CHAPTER-V

DISCUSSION

5.1 Sequential extraction of *E.operculata* leaves

Plants are used in the treatment of human diseases all the time as it contains a variety of natural product i.e. secondary metabolites. Although the impacts of secondary metabolites are certain, their identification and isolation is still a tedious job due to their complexity. Therefore various solvents were evaluated to understand the solubility of phytoconstituents. And suitable extraction of bioactive compounds was carried out by successive extraction with solvents of different polarities. As the solvents, nature, age of the plant materials and the conditions used for extraction greatly influence the yield and bioactivities of the extracts (Weisiger and Fridovich, 1973).

In the current study, a sequential extraction involving solvents of increasing polarity (petroleum ether, ethyl acetate, ethanol, methanol and water) was used to extract bioactive compounds from leaves of *E.operculata*. A sequential extraction procedure was chosen mainly because the nature, polarity, and hence the solubility of the bioactive compounds present in the leaves of *E.operculata* were unknown. From all the solvent used, the highest yield was obtained from the solvent of aqueous and ethanol and the least from ethyl acetate.

5.2 In vitro screening of *E.operculata* leaves extracts for α-amylase and α-glucosidase enzyme inhibitory activities.

In-vitro studies have a pivotal role in the initial screening of medicinal plants and as a preliminary step in human or other clinical trials. α-amylase and α-glucosidase are the enzymes that plays an important role in carbohydrate digestion and glucose adsorption leading to high blood glucose level. α-amylase catalyses digestion of polysaccharides (starch) into various oligosaccharides and disaccharides. α-Glucosidase enzymes in the intestinal lumen and in the brush border membrane further hydrolyses disaccharides produced by α-amylase to glucose and other monosaccharide's before they can be absorbed readily in the small intestine (Puls *et al*., 1997). One therapeutic approach to decrease postprandial hyperglycemia for the treatment of diabetes is to

suppress the production and/or absorption of glucose from the gastrointestinal tract through inhibition of either α-amylase or α-glucosidase enzymes (Cheng and Funtus, 2005; Kim *et al*., 2005; Matsui *et al*., 2007). As suppression of the activity of such digestive enzymes would delay the degradation of starch and oligosaccharides, which would in turn cause a decrease in the absorption of glucose and consequently the reduction of postprandial blood glucose level elevation. Furthermore some competitive inhibitors of α-amylase and α-glucosidase such as acarbose and miglitol are currently used to suppress postprandial blood glucose level in diabetic patients (Kim *et al*., 2005). Acarbose is complex oligosaccharides that delay the digestion of carbohydrates. It inhibits the action of pancreatic amylase in breakdown of starch.

5.2.1 Alpha amylase inhibition

High intake of carbohydrate and sucrose rich food is one of the main causes of non-insulin diabetes mellitus. Potent inhibitors of mammalian α-amylase found in medicinal plants involved in digestion of carbohydrates can significantly decrease the post prandial increase of blood glucose. *E.operculata* leave extracts (petroleum ether, ethyl acetate, ethanol, methanol and water) investigated in the current study demonstrated dose dependent α-amylase inhibitory activity. This observation suggests that porcine pancreatic α-amylase is inhibited by both polar and nonpolar components of the leave extract of *E.operculata*. Ethanol extract showed better inhibitor of porcine pancreatic α-amylase than the reference drug acarbose with IC_{50} 28.32 µg/ml, an indication that this extract of the leave of *E.operculata* may be more beneficial in preventing postprandial hyperglycemia than acarbose. Furthermore, the mode of inhibition of acarbose towards α –amylase has been reported to be mixed non-competitive (Youn, 2004). In the current study, the mode of inhibition of *E.operculata* ethanol extract was found to be competitive suggesting that some of the α – amylase inhibitory components in extract may be structural analogs of the substrate of α-amylase or may be due to the changes of the conformation of carbohydrate binding regions of α – amylase, that catalyze hydrolysis of the internal α-1,4 glucosidic linkages in starch and other related polysaccharides. (Kim *et al*., 2000; Jiju *et al*., 2013).

