr>
<td>12</td>
<td>45:55 (Sub fraction 3)</td>
<td>0.90</td>
</tr>
</tbody>
</table>

Among these fractions, fraction-3 (3\textsuperscript{rd} sub fraction of 60:40) and fraction-5 (2\textsuperscript{nd} sub fraction of 55:45) showed the optimum insulin stimulating action. These two fractions were subjected to further structural elucidation by GC-MS analysis followed by IR, H\textsuperscript{1} NMR analysis.
6.3 Identification of compounds from fraction 60:40 (sub fraction 3)

6.3.1 GC-MS analysis

The main chromatogram of GC-MS analysis was shown in Figure 3. In GC-MS, four peaks at a retention time (RT) of 15.6, 16.9, 17.7 and 21.8 were observed.

Figure 3. GC-MS analysis of fraction 60:40 (sub fraction 3)

The most prominent component was observed with the RT of 15.6 and predicated as cuminaldehyde by NIST MS library. The mass fragmentation of this compound was shown in Figure 4 and the fragmentation matches with the cuminaldehyde. Hence, the compound at RT 15.6 may be cuminaldehyde. The possibility was confirmed by further structural elucidation by means of FTIR and H$^1$NMR analysis. The other component present RT 16.9 was predicted as safranal.
Figure 4. Fragmentation pattern of major compound at RT 15.6

6.3.2 FTIR analysis

The FTIR analysis was performed to predict probable functional groups present in the sample. The FTIR spectrum was shown in Figure 5.

Figure 5. FTIR spectra for 60:40 (Sub fraction 3)

The major peaks observed in FTIR spectra and probable functional group for respective peak is shown in Table 6.
Table 6. The peak observed and probable functional group in the fraction

<table>
<thead>
<tr>
<th>Peak observed in FTIR</th>
<th>Probable functional group</th>
</tr>
</thead>
<tbody>
<tr>
<td>2963.41</td>
<td>aldehyde group C-H stretch (H–C=O: C–H stretch)</td>
</tr>
<tr>
<td>1702.42</td>
<td>C=O stretch</td>
</tr>
<tr>
<td>1607.90</td>
<td>C–C stretch (in aromatic ring)</td>
</tr>
<tr>
<td>770.41</td>
<td>Para distribution</td>
</tr>
</tbody>
</table>

6.3.3 NMR analysis

The $^1H$ NMR analysis of the compound was performed. The spectrum is shown in Figure 6.

Figure 6. $^1H$ NMR analysis of fraction 60:40 (sub fraction 3)
The major shifts observed in $^1$H NMR analysis with possible positions of ‘H’ atoms are shown in Table 7.

Table 7. Major shifts with probable positions and numbers of ‘H’ atoms

<table>
<thead>
<tr>
<th>Shift</th>
<th>‘H’ numbering in figure</th>
<th>Total ‘H’ at a shift</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.244</td>
<td>H8 a, 8 b, 8 c and 11 a,11b, 11c</td>
<td>6</td>
</tr>
<tr>
<td>3.015</td>
<td>H7a</td>
<td>1</td>
</tr>
<tr>
<td>7.356</td>
<td>H2a and H6a</td>
<td>2</td>
</tr>
<tr>
<td>7.782</td>
<td>H3a and H5a</td>
<td>2</td>
</tr>
<tr>
<td>9.957</td>
<td>aldehyde group H (H 9a)</td>
<td>1</td>
</tr>
</tbody>
</table>

The $^1$H NMR of the compound matches with the reported NMR of standard cuminaldehyde. Hence, it was confirmed that the compound isolated is cuminaldehyde.

6.4 Identification of compounds from fraction 55:45 (Sub fraction 2)

6.4.1 GC-MS analysis

The main chromatogram of GC-MS analysis was shown in Figure 7. In GC-MS, only two peaks at a retention time (RT) of 17.0 and 20.7 were observed.
Figure 7. GC-MS analysis of fraction 55:45 (sub fraction 2)

![GC-MS analysis graph]

Figure 8. Fragmentation pattern of major compound at RT 17.0

![Fragmentation pattern graph]

6.4.2 FTIR analysis

The FTIR analysis was performed to predict probable functional groups present in the sample. The FTIR spectra was shown in Figure 9.

Figure 9. FTIR spectra for 55:45 (sub fraction 2)
The major peaks observed in FTIR spectra and probable functional group for respective peak is shown in Table 8.

