CHAPTER 5

DISCUSSION

5.1. Preliminary phytochemical screening of CAEE, CAME and CAAE

Phytochemicals are natural bioactive compound widely distributed in plants. The principal examples of natural products including alkaloids, terpenoids, steroids, polyphenols, and flavonoids have rational uses and are found in varying amounts in different species. Their presence in the food chains has significant role which is to work with nutrients and dietary fibre to protect against disease (Shad *et al.,* 2014; Sanjoaquin *et al.,* 2004). Most of the traditionally used antidiabetic plants have proved experimentally. Among those some studies have reported that compounds like phenols (Zakłos-Szyda *et al.,* 2015; Phoboo *et al.,* 2013), flavonoids (Li *etal.*,2009; Babu *et al.,* 2013; Salib *et al.,* 2013), alkaloids (Tiong *et al.,* 2013; Agrawal *et al.,* 2013), steroids (Lauro *et al.*, 2014; Daisy *et al.*, 2009; Schimizu *et al.*,1984), etc are responsible for antidiabetic properties. Priliminary phytochemical screening results (Table.4.2) suggested CA as a good source of natural compounds like phenols, alkaloids, flavonoids, terpenoids, steroids, cardiac glycosides, tennins, proteins and amino acids, diterpenes, etc. Glycosides have been used for over two centuries as stimulant in cases of cardiac failure and diseases (Abidemi *et al.,* 2009) which is one of the major complications of diabetes. Due to the presence of the above said compounds in CA, a significant antihyperglycemic property of the plant is predicted, which might be due to the single/combined activities of the compound/compounds present.

5.2. Quantification of Total Phenolic Contents (TPC)

Polyphenols possess multiple biological activities and constitute an important part of the human diet; consequently they have recently emerged as critical phytochemicals in diabetes prevention and treatment (Zakłos-Szyda *et al.,* 2015). Polyphenols are very popular because of their anti-hyperglycemic effects, safety and non side-effects. Potential efficacy of polyphenols on carbohydrate metabolism and glucose homeostasis has been well investigated in *in vitro*, *iv vivo* and clinical trials (Hanhineva *et al.,* 2010). The hypoglycemic effects of polyphenols are mainly attributed to reduce intestinal absorption of dietary carbohydrate, modulation of the enzymes involved in glucose metabolism, improvement of β-cell function and insulin action, and stimulation of insulin secretion (Iwai *et al.,* 2006; Iwai, 2006). Hence, the presence of the phenolic compounds in the extracts might be the reason behind the antidiabetic activity of the plant.

Information of the presence and/or quantification of phenolic compounds in CA were provided by many researchers (Da *et al.*, 2014; Isah *et al.*, 2015). The result of TPC, in the present research, have shown the presence of 31.56, 35.08 and 26.46 (mg GAE/g dry extract) TPC in CAEE, CAME and CAAE respectively. Sarkar *et al.*, (2014) reported higher level of total phenolics (74.35±0.89 mg GAE/g extract) in methanol extracts of CA than the present result, which could be due to difference in concentration of the samples. Additionally, Devendra *et al.* (2013) have reported the presence of 33.65 mg GAE/g extract and 36.23 mg GAE/g extract TPC in alcoholic and water extracts of CA respectively, which is more or less consistent with the present result. Da *et al.* (2014) reported 145.36 µg TAEs/mg of total phenolic in aqueous extract of CA. The differences in total phenolic content in the same plant species might be due to the fact that quantity of phenolic compounds present in a plant depend on the factors such as genotype, site of location, climatic conditions, technological measures, year, etc (Mikulic-Prtkovsek *et al.*, 2012).

3. *Antioxidant activity assessment*

Oxidative stress is defined in general as excess formation and/or insufficient removal of highly reactive molecules such as reactive oxygen species (ROS) and reactive nitrogen species (RNS). ROS includes free radicals such as superoxide ($\bullet O^2$-), hydroxyl (•OH), peroxyl ($\bullet RO^2$), hydroperoxyl ($\bullet HRO^2$-) as well as nonradical species such as hydrogen peroxide (H^2O^2) and hydrochlorous acid (HOCl). RNS includes free radicals like nitric oxide (•NO) and nitrogen dioxide ($\bullet NO^2$-), as well as nonradicals such as peroxynitrite (ONOO-), nitrous oxide (HNO^2) and alkyl peroxynitrates (RONOO) (Johansen *et al.*, 2005). A free radical can be defined as any molecular species that contain an unpaired electron in an atomic orbital that makes it unstable and highly reactive, produced by all aerobic organisms, primarily as a consequence of aerobic respiration. They can either donate an electron to or accept an electron from other molecules (Lobo *et al.*, 2010; Cheeseman and Slater, 1993), thereby attacking/destroying important macromolecules leading to cell damage and homeostatic disruption. Targets of free radicals include all kinds of molecules in the body. Among them, lipids, nucleic acids, and proteins are the major targets (Lobo *et al.*, 2010). An antioxidant is a molecule stable enough to donate an electron to a rampaging free radical and neutralize it, thus reducing toxic/cell-damaging effect of

free radicals. Antioxidants delay or inhibit cellular damage mainly through their free radical scavenging property (Halliwell, 1995; Lobo *et al.,* 2010).