5.2.2 Alpha glucosidase inhibition

Inhibition of the activity of carbohydrate-hydrolyzing enzymes plays an important role in the prevention and treatment of diabetes. α-glucosidase inhibiting activity of *E.operculata* extract was determined by measuring the release of P-nitrophenol from P-nitrophenyl–α-D-glucopyranose. *E.operculata* leave extracts (petroleum ether, ethyl acetate, ethanol, methanol and water) investigated in the current study demonstrated dose dependent yeast α-glucosidase inhibitory activity. This observation suggests that yeast α-gucosidase is inhibited by both polar and nonpolar components of the leave extract of *E.operculata*. Of all the extract tested, aqueous extract showed highest inhibition activity with IC50 38.61µg/ml followed by ethanol extract, IC50 54.25 µg/ml but lesser than the reference drug acarbose with IC50 30.57µg/ml. Previous studies also showed inhibitory activity of *E.operculata* bud extract against the α-glucosidase, rat-intestinal maltase and sucrase activities (Mai and Chuyen, 2007). Preliminary phytochemical screening of E.o ethanol and aqueous extract suggested the presence of alkaloids, phenols, flavonoids and terpernoids. Taking in to consideration the results of other similar *in vitro* studies, which have attributed the α-glucosidase inhibitory activity of some plant material extracts to the presence of flavonoids, polyphenols as well as their glycoside derivatives (Jung *et al*, 2006), it is reasonable to suggest that the α-glucosidase inhibitory effect of *E.o* extract observed in the current study could also be due to the presence of less polar flavonoids and/or other phenolic compounds. Thereby considering *E.operculata* as a promising material for preventing and treating diabetes.

5.3 In vivo antidiabetic effects of *E.operculata* extract

In order to determine whether the observed in vitro effects of *E.operculata* leaves extracts are applicable to in vivo and to study the possible hypoglycemic mechanism of action of *E.operculata* leaves, the effect of *E.operculata* ethanol and aqueous extract on postprandial glucose level after an oral administration of glucose as well as the long term treatment effects on fasting blood glucose levels, plasma triglycerides, serum lipid profile and cholesterol and their impact on histology were studied in normal and STZ induced diabetic mice. The ethanol and aqueous extract were chosen because of their potent α-amylase and α-glucosidase activity.

5.3.1 Induction of diabetes

For testing antidiabetic potential of plants, STZ induced hyperglycemia in rodents is considered to be a good preliminary screening model and is widely used because of its simple, inexpensive and available method (Ivorra *et al* .,1989). In the present study diabetes is induced to mice by giving freshly prepared multiple low dosage of STZ, 45mg/kg b.w daily for 5 consecutive days. STZ is a synthetic nitrosoureido glucopyranose derivative isolated from fermentations of *Streptomyces acromogenes*. STZ induced diabetes by selectively destroying the insulin producing β cells by inducing necrosis. The selective β cells toxicity in STZ is related to the glucose moiety in its chemical structure, which enables STZ to enter the cell via the low affinity glucose transporter GLUT2 in the plasma membrane and causes alkylation of DNA thereby damaging the pancreatic β cells leading to high blood glucose level (Elsner *et al*., 2007; Njomen *et al*., 2009).