Table 8. The peak observed and probable functional group in the fraction

<table>
<thead>
<tr>
<th>Peak observed in FTIR</th>
<th>Probable functional group</th>
</tr>
</thead>
<tbody>
<tr>
<td>3413</td>
<td>O–H stretch</td>
</tr>
<tr>
<td>2924</td>
<td>Aromatics C–H stretch</td>
</tr>
<tr>
<td>1019</td>
<td>C–O stretch</td>
</tr>
<tr>
<td>1607.90</td>
<td>C–C stretch (in aromatic ring)</td>
</tr>
<tr>
<td>770.41</td>
<td>Para distribution</td>
</tr>
</tbody>
</table>

6.4.3 \textbf{H}^1 \textbf{NMR analysis}

The H$^1$ NMR analysis of the compound was preformed. The spectrum is shown in Figure 10.
Figure 10. H\textsuperscript{1} NMR analysis of fraction 55:45 (sub fraction 2)

The major shifts observed in H\textsuperscript{1} NMR analysis with possible positions of ‘H’ atoms are shown in Table 9.

Table 9. Major shifts with probable positions and numbers of ‘H’ atoms.

<table>
<thead>
<tr>
<th>Shift</th>
<th>‘H’ numbering in figure</th>
<th>Total H at a shift</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.228</td>
<td>H 10a, 10b, 10c and 11 a, 11b, 11c</td>
<td>6</td>
</tr>
<tr>
<td>2.914</td>
<td>alcoholic group H (8a)</td>
<td>1</td>
</tr>
<tr>
<td>4.648</td>
<td>H7a and 7b</td>
<td>2</td>
</tr>
<tr>
<td>7.325</td>
<td>H3a and H5a</td>
<td>2</td>
</tr>
</tbody>
</table>
The $^1H$ NMR of the compound matches with the reported NMR of standard cuminic alcohol. Hence, it was conformed that the compound is cuminic alcohol.

From the structural elucidation by GC-MS, FTIR and $^1H$ NMR it was predicted that the isolated insulin stimulatory compounds present in pet ether fraction of *Cuminum cyminum* are cuminaldehyde and cuminic alcohol. Cuminaldehyde (Syn. cuminal) and cuminic alcohol (syn. cuminol; cuminyl alcohol; p-cymen-7-ol; (4-propan-2-ylphenyl) methanol etc.) are as below (Figure 11).

Figure 11: Structures of cuminaldehyde and cuminic alcohol are as below
7  **In vitro** insulin secretion studies

After isolation of compounds from the pet ether fraction, the two isolated compounds showing maximum insulin secretagogue activity were identified as cuminaldehyde and cuminic alcohol. The detailed effect of these compounds was studied further on isolated pancreatic islets to elucidate probable mechanism of insulin secretion.

7.1  **Dose dependent effect of cuminaldehyde and cuminic alcohol on insulin secretion**

To evaluate dose dependent effect and to know maximum concentration showing optimum stimulatory action, islets were treated with both cuminaldehyde and cuminic alcohol using varying concentration in presence of stimulatory glucose concentration (11.8 mM). The results are shown in Figure 12.

Figure 12. Dose dependent effect of cuminaldehyde (cuminal) and cuminic alcohol (cuminol) on insulin secretion.

![Insulin secretion induced by 11.8 mM glucose was considered as a negative control while in presence of glibenclamide (10 µg/ml of reaction mixture) was considered as a positive control. Insulin stimulatory effect of cuminaldehyde and cuminic alcohol (at concentration of 6.25, 12.5, 25 and 50 µg/ml) was evaluated in presence of 11.8 mM glucose. Results are mean ± S.D.; n=6. *p<0.005 significant from 11.8 mM glucose control.](image-url)
Four concentrations of each component (6.25, 12.5, 25 and 50 µg/ml reaction mixture) in presence of 11.8 mM glucose were used. Insulin secretion in presence of 11.8 mM glucose was considered as negative control while 10 µg/ml of glibenclamide (a commercially used sulfonylurea) in presence of 11.8 mM glucose was used as positive control. Insulin secretion in each test was expressed in terms of µIU/10 islets/60 min. Glibenclamide strongly stimulates insulin which was 2.96 fold greater than 11.8 mM glucose control. Both cuminaldehyde and cuminic alcohol showed dose dependent insulin secretion. At each concentration, the insulin stimulatory activity of cuminic alcohol was more prominent in comparison to cuminaldehyde. At a concentration of 25 µg/ml reaction mixture, cuminaldehyde showed 3.29 folds while cuminic alcohol showed 4.05 folds higher insulin secretion than the 11.8 mM glucose. No further stimulatory effect was observed at higher concentration (i.e. 50 µg/ml) with cuminaldehyde and cuminic alcohol in the above mentioned test conditions. Hence, a concentration of 25 µg/ml reaction mixture was used for further studies.