Hyperglycemia induces free radicals and it impairs the endogenous antioxidant defence system in patients with diabetes, thereby increasing oxidative stress which inturn contributes to progression of diabetes complications (Rosen *et al.,* 2001; Johansen *et al.,* 2005; Matough *et al.,* 2012). It became clear that ameliorating oxidative stress through treatment with antioxidants might be an effective strategy for reducing diabetic complications (Johansen *et al.,* 2005).

DPPH is nitrogen centered free radical with a dark purple colour which becomes colourless when it received hydrogen atom from an antioxidants. The rate of disappearance of purple colour (which can be measured by spectrophometrically determining the absorbance at 517 nm) is directly proportional to antioxidant or hydrogen donating ability of an antioxidant/extract sample (Kedare and Singh, 2011). The antioxidant activity of the extracts, in DPPH assay (Fig.4.3) might be due to its hydrogen atom donating ability that reduces the DPPH radical. CAME (IC_{50} - 2.4 µg/ml) have shown the highest antioxidant activity followed by CAEE (IC_{50} - 2.4 µg/ml) then CAAE (IC_{50} – 7.06 µg/ml) indicating a potent hydrogen donating ability of CAME than the remaining. The reason behind the extract's ability to donate hydrogen atom might be due to the presence of high quantity of phenolic compounds, based on the following accepted fact: phenolic compounds can lose hydrogen atom easily due to lower bond dissociation energies of O-H (Bendary *et al.,* 2013); redox properties of the phenolic compounds allow them to act as reducing agent, hydrogen donators, and singlet oxygen quenchers (Rice-Evans *et al.,* 1997). Considering previous studies on DPPH radical scavenging activity of CA, it was found that: Chatterjee *et al.,* (2013) have reported a strong DPPH radical scavenging activity (IC_{50}=54g/ml) of methanol leaf extract; methanol extract with ED_{50} 28.50µg/ml was reported by Panichayupakaranant and Kaewsuwan (2004); 80% inhibition at 100µg/ml of methanolic extract was described by Johna *et al.* (2012); methanol extract with IC_{50} 23.69 µg/ml was reported by Sarkar *et al.* (2014); Olarte *et al.* 2010 isolated a new indole alkaloid, 1-(4'-hydroxyphenyl)-2,4,6-trihydroxy-indole-3-carboxylic with an IC50 of 0.0311 µM ± 0.002. However the activity of the extracts of the present work might be different from the reported work as degree of

effectiveness of plants varied base on the climatic condition of the plant sample collected.

Reducing power of a compound is related to its electron transfer ability and may serve as a significant indicator of its potential antioxidant activity (Mayakrishnan *et al.,* 2013). The principle behind RPA is that an unstable Fe^{3+} can be reduced to Fe^{2+} by accepting an electron from an antioxidant. The presence of antioxidants in the extracts resulted into reduction of the ferric cyanide complex (Fe3+) to the ferrous cyanide form (Fe2+) with a significant colour change. The yellow colour of the test changes into various shades from green or deep blue depending on the reducing power of the test sample. A strong antioxidant, however gives a deep Perl's Prussian blue. The colour changes can be spectrophotometrically measured at 700nm (Irshad *et al.,* 2012). In the result of the RPA (Fig.4.4), the reducing power of CAME is higher than the remaining extracts throughout the range of concentration used. The result can be further interpreted that CAME showed higher degree of Fe3+ reduction than CAEE and CAAE but not more than that of standard AA. Literature review on reducing power of CA reveals similar findings: Akinmoladun *et al.* (2010) reported a fair reductive potential of CA methanol leaf extract. Priyadharshini *et al.* (2011) reported a reasonable increase in reducing power with a range of concentration (0.2 - 1µg/ml) in various solvent flower extracts of CA. The reducing ability of the extracts is probably due to presence of reductones like phenols, flavonoids, alkaloids, etc (Samad *et al.,* 2014), which is concomitant with the phytochemical screening result (Table.4.2).

In the FRAP assay, reductants/antioxidants in the sample reduce Fe3/tripyridyltriazine complex, present in FRAP, to the blue coloured ferrous form. The absorbance of change in colour can be spectrophometrically measured at 593 nm. The change in absorbance is proportional to the combined (total) ferric reducing/antioxidant power (FRAP value) of the antioxidants in the sample (Benzie and Szeto, 1999). The reducing properties of the extracts, associated with the presence of compounds exert their action by breaking the free radical chain through donating a hydrogen atom (Duh *et al.,* 1999; Rice-Evans *et al.,* 1997). Da *et al.* (2014) have reported FRAP value 12.99µmolTE/mg in aqueous extract of CA.