5.3.2 Effect on postprandial glucose level after glucose loading

Administration of *E.operculata* ethanol and aqueous extract significantly suppressed rise in postprandial blood glucose level in both normal and STZ induced diabetic mice. The maximum rise in blood glucose occurred 30 minutes after the oral glucose challenged in all the groups. After 30 minutes the groups treated with *E.operculata* extract and glibenclamide significantly suppressed ($p<0.05$) rise in postprandial blood glucose level. The decline in the level of blood glucose reached its maximum after 120 minutes ($p<0.01$). In normal and diabetic control group the fall of blood glucose level is not significant while comparing to the rest of the groups. In this study the in vitro study reveals the inhibitory activities of *E.operculata* extract in concomitant to acarbose. Since acarbose is known to exert its effect on postprandial hyperglycemia through inhibition of the intestinal brush border glucosidase (Kim *et al*.,2005), it can be speculated from the result of the current study that the ethanol and aqueous extract of *E.operculata* leave may also exert its postprandial glucose lowering effect through inhibition of intestinal brush border α-glucosidases or probably by increasing peripheral utilization of glucose (Whitton and Hems, 1975). On the basis of these finding it is reasonable to conclude that the observed in vitro inhibitory effects of *E.operculata* leave extracts on α-glucosidase enzymes are also applicable in vivo. The

results of the present study are similar to the one in which *Eriobotrya japonica* seeds effectively improve glucose tolerance in the KK-Ay mice (Tanaka *et al.*, 2008). Similarly the glucose tolerance report of Jie Yang *et al*., (2009) states that, treatment with *Potentilla discolour* extract in alloxan-induced diabetic mice could significantly increase the rate of glucose disposal.

5.3.3 In vivo long term treatment effects of *E.operculata* ethanol and aqueous extract

5.3.3.1 Effect of *E.operculata* on STZ induced diabetic mice

STZ induced diabetes by damaging the insulin secreting cells of the pancreas leading to hypoinsulimenia and hyperglycemia (Njomen *et al*., 2009). In agreement with these known effect of STZ, the blood glucose levels of untreated STZ induced diabetic mice were significantly increased throughout the study compared to those of normal control mice. Also the plasma insulin levels of diabetic control mice was significantly decreased compared to that of normal control mice at the end of the experiment. Daily administration of *E.operculata* ethanol and aqueous extract to diabetic mice significantly reduced their fasting blood glucose level compared to normal diabetic control at the end of the experiment. Of all the groups treated both with extract and glibenclamide, the group treated with 250mg/kg b.w of ethanol extract shows maximum significance throughout the experiment in comparing with diabetic control. The antihyperglycemic poeperties of *E.operculata* were previously reported from the flower bud aqueous extract. Also it is worth to recall the report of Noor *et al*., (2008), who postulated two possible mechanisms for antidiabetic activity of *Aloe vera* in streptozotocin-induced diabetic rats. First, by preventing the death of β-cells and second, by permitting the recovery of partially destroyed β-cells. Burcelain *et al*., (1995) reported that the hypoglycemic action of the extract of herbal plants in diabetic rats may be possible through the insulin mimetic action or by other mechanism such as stimulation of glucose uptake by peripheral tissue, inhibition of endogenous glucose production or activation of gluconeogenesis in liver and muscles.

The overall results indicate that treatment with *E.operculata* extract has significant ($p<0.001$) effects on normalization of blood glucose levels. The normalization can be due to the effects on normalizing the pancreatic cells or can also

be due to the effect on sensitizing insulin and insulin receptors in diabetic mice, stimulated glycogenesis in liver, increased glucose utilization by the body or insulin like activity of the compounds.

In concordance with our results, oral administration of aqueous extract of leaves of *G. sylvestre* normalized blood sugar levels of diabetic animals through β–cell regeneration (Shanmugasundaram *et al.*, 1981, 1983, 1988, 1990 a & b). Kamble *et al.*, (1998) demonstrated that the *Coccinia grandis* extract mimics insulin like activity and improved the functional status of enzymes in glycolytic pathway and lypolytic pathway. Aqueous leaf-extract of *Annona squamosa* Linn significantly reduced the levels of blood glucose and increased the activity of plasma insulin and antioxidant enzymes (Kaleem *et al.*, 2006). The methanolic extracts of *Sinularia firma* and *Sinularia erecta* were found to be effective in lowering blood glucose level at the dose of 250mg/kg body weight (Tamrakar *et al*., 2008). These studies supported our results that plant extract treatment would decrease the plasma glucose level.