Though both the compounds showed dose dependent secretagogue activity, the action of cuminic alcohol was more prominent in comparison to cuminaldehyde. The viability of islets after the experiment was assessed by MTT conversion. The viability of islets in 11.8 mM glucose stimulated group was considered as 100%. In each remained test groups, the viability was more than 95%. The concentration of 25 µg/ml reaction mixture of both the compounds showing optimum insulin secretion was used in further experiments.

Insulin secretion from the β-cell is tightly regulated by the extracellular glucose concentration. The metabolism of glucose causes an increase in the intracellular ATP/ADP ratio, which closes K⁺-ATP channels leading to events as depolarization of β-cell membranes, activation of voltage-gated Ca²⁺ channels, Ca²⁺ influx, and an increase in the free cytosolic Ca²⁺ concentration. The increased [Ca²⁺]i stimulates a cascade of events that culminate in the exocytosis of insulin. The extent of this response depends on the extracellular glucose concentration. The sulfonylureas are the commercially used drugs in treatment of diabetes mellitus. A sulfonylurea drug directly bind and inhibits the K⁺-ATP channel, thereby depolarizes the beta cell membrane and stimulating Ca²⁺ influx
which ultimately leads to insulin secretion (Ashcroft and Gribble 1999). In contrast to glibenclamide, the non-sulfonylurea insulin secretagogue like repaglinide did not stimulate insulin secretion in absence of glucose (Fuhlendorff et al. 1998). Hence, there is need to determine whether the insulin stimulatory activity of cuminaldehyde and cuminic alcohol is dependent or independent of the extracellular glucose.

### 7.2 Glucose dependent insulin secretion studies

The effect of increasing glucose concentration on insulin stimulatory activity of cuminaldehyde and cuminic alcohol (a concentration of 25 µg/ml reaction mixture) was demonstrated in Figure 13.

Figure 13. Effect of dose dependent glucose concentration on insulin secretagogue action of cuminaldehyde (cuminal) and cuminic alcohol (cuminol).

![Figure 13](image)

Figure 13. Insulin secretion in presence of 2.8, 5.6 and 11.8 mM glucose was considered as control. Results are mean ± S.D.; n=6. *p<0.01 and **p<0.001 significant from 2.8 mM glucose control.
In control group, insulin secretion increases as with the increase in extracellular glucose concentration. Both cuminaldehyde and cuminic alcohol were not able to stimulate the insulin secretion at 2.8 mM glucose concentration. At 5.6 mM glucose concentration, there was increased insulin stimulation in presence of both cuminaldehyde (54.53 µIU) and cuminic alcohol (58.55 µIU) as compared to 5.6 mM glucose control (29.32 µIU). An elevated glucose concentration to 11.8 mM further potentiates the stimulatory action of cuminaldehyde and cuminic alcohol. Cuminaldehyde showed 3.10 folds while cuminic alcohol showed 3.85 fold insulin secretion than 11.8 mM glucose control.

7.3 Evaluation of mechanism of insulin secretagogue action

Insulin secretagogues can stimulate insulin secretion in number of ways as (a) K⁺-ATP channel-dependent pathway (depolarization or triggering pathway) (b) K⁺-ATP channel-independent Ca²⁺-dependent pathway and K⁺-ATP channel-independent Ca²⁺-independent pathway. The former one was most extensively studied and characterized (Bratanova-Tochkova et al., 2002). To illustrate probable pathway for insulin secretagogue action of cuminaldehyde and cuminic alcohol, their dependency on K⁺-ATP channel and its dependency on Ca²⁺ channel was assessed.

7.3.1 Role of K⁺-ATP channel in cuminaldehyde and cuminic alcohol mediated insulin secretion

The K⁺-ATP channel plays pivotal role in insulin secretion (Ashcroft and Gribble, 1999). Diazoxide is best known K⁺ATP channel opener which prevents the closure of K⁺ATP channel and inhibits the insulin secretion (Henquin et al., 1982; Dunne and Petersen, 1991). The insulin stimulatory effect of glucose and sulphonylureas was reduced by diazoxide. The effect of diazoxide (an established opener of K⁺-ATP channel) on insulin stimulatory activity of cuminaldehyde and cuminic alcohol was shown in figure 14. Both the compounds are used at a concentration 25 µl/ml of reaction mixture.
Figure 14. Effect of diazoxide on insulin stimulatory activity of cuminaldehyde and cuminic alcohol

Results are mean ± S.D.; n=6. *p<0.05 and **p<0.005 significant from 11.8 mM glucose control.