5.3.3.2 Effect on body weight

Induction of diabetes with STZ is associated with a characteristic loss of body weight, which is probably due to muscle wasting or decreased protein content in muscular tissue by proteolysis (Swanston-Flatt *et al*., 1990; Vats *et al*., 2004). In our study there was a significant weight loss in the vehicle treated diabetic mice, whereas treatment with *E.operculata* extract of leaves showed improvement in their body weight, indicating that the aqueous extract had beneficial effect in preventing loss of body weight of diabetic mice. The probable mechanism of this benefit is due to its effect in controlling muscle wasting i.e.by reversal of antagonism (Whitton and Hems, 1975). The metabolic disturbances were corrected after the plant extract was administered for 21 days. This effect may be due to increased secretion of insulin from the regenerated β-cells of the pancreas and insulin mimetic activity of the compounds

5.3.3.3 Effect on serum lipid profile

Cholesterol and triglycerides are transported in the blood by combinations of lipids and proteins called lipoproteins. HDLs, the so-called “good” or “healthy” cholesterol, are lipoproteins made mostly of protein and little cholesterol. HDLs can

help to clear cholesterol deposits in blood vessels left by another blood component called low-density lipoproteins, or LDLs. According to Ravi *et al*., (2005) abnormalities in lipid profile are one of the most common complications in diabetes mellitus found in 40% of diabetic cases. Diabetes causes an increase in the cholesterol, triglycerides, LDL and VLDL (Soltani *et al*., 2007). The abnormal high concentration of serum lipids in the diabetic subjects is due mainly to the increase in the mobilization of free fatty acids from the peripheral fat depots, since insulin inhibits the hormone-sensitive lipase. Insulin deficiency or insulin resistance may be responsible for dyslipidemia, because insulin has an inhibitory action on HMG-CoA reductase, a key rate-limiting enzyme responsible for the metabolism of cholesterol-rich LDL particles. Acute insulin deficiency initially causes an increase in free fatty acid mobilization from adipose tissue. This results in increased production of cholesterol-rich LDL particle (Balasee *et al*., 1972; Taskimen, 1987; Murali *et al*., 2002). With longer insulin deficiency, the liver converts free fatty acids into ketone bodies (Basso and Havel, 1970; Bainton *et al*., 1992) and reduces the lipoprotein lipase activity resulting in impaired clearance of VLDL and chylomicrons from blood (Bagdade *et al*., 1968; Nikkila *et al*., 1977; Taskimen, 1987; Chakrabarti *et al*., 2003). VLDL, which is a major carrier of plasma triglycerides in blood, becomes rich in cholesterol and acts as a carrier of cholesterol (Mizuguchi, 1968; Miller, 1980; Shanmugasundaram *et al*., 1983).

Insulin inhibits lipolysis with an increase in the uptake of fatty acids into adipose tissue and triglyceride synthesis. HDL is an antiatherogenic lipoprotein. It transports cholesterol from peripheral tissues to the liver and thereby acts as a protective factor against coronary heart disease. The level of HDL-cholesterol, which increased, might be due to the increase in the activity of lecithin cholesterol acyl transferase (LCAT), which may contribute to the regulation of blood lipids (Patil *et al*., 2004). Hyperglycemia produced by STZ exhibited marked increase in serum triglycerides and total cholesterol. Under normal conditions the enzyme lipoprotein lipase hydrolyses triglycerides. Diabetes mellitus results in failure to activate this enzyme thereby causing hypertriglyceridemic. Elevated serum total cholesterol, triglycerides and decreased high density lipoprotein level were observed in diabetic control mice. In contrary diabetic mice treated with *E.operculata* extract at a dose of 250 mg/kg for 21 days significantly ($p<0.01$) reduced total cholesterol, triglycerides,

low density lipoprotein and very low density lipoprotein associated with significant increase in HDL and the same has been obtained from glibenclamide treated group. Normalization of serum lipid profile by *E.operculata* extract and glibenclamide might be due to stimulation of insulin secretion from beta cells or may be due to low activity of cholesterol biosynthesis enzymes and or low level of lipolysis, which are under the control of insulin (Sharma *et al.,* 2003). The observed hypolipidaemia effect may be because of decreased cholesterogenesis and fatty acid synthesis. Significant lowering of total cholesterol and elevation of HDL cholesterol are very desirable biochemical states for prevention of atherosclerosis and ischemic conditions (Tripathi *et al*., 2012). There are reports that other medicinal plants have hypoglycemic and hypolipidemic effects that could prevent or be helpful in reducing the complications of lipid profile seen in some cases of diabetes in which hyperglycemia and hypercholesterolemia coexist (Sharma *et al.,* 2003). The impairment of insulin secretion results in enhanced metabolism of lipids from the adipose tissue to the plasma. A variety of derangements in metabolic and regulatory mechanisms, due to insulin deficiency, are responsible for the observed accumulation of lipids (Rajalingam *et al.,* 1993). Further it has been reported that diabetic rats treated with insulin shows normalized lipid levels (Pathak *et al.*, 1981). Thus, the results indicate that *E.operculata* also may possess insulin like action by virtue of the ability to lower the lipid levels, which increased the activity of lipoprotein lipase and decreased fatty acid synthesis. These results are similar to earlier reports observed with the other plant (Kaleem *et al*., 2006).

5.3.3.4 Histopathological study of mouse pancreas

The islets exposed to toxic chemicals introduced into the environment are known to undergo destruction particularly in respect to their β–cells. Similarly, loss of islet mass is observed in experimental diabetes brought about by chemicals, STZ (Szkudelski, 2001). STZ-diabetes results in degenerative and lytic changes in the islets of Langerhans of the pancreas. The islet is considerably reduced and shrunken, there is destruction of some β-cells with central hyalinization, a few cells show pyknotic nuclei and the number of cells is lower (Chatterjee *et al.,* 1980; Bora *et al.,* 1985, 1989; Shanmugasundaram *et al.,* 1990a; Kavalali *et al.,* 2003). In this study the histological section of pancreas was observed to know the protective effect of *E.operculata*. In normal control group no alterations were observed, the structure of pancreas remained

intact. In diabetic control mice, the islets were less and their shape was destroyed and insulin-producing β-cells were degranulated, degenerated or necroses compared to normal. However in *E.operculata* treated mice there were more islets and they were comparable to normal mice islets, although there were individual differences that show a significant antihyperglycemic activity. The result of this present study indicated that decreased in the blood glucose concentration of diabetic mice by *E.operculata* treatment may be due to the regeneration/proliferation in the pancreatic β-cells. Histopathological examination of pancreas in streptozotocin-induced diabetic rat treated with D-400 (a herbomineral formulation) revealed that the treatment restored the activity of the islets of Langerhans (Mitra *et al.*, 1995, 1996).

5.3.3.5 Electron microscopical studies of pancreas

Electron microscopic studies have played a key role in the evolution of our understanding of the biology of pancreatic islets as it clearly revealed the variety of cell types comprising the pancreatic islets. (Munger *et al.,* 1965; Like and Orci, 1972; Slavin *et al.,* 1977; Sato and Herman, 1981; Polak and Bloom, 1992; Delfino *et al.,* 1993; Bertelli *et al.,* 1994; Mythili *et al.,* 2003). The total volume of the endocrine part of the mammalian pancreas is only a small percentage of the whole gland and consists of different types of parenchymal cells. Usually, the pancreatic islets consist of all endocrine cell types of the pancreas, but it is not rare to find some islets composed of only one or two cell types. The ratio between the different cell types can vary in the islets according to the pancreatic lobe. Appropriate fixation and staining techniques reveal the presence of several cell types. The two most common are the larger, flame shaped β-cells, which constitute about 20%, and the smaller β-cells which constitute about 75% of the islet cells. The β-cells are sometimes absent in the smaller islets and, when present tend to be located peripherally (Like, 1967; Larson *et al.,* 1976; Pelletier and Leclerc, 1977; Baetens *et al.,* 1979; Jorns *et al.,* 1988).