In present study, the insulin stimulation due to elevated glucose was suppressed by presence of diazoxide (300 µM) and insulin secretion dropped from 39.51 to 21.57 µIU. However, the diazoxide does not completely abolish the insulin stimulation at high glucose concentration. This may be due to the augmentation effect of the K⁺-ATP channel-independent pathway of glucose signaling on the Ca²⁺-induced release (Sato et al. 1992; Gembal et al. 1992). The cuminaldehyde or cuminic alcohol was not able to stimulate further insulin secretion in presence of diazoxide. Hence it can be postulated that the K⁺-ATP channel plays vital role in cuminaldehyde and cuminic alcohol mediated insulin secretion.

7.3.2 Effect of artificial membrane depolarization on insulin stimulatory activity of cuminaldehyde and cuminic alcohol

High concentrations of extracellular KCl directly depolarizes the cell membrane which induces an activation of L-type Ca²⁺ channels and a rapid increase in [Ca²⁺].
(Yaekura et al., 1996; Geng et al. 2007). Hence, the depolarizing concentration of KCl which acts as a non-nutrient secretagogue stimulates insulin secretion through K⁺-ATP channel independent and ultimately Ca²⁺ channel mediated signaling (Yada et al., 1999). Any component acting independent of K⁺-ATP channel should further stimulate insulin as compared to the control.

Figure 15. Effect of depolarizing concentration of KCl on insulin stimulatory activity of cuminaldehyde (cuminal) and cuminic alcohol (cuminol)

![Figure 15](image)

Figure 15. Results are mean ± S.D.; n=6. **p<0.0005 significant from 11.8 mM glucose control.

In present study, challenge of 30 mM KCl along with 11.8 mM glucose stimulates the insulin secretion almost 2.5 times as compared to glucose control alone. The cuminaldehyde or cuminic acolhol did not augment insulin secretion from depolarized β-cells exposed to 30 mM KCl. The data further supports the initial results obtained with diazoxide.

Hence, from the experiments with diazoxide (an established opener of K⁺-ATP channel) and depolarizing concentration of KCl, it can be concluded that K⁺-ATP channel and Ca²⁺ influx through the voltage gated L-type Ca²⁺ channel plays important role in the insulin secretagogue action of cuminaldehyde (cuminal) and cuminic alcohol (cuminol).
7.3.3 Role of extracellular \( \text{Ca}^{2+} \) in cuminaldehyde and cuminic alcohol mediated insulin secretion

Increase in \([\text{Ca}^{2+}]\), whether by influx of extracellular \( \text{Ca}^{2+} \) or by release of \( \text{Ca}^{2+} \) from intracellular stores plays important role in insulin secretion (Henquin, 2004; Tengholm and Gylfe 2009). To evaluate the role of extracellular \( \text{Ca}^{2+} \) in insulin secretion by cuminaldehyde (cuminal) and cuminic alcohol (cuminol), islets were treated with 11.8 mM glucose in presence of 20 µM nifedipine (the L-type \( \text{Ca}^{2+} \) channel blocker) with or without cuminaldehyde or cuminic alcohol (Figure 16). Nifedipine is L-type \( \text{Ca}^{2+} \) channel blocker which prevents the influx of extracellular \( \text{Ca}^{2+} \) and hence reduces the insulin secretion (Giugliano 1980).

Figure 16. Effect of nifedipine (the L-type \( \text{Ca}^{2+} \) channel blocker) on insulin stimulatory activity of cuminaldehyde (cuminal) and cuminic alcohol (cuminol)

![Figure 16](image)

Figure 16. Results are mean ± S.D.; n=6. *\( p<0.005 \) and **\( p<0.001 \) significant from 11.8 mM glucose control.

In present study, nifidipine abolishes the insulin secretion and drops it from 39.51 µIU to 21.04 µIU. Nifedipine reduces however not completely abolishes the insulin stimulation by 11.8 glucose challenge. The presence of cuminaldehyde or cuminic alcohol (both at concentration 25 µl/ml reaction mixture) was not able to overcome the inhibition mediated by nifedipine. Hence, it further strengthen the importance of \( \text{Ca}^{2+} \) influx through the voltage gated L-type \( \text{Ca}^{2+} \) channel in insulin secretagogue action of cuminaldehyde and cuminic alcohol.
7.3.4 Effect of 3-Isobutyl-1-methylxanthine on insulin secretagogue action