The β-cells were originally characterized as having a uniform population of extremely electron–opaque secretion granules (Like, 1967; Jorns *et al.,* 1988; Bertelli *et al*., 1994). The β-cells are roughly same size, the only distinguishing feature being the nature of the core. The structure of β-cells granules appears to be a relatively consistent characteristic among all mammals. Clusters of granular endoplasmic reticulum are

commonly observed in the β-cells. The cytoplasm of β-cells contains a well-developed Golgi complex, a moderate amount of rough endoplasmic reticulum and free ribosomes. A few small filamentous mitochondria are present in the cytoplasmic matrix. The nucleus of β-cells tends to be deeply indented or lobular (Lacy, 1972; Like and Orci, 1972; Unger, 1976; Kodama, 1983).

The β-cells are the easiest cells to identify in electron micrographs in that they usually have very distinctive cytological characteristics (Lacy, 1962). The β-cells of most of the animals are characterized by the presence of an electron–opaque para-crystalline granule core. This electron-opaque, somewhat angular, mass is separated from a granular limiting membrane by an electron–lucent space. These characteristic secretion granules are usually massed towards the secretory pole. The cytoplasm of β-cells between the numerous secretion granules contains the organelles including the Golgi apparatus, rough and smooth endoplasmic reticulum, mitochondria, microtubules and cytoplasmic microfilaments (Greider *et al.,* 1969; Pipeleers, 1987, 1992; Delfino *et al.,* 1993). As mentioned earlier in the present study also, STZ induced diabetes produced severe alteration of beta cells, with vacuolization and lysis of the entire cytoplasm and the absence of insulin granules. Also it showed advanced fibrosis which originated in the surrounding capsules with collagen fascicles that intruded among cells, isolating and in some area destroying them (Petkov *et al*., 1985). In *E.operculata* extract treated group more than moderate protection against the STZ induced beta cells destruction are observed even though the destructive effect are still present as dilations of granular endoplasmic reticulam (GER) and of the perinuclear space and some nuclei with irregular outline or abnormal space. Numerous insulin granules both mature and immature and many mitochondria were seen in the cytoplasm of beta cells. There are two possible explanations of these findings. First, *E.operculata* extract may exert its effect by preventing the death of beta cells or second, it may permit recovery of partially destroyed beta cells thereby increasing insulin secretion. The ultrastructure of pancreatic islets in *Vinca rosea* flower and leaf–treated diabetic rats showed considerable improvement in β-cells activity. This is probably due to regeneration and rejuvenation of β-cells leading to increased insulin production and secretion (Ghosh and Suryawanshi, 2001).

5.3.3.6 Effect on liver glycogen content

Glycogen, the preliminary intracellular storable form of glucose in various tissues is a direct reflection of insulin activity, as insulin promotes its deposition by stimulating glycogen synthetase and inhibiting glycogen phosphorylase. The observed reduction of liver glycogen level in diabetic mice was consistent with the earlier result indicating that it was possibly due to lack of insulin as, STZ caused selective destruction of beta cells of the islet of langerhans resulting in a marked decrease of insulin level resulting in the inactivation of glycogen synthase enzyme (Whitton and Hems, 1975). Furthermore, earlier studies also showed that the reduced hepatic glycogen content was normalized by insulin treatment (El-Shenawy and Abdel-Nabi, 2006). In this study, administration of *E.operculata* extract for 21 days showed a trend towards the significant increase in glycogen content towards its near initial values when compared to diabetic control group. The significant increase in the glycogen content of the treated groups may be due to increased insulin secretion or reactivation of the glycogen synthase enzyme thus confirming its insulin potentiating effect to a certain level.