Besides the use of depolarizing secretagogue KCl, the stimulatory effect of cuminaldehyde (cuminal) and cuminic alcohol (cuminol) was assessed in presence of non-depolarizing insulin secretagogue such as 3-Isobutyl-1-methylxanthine (IBMX). IBMX is an inhibitor of phosphodiesterase that elevates the intracellular cAMP levels and activating the PKA mediated signal transduction pathway, increases the release of free Ca\(^{2+}\) and consequently the potentiates glucose-induced insulin secretion (Sharp 1979; Siegel et al. 1980). IBMX significantly increased the glucose stimulated insulin secretion of islets of all rat strains (Groot 2004). The effect of 3-Isobutyl-1-methylxanthine (IBMX; phosphodiesterase inhibitor) on insulin stimulatory activity of cuminaldehyde and cuminic alcohol is shown in Figure 17.

![Figure 17. Effect of IBMX on insulin stimulatory activity of cuminaldehyde and cuminic alcohol](image)

Figure 17. Results are mean ± S.D.; n=6. **p<0.001 significant from 5.6 mM glucose control.

In current studies IBMX potentiates the 5.6 mM glucose stimulated insulin secretion almost 2.18 times higher than the 5.6 mM glucose control. Both the cuminaldehyde and cuminic alcohol augment the stimulatory effect of IBMX. Insulin secretion due to the presence of cuminaldehyde and cuminic alcohol was 113.58 µIU and 115.30 µIU respectively as compared to IBMX control which shows 63.82 µIU insulin releases. Hence, it can be concluded that cuminaldehyde and cuminic alcohol shows additive effect to IBMX.
7.3.5 Effect of cuminaldehyde and cuminic alcohol on functionality of islets through L-alanine metabolism

The amino acid L-alanine promotes the insulin secretion through changes in Na\(^+\) transport (Yada 1994). Alanine is a gluconeogenic amino acid converted to pyruvate and enters the TCA cycle. Therefore, the functionality of islet is conformed through process of operative glycolysis and TCA cycle. Deleterious effect of unknown compound can be evaluated through the impaired metabolism of L-alanine by β-cells. To evaluate the effect cuminaldehyde or cuminic alcohol on islet functionality through amino acid metabolism, islets were incubated in presence of cuminaldehyde (cuminal) or cuminic alcohol (cuminol) 10 min prior to addition of 10 mM L-alanine at basal glucose concentration (Gray and Flatt 1999). The results are demonstrated in Figure 18.

Figure 18. Effect of L-alanine on insulin stimulatory activity of cuminaldehyde (cuminal) and cuminic alcohol (cuminol)

![Figure 18](image)

Figure 18. Results are mean ± S.D.; n=6. **p<0.001 significant from 2.8 mM glucose control.

In presence of 2.8 mM glucose L-alanine (10 mM) stimulates the insulin secretion almost 2.42 times than 2.8 mM glucose control. Both the cuminaldehyde and cuminic alcohol does not abolish the stimulatory effect of L-alanine. The insulin secretion in presence of cuminaldehyde was 50.74 µIU and with cuminic alcohol was
48.47 µIU as compared to 30.22 µIU insulin secretion of L-alanine control alone. Hence, both the cuminaldehyde and cuminic alcohol show an additive effect as alanine would attribute to increase in ATP and the two components contribute to K⁺-ATP channel closure hence augmenting alanine activity.

8. **Viability of islets by MTT conversion**

Besides the functionality, the viability of islets of islets after each test was assessed by the MTT conversion test. MTT (3-[4,5-dimethylthiazol-2-y1]-2, 5-diphenyltetrazolium bromide) is a yellow water soluble tetrazolium salt which is converted to water-insoluble purple formazan by living cells. Hence, the amount of formazan formed serves as the number of living cells in the sample. The viability of islets after each test was confirmed and expressed in terms of percent viability (% viability) relative to control group cells which were considered as 100% viable. The viability cell in each treatment group was found to be higher than 90%. Hence, it was confirmed that there is no toxic or detrimental cellular effects are responsible insulin stimulatory activity of both cuminaldehyde and cuminic alcohol.

In bioactivity guided isolation of insulin stimulatory compounds from pet ether fraction of cumin, we serendipitously found inhibition of glucose stimulated insulin secretion by 2nd fraction (gradient Hexane: Ethyl acetate 100:0). Hence, attempt was made to isolate and identify the insulin inhibitory compound from 2nd fraction.