3.7 Elevated glycosylated hemoglobin

Glycosylated hemoglobin has been found to be increased over a long period of time in diabetes (Bunn *et al.,* 1978). During diabetes, the excess of glucose present in blood reacts with hemoglobin to form glycosylated hemoglobin (Alyassin *et al.,* 1981). The rate of glycation is proportional to the concentration of blood glucose (Sheela *et al.,* 1992). In the present study, the diabetic rats had shown higher levels of HbAIC compared to those normal mice. Treatment with *E.operculata* and glibenclamide showed a significant decrease in HbAIC levels in diabetic mice that could be due to an improvement in glycemic status. Similar results were noted by Chandramohan *et al*., (2008), when the diabetic rats treated with 3-hydroxymethyl xylitol brought back Hb and HbA1c values to near normal levels. Administration of bark extracts of *Helicteres isora* (100, 200mg/kg) to diabetic rats decreased the level of glyosylated haemoglobin and increased the level of hemoglobin (Kumar and Murugesan, 2008).

5.4 Antioxidant activity of *E.operculata* extract

Consistent elevation of glucose level in diabetes results in a cascade of biochemical reactions which increases the level of free radicals in various tissues. The free radicals such as superoxide anion radicals (O_2^-), hydroxyl radicals (OH.), non free-radical species like H_2O_2 and singlet oxygen ($^1O_2$), are various forms of activated oxygen collectively known as Reactive Oxygen Species (ROS) (Friedman *et al*., 2003). Abnormally high levels of free radicals cause membrane damage due to peroxidation of membrane lipids and protein glycation and the simultaneous decline of antioxidant defense mechanisms leads to cell and tissue damage which subsequently leads to the development of various associated complication of diabetes (Kesavulu *et al*., 2000;; Maritim *et al*., 2003). Pancreatic β-cells are particularly susceptible to the deleterious effects of reactive oxygen species (ROS), because of their low expression of the antioxidant enzymes genes as compared to other tissues. Thus, the increase of ROS leads to damage of β-cells through the induction of apoptosis and suppression of insulin biosynthesis (El-Alfy *et al*., 2005; Vijayakumar *et al*., 2006). As a new strategy for alleviating the oxidative damage in diabetes, interest has grown in the usage of natural antioxidants. It has been postulated that many of the negative effect of oxidative stress are diminished upon supplementation with certain dietary antioxidants such as vitamin E, C and other non-nutrient antioxidant such as flavonoids (Al-Azzawie *et al*., 2006).

5.4.1 DPPH assay

The antioxidant activity of the extracts was determined using a DPPH scavenging assay. The DPPH assay is often used to evaluate the ability of antioxidants to scavenge free radicals which are known to be a major factor in biological damages caused by oxidative stress. This assay is known to give reliable information concerning the antioxidant ability of the tested compounds (Huang *et al*., 2005). The principle of the assay is based on the color change of the DPPH solution from purple to yellow as the radical is quenched by the antioxidant resulting in a decrease in absorbance at 517 nm (Karagozler *et al*., 2008). The addition of the extracts of *E.operculata* to the DPPH solution caused a rapid decrease in the optical density at 517nm indicating the good scavenging activity of the extract. The extract showed substantial antioxidant activity in

a dose dependent manner similar to that of ascorbic acid which was used as a control standard antioxidant.

5.4.2 Reducing power assay

Reducing power is associated with antioxidant activity and may serve as a significant reflection of the antioxidant activity (Oktay *et al*., 2003). It is a measure of reductive ability of antioxidants and it is evaluated by the transformation of Fe^{3+} to Fe^{2+} in the presence of extracts. In this assay, the yellow color of the test solution changes to various shades of green and blue depending on the reducing power of each compound. Results show that the extract of *E.operculata* possesses reducing power capabilities and acts as a potent antioxidant.