9. **Inhibitory effect of unknown compound isolated from pet ether fraction on insulin secretion.**

For the isolation of insulin stimulation inhibitory compound, the 100:0 fraction was loaded on a column and successively eluted with stepwise gradient 0f Hexane: ethyl acetate system (100:0, 95:5, 90:10 and 85:15). Three subfractions were collected for each gradient. Hence total 12 fractions were collected for this step. The 2nd fraction (gradient Hexane: Ethyl acetate 100:0) showed the maximum insulin inhibitory activity. The fraction was further proceeded for GC-MS analysis.
9.1 GC-MS of insulin secretion inhibitory compound from fraction 100:0 (Sub fraction 2)

The main chromatogram of GC-MS analysis was shown in Figure 19. In GC-MS, only a single major peak was observed at a retention time (RT) of 27.199.

Figure 19. GC-MS of insulin secretion inhibitory compound from fraction 100:0 (sub fraction 2)

The mass fragmentation of the compound is shown in Figure 20. The fragmentation pattern did not match with to any compound present in the NIST library. The mass of this unknown compound was postulated to be 493.

Figure 20. Mass fragmentation pattern of compound at RT 27.199

The dose dependent insulin secretion inhibitory activity of unknown compound (fraction 2nd of gradient Hexane: Ethyl acetate 100:0) is shown in Figure 21.
Figure 21. Dose dependent insulin secretion inhibitory action of fraction 2\textsuperscript{nd}.

At a concentration of 1.25, 2.5 and 5 µg/ml, the compound showed dose dependent decrease in insulin secretion. Maximum inhibition was observed at 5 µg/ml concentration which drops the insulin secretion to 16.57 µIU as compared to 11.8 mM glucose control (41.91 µIU). At an increased concentration (10 µg/ml), no further inhibition was observed. The viability of islets after the experiment was assessed by MTT conversion. The viability of islets in control group was considered as 100%. The viability of islets in presence of unknown insulin secretion inhibitory compound. The % viability at a concentration of 1.25, 2.5, 5 and 10 µg/ml was found to be 114, 112, 124 and 117% respectively as compared to control group. From the data it is clear that though the compound inhibits but not totally abolishes the glucose stimulated insulin secretion.

Similar to the above mentioned unknown compound, there are several natural compounds such as peptides e.g. galanin (Ahren \textit{et al.} 1988; Sharp \textit{et al.} 1989), leptin (Fehmann \textit{et al.} 1997; Zhao \textit{et al.} 1998), ghrelin (Reimer \textit{et al.} 2003) or synthetic...
compounds such as 3, 3-Diamino-sulfonylacrylonitriles (Tagmose et al. 2003) and Y-26763 (Cosgrove et al. 2004) that are reported to inhibit the insulin secretion. Only few plant natural compounds such as resveratrol were reported to inhibit the insulin secretion (Szkudelski 2007). However, this is the first finding of both insulin stimulatory and inhibitory compounds in the same plant in the present study. As like that of insulin stimulatory compounds which are used in diabetes mellitus, the insulin stimulatory compounds are also used in used in disorders such as “hyperinsulinism in infancy” (HI). Hyperinsulinism infancy is also known as hyperinsulinemic-hypoglycemia, including persistent hyperinsulinemic hypoglycemia of infancy (PHHI), congenital hyperinsulinism in infancy (CHI). There is an inappropriate or uncontrolled insulin release for the level of glycemia.

Acute treatment regimens for “hyperinsulinism in infancy” (HI) are targeted at either the inhibition of insulin release or glucagon to promote mobilization of hepatic glucose. For early-onset HI the drugs of preference are those that can be administered orally, followed by agents that are delivered intravenously or subcutaneously. Numbers of drugs such as pinacidil, diazoxide, nicorandil, nifedipine etc. are used to treatment of HI. However, none of the agents that are currently used is specific for the inhibition of insulin release (Dunne 2004). Among these drugs, diazoxide (given within the range 10–20 mg/kg/day) is the most widely used in medical treatment for hyperinsulinism, since the drug is an effective inhibitor of insulin secretion and can be administered orally. However, despite the widespread use of diazoxide, the agent is poorly tolerated by a number of patients due mainly to adverse side effects. Particular note are those complications related to nausea and vomiting or sodium and water retention, which can lead to further problems in patients with congestive heart defects or poor cardiac reserve, hyperuricemia, hypotension, hypertrichosis and on occasions blood dyscrasias, leucopenia, and thrombocytopenia. In addition, diazoxide therapy is associated with decreased serum immunoglobulin G levels, and this can lead to problems associated with infection. Long-term use also reported with hyperosmolar non-ketotic comas (Touati et al. 1998; Aynsley-Green et al. 2000). The availability of more potent and more selective diazoxide analogs for the inhibition of insulin release would seem to be a logical progression of the K⁺-ATP channel-based treatment option.