5.5 Phytochemical screening

The dried powder leaves of *E.operculata* were extracted with different solvent of increasing polarity and the yield obtained were analyzed for antidiabetic activity. Of all the extract examined ethanol and aqueous showed maximum inhibitory against α-amylase and α-glucosidase. The phytochemical studies of ethanol and aqueous reveals the presence of flavonoids, carbohydrates, resins, terpenoids, phenol, tannins in the both extract. The presence of alkaloids and cardiac glycoside were seen in aqueous extract only. Flavonoids and phenol were present in bulk amount in both the extract.

5.6 Bioassay guided isolation and characterization of antidiabetic compound(s)

Spectral analysis of the compounds which was eluted from active fraction 3 of ethanol extract of *E.operculata* in this study revealed the presence of four flavonoid compounds, (1) Quercetin-3-O-β-D-glucopyranosyl-(1→4)-α-L-rhamnopyranoside, (2) Kaempferol-3-O-β-D-glucopyranosyl-(1→4)-α-L-rhamnopyranoside, also known as rutin (3) Quercetin-3-O-β-D-glucopyranosyl-(1→6)-α-L-rhamnopyranoside and (4) kaemferol-3-7-O-α-diahamnoside, commonly known as kaempferitrin (Pizzolatti *et al*., 2003; kucukislamoglu *et al*., 2000; Guyenalp *et al*., 2006; Agrawal 1989; Dongmo *et al*., 2007). Among these four compounds previous reports revealed the antidiabetic properties of Kaemferol-3-7-O-α-diahamnoside and kaemferol-3-O-β-D-glucopyranosyl-(1→4)-α-L- rhamnopyranoside. Previous epidemiological studies suggested that flavonoids have various biological activities such as antiinflamatory,

decrease risk of cardiovascular, hypoglycemic, antioxidant also it acts as potential antidiabetic agents because they exert multiple actions that are both hypoglycemic (insulinomimetic action) and antihyperglycemic (insulin secretagogue) (Cazarolli *et al*, 2008). Antihyperglycemic activity of these compounds has been reported on insulin-mimetic activities on regulation of adiponectin secretion, tyrosine phosphorylation of insulin receptor-β and glucose transporter 4 in mouse 3T3-LI adipocytrase (Tzeng *et al*., 2009; Lee *et al*., 2009). Similar results were also obtained from flavonoid compounds of ethanol extract of *C.sinensis* leaves (Li *et al*., 2007). In addition, the hypoglycemic effect and antioxidant potential of kaemferol-3-7-O- α-diahamnoside was also illustrated in normal and alloxan induced diabetic rats (Eliandra de Sousa *et al*., 2004). Previous report also revealed non-toxic and glucose effect of kaempferitrin isolated from *Justicia spicigera* in normoglycemic and STZ induced diabetic rats (Andrade *et al*., 2012).

Quercetin-3-O-β-D-glucopyranosyl-(1→6)-α-L-rhamnopyranoside (rutin) exhibit multiple pharmacological activities including antibacterial, antitumor, anti-inflammatory, antidiarrhoel, antiulcer, antimutagenic, myocardial protecting, vasodilator, immunomodulator and hepatoprotective activities (Janbaz *et al.* 2002). Rutin administration to diabetic rats decreased food consumption and improved body weight and this could be due to a better control of the hrperglycemic state in the diabetic rats. Decreased level of blood glucose could improve body weight in STZ induced diabetic rats (Kamalakkanan *et al.* 2003). Rutin treated STZ diabetic rats exhibited a decrease in plasma glucose level and an increase in insulin and c-peptide levels. A decrease in blood glucose might also contribute to decrease level of glcycosylated hemoglobin. Rutin with its free radical scavenging capacity effectively reduced the formation of glycated haemoglobin and increased the haemoglobin levels in diabetic rats (Liao and Yin, 2000; Kamalakkanam and Prince, 2006).