The insulin secretion inhibitory compound found in 2nd fraction was may prove as a new compound in treatment of hyperinsulinism. It was observed that the compound
showed dose dependent inhibition of glucose stimulated insulin secretion. Further, though the compound inhibits the insulin secretion, it does not completely abolish the glucose stimulated insulin secretion at even more than the optimum concentration of insulin secretion inhibitory compound. Complete inhibition of insulin secretion can lead to side effect such as hyperglycemic shock. Hence, the unidentified compound from the 2\textsuperscript{nd} fraction (gradient Hexane: Ethyl acetate 100:0) can be ideally used in treatment of hyperinsulinism.

The presence of insulin inhibitory compound can be greatly useful in treatment of diabetes mellitus. The overstimulation of β-cells by excessive glucose and use of sulfonylureas results in negative consequences on β-cells such as apoptosis and reduction in their mass (Donath \textit{et al.} 2005). Hence, rest for β-cells was beneficial in prevention of diabetes. In contrast to sulfonylureas, K\textsuperscript{+}-ATP channels channel openers may exert protective effects on β-cells (Maedler \textit{et al.} 2004, Ritzel \textit{et al.} 2004, Kullin \textit{et al} 2000). In 1976 for the first time, Greenwood \textit{et al}. reported an improvement in insulin secretion after administration of diazoxide to diabetic subjects for 7 days (Greenwood \textit{et al}. 1976). Similar protective effects were observed more recently in patients classified with type 1 and type 2 diabetes (Guldstrand \textit{et al}. 2002). Hence, use of insulin secretion inhibitors in diabetes therapy will be beneficial.

To know whether the insulin secretion inhibitory compound (isolated from fraction 2\textsuperscript{nd} of gradient Hexane: Ethyl acetate 100:0) also inhibits the insulin secretagogue compounds, the islets were incubated in presence of such compounds at elevated glucose concentration and with or without presence of inhibitory compound. Change in the insulin secretagogue activity of insulin secretagogue compounds in presence of insulin secretion inhibitory compound (fraction 2\textsuperscript{nd} of gradient Hexane: Ethyl acetate 100:0) is shown in Figure 22. The insulin secretion in presence of 11.8 mM glucose alone was considered as negative control while that of in presence of inhibitory compound with 11.8 mM glucose was considered as positive control. Insulin secretion in presence of 60:40 pet ether fraction, cuminaldehyde or cuminic alcohol (each with concentration of 12.5 µg/ml) in presence or absence of insulin inhibitory compound (2.5 µg/ml) was evaluated.
Figure 22. Reduced action of insulin secretagogue compounds in presence of insulin secretion inhibitory compound isolated from fraction 2nd.

Isolated islets incubated with elevated glucose (i.e. 11.8 mM) showed 44.72 μIU insulin secretion while in presence of insulin inhibitory compound the insulin secretion was decreased to 29.70 μIU. At the elevated glucose challenge, the previously mentioned insulin secretagogue fraction and its isolated compounds viz. fraction 60:40, cuminaldehyde and cuminic alcohol showed 65.34, 73.22 and 79.05 μIU insulin secretion respectively. Insulin secretion due to fraction 60:40, cuminaldehyde and cuminic alcohol was decreased by 32.90%, 26.27%, and 30.55% respectively when incubated in presence of insulin secretion inhibitory compound.

The viability of islets after the experiment was assessed by MTT conversion. Viability of islets in control group (11.8 mM glucose) was considered as 100%. The viability of islets was observed to increase in presence of unknown insulin secretion inhibitory compound i.e. 122%. The viability of cells in presence of fraction 60:40, cuminaldehyde and cuminic alcohol was observed to be 96.44%, 98.28% and 96.93% respectively. A better viability was observed when these compounds were incubated in
presence of inhibitory compound. The viability of cells in presence of fraction 60:40, cuminaldehyde and cuminic alcohol along with inhibitory compound was observed to be 102.75%, 106.33% and 103.69% respectively.

The data demonstrates that the insulin secretion inhibitory compound isolated from pet ether fraction of Cuminum cyminum improves the viability of islets in presence of elevated glucose and insulin secretagogue compounds.

The presence of insulin inhibitory compound can be greatly useful in treatment of diabetes mellitus. The use of inhibitor prevents the overstimulation of β-cells by excessive glucose and use of sulfonylureas which ultimately prevents the negative consequences such as apoptosis and reduction in β-cells mass. Hence, rest for β-cells was beneficial in prevention of diabetes.

10. Conclusion

In present study, the antihyperglycemic activity of petroleum ether, chloroform, dichloromethane and aqueous extracts isolated form Cuminum cyminum steam distillate was evaluated through oral glucose tolerance test (OGTT). Among the four fractions, pet ether fraction showed most prominent antihyperglycemic action. Hence, the antihyperglycemic activity was supposed due to pet ether fraction of Cuminum cyminum seeds distillate.

The activity of pet ether fraction was further confirmed in prolonged studies. In pet ether treated rats, both 5 mg/kg and 10 mg/kg body weight doses showed dose dependent lowering of fasting blood glucose levels, improved GHb values and serum insulin levels. Treatment with pet ether fraction further improves the lipid profile in dose dependent manner and confirmed the antihyperlipidemic effect of cumin seeds.

In vitro insulin secretion studies, pet ether fraction showed maximum insulin secretion as compared to other fractions. Hence, pet ether fraction was carried further for isolation and characterization of compounds. The ultimate purification and structural elucidation revealed that the most active insulin secretagogue compounds were cuminaldehyde and cuminic alcohol. Further study was performed to elucidate probable
mechanism for insulin secretagogue of cuminaldehyde and cuminic alcohol. Both cuminaldehyde and cuminic alcohol showed dose dependent insulin secretion. The stimulus due to both the compounds was even more pronounced than that of glibenclamide which is a commercially used sulfonylurea. Though both the compounds showed dose dependent stimulatory activity, the action of cuminic alcohol was more prominent in comparison to cuminaldehyde. The $K^+$-ATP channel and $Ca^{2+}$ influx through the voltage gated L-type $Ca^{2+}$ channel plays important role in the insulin secretagogue action of cuminaldehyde and cuminic alcohol.

It is very important to note that the same plant showed the presence of both an insulin secretagogue as well as an inhibitor of insulin secretion. It is also to be noted that though cuminaldehyde and cuminic alcohol showed higher insulin secretion at 25 $\mu$g/ml concentrations as compared to glibenclamide at 10 $\mu$g/ml. Further increase in insulin secretion beyond the 50 $\mu$g/ml was not a dose dependent. Likewise, insulin inhibitory effect was also optimum at 5 $\mu$g/mg it did not enhance at 10 $\mu$g/ml. Both these properties are very important in proper maintenance of insulin secretion. In conclusion, the activator and inhibitor will neither allow excess insulin secretion nor totally abolish it. This will permit an excellent control of glycemia without fear of hypoglycemic shock. Moreover, the inhibitor also clearly demonstrated a $\beta$-cell protective action through showing optimum viability in the MTT assay. This would prevent progression of $\beta$-cell loss and thereby overcome the major drawback of insulin secretagogues currently available is causing $\beta$-cell damage leading to progression of the diabetes to type I like situation and necessitating insulin therapy as well as progression of the secondary complications. This combined effect is very obvious in the long term studies. Although, a single dose OGTT reveled better lowering of blood glucose by glibenclamide (280 mg/dl) as compared to pet ether fraction (324 mg/dl). Prolonged treatment for 45 days has shown a much better lowering of glucose content as well as better glycated Hb values as compared to glibenclamide proving that the total pet ether fraction has the capacity to maintenance a better glycemic control than the standard drug glibenclamide. The serum insulin levels were also improved implying the better health of the pancreatic $\beta$-cells. These findings therefore have immense implications in evolving a better therapy in treatment of diabetes mellitus.
The significance of the present study lies in the part that cuminaldehyde and cuminic alcohol as components of *Cuminum cyminum* are in use for hundreds of years. The benefits of cuminaldehyde and cuminic alcohol are follows:

i) Being a food component and palatable, both are easy to administer

ii) No toxicity reports are available.

iii) The compounds can easily manufacture artificially hence are cost effective.

iv) Both the compounds show good insulin secretagogue action that is comparable even better than glibenclamide.

v) The insulin secretagogue activity of cuminaldehyde and cuminic alcohol is found to be glucose dependent therefore there is no possibility of hypoglycemic shock.

vi) Cuminaldehyde was also reported to possess significant aldose reductase inhibitory and alpha-glucosidase inhibitory action thereby can prevent progression of secondary complications (Lee 2005). We have observed that after prolonged treatment with pet ether fraction the animals showed no evidence of cataract or nephrotoxicity.

vii) Moreover the simultaneous presence of a secretagogue and an inhibitor suggests an inbuilt protection that can avoid overstimulation of beta cells and help in their protection.

Hence, in conclusion *Cuminum cyminum* pet ether fraction is a better prospective option in treatment of diabetes mellitus in comparison to the currently available insulin secretagogues.

11. References


Chapter III