The principle behind NRSA is that sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitrite oxide which interacts with oxygen to

produce Nitrite ions, which can be measured at 550nm by spectrophotometer in the presence of Griess reagent (Kumar *et al.,* 2008; Singh *et al.,* 2012). The chronic expression of nitric oxide radical is associated with various carcinoma and inflammatory conditions including diabetes. The toxicity of NO increases greatly when it reacts with superoxide radical forming the highly reactive peroxynitrite anion (ONOO-) (Ashok-Kumar *et al.,* 2011). The nitric oxide generated from sodium nitroprusside reacts with oxygen to form nitrite. The nitric oxide scavengers from the extracts/antioxidants inhibit nitrite formation by directly competing with oxygen in the reaction with nitric oxide. The plant/plant products with their property to counteract the effect of NO formation are sure to help in preventing the ill effects of excessive NO generation in vivo (Jagetia *et al.,* 2005). In NRSA result (Fig: 4.7), CA extracts proved to decrease in amount of NO generated from the decomposition of sodium nitroprusside in vitro. The result evidenced the inhibitory effect of the extracts on NO radical generated from sodium nitroprusside, which is indicative of its NO scavenging potential. Afolabi *et al.* (2010) reported a generally low NO radical scavenging activity of methanol extract of CA.

In all the four assays carried out the antioxidant activity of CAME is greater than CAEE and CAAE. Different extracts have exhibited varying degree of scavenging activity against different systems or assays. The underlying reason might be due to the fact that antioxidant activities of an extract depend on: 1). The nature of extracting solvent, as the solubility of different antioxidant compounds with varied chemical characteristics depends on the range of solvent polarity used (Sultana *et al.,* 2009). 2) varied phytochemical contents (Nagarani *et al.,* 2014). 3). Difference in the stoichiometry of reactions between the antioxidant compounds in the extracts and the various radical in the different assays (Khan *et al.,* 2012), 4). Molecular weight, the number of aromatic rings and nature of hydroxyl groups of the antioxidant associated compounds (Hagerman *et al.,* 1998). However, the antioxidant potency range of the extracts was consistent, i.e. highest activity of CAME followed by CAEE, then CAAE, in all the four assays. The said consistency can be further detailed by correlating with the Total Phenolic Content (TPC) of the extracts (Table.4.3). CAME which was shown to have highest TPC, followed by CAEE and CAAE was also exhibited highest antioxidant activity followed by CAEE and CAAE, and is supported by the fact that total phenolics is one of the major contributors to the antioxidant

activity (Sasipriya *et al.,* 2014; Nagarani *et al.,* 2014). As a further agreement with the above statement, a strong correlation between the antioxidant activity and TPC of the extracts are shown in Fig.4.8. With reference to Fig.4.8., TPC gave a significant correlation with DPPH (IC_{50}), FRAP value, Reducing potential and NO radical scavenging activity of the extracts, with R^2=0.999, 0.996, 0.955 and 0.887 respectively. DPPH (IC_{50}) correlated well with TPC (R^2=0.999) attributing the radical scavenging activity of the extracts to its phenolic contents. The correlation between TPC and NO radical scavenging activity was comparatively less suggesting that the antioxidant activity in NRSA was at least partially achieved by bioactive substances other than phenolic compounds. The statement of correlation between TPC and antioxidant activity was similarly reported by many researchers (Piluzza and Bullitta, 2011; Sadeghi *et al.,* 2015; Hatami *et al.,* 2014). However, on the other hand, a contradictory statement was made by Terpinc *et al.* (2012) by reporting a negative correlation between TPC and antioxidant activity of different oil cake extracts.

4. In vitro antidiabetic activity assessment

Alpha-amylase is a digestive enzyme found in the pancreatic juice and saliva which breaks down large insoluble starch molecules into absorbable molecules while the mammalian α-glucosidase in the mucosal brush border of the small intestine that catalyzes the end step of digestion of starch and disaccharides that are abundant in human diet (Afifi *et al.,* 2008; Manohar *et al.,* 2002). This enzyme activities increase the bioavailability of glucose in the blood thus increasing postprandial blood glucose level. Inhibitors of α-amylase and α-glucosidase delay the breakdown of carbohydrates in the small intestine and diminish the postprandial blood glucose excursion (Kazeem *et al.,* 2013). For a substance to be antidiabetic, it should be able to reduce the amount of glucose in the blood or increase the efficacy of insulin (Kumar *et al.,* 2012). The results of *in vitro* α-amylase (Table.4.4) and α-glucosidase (Table.4.5) inhibition test revealed that the extracts have fair potent inhibitory action in a dose dependent manner, though less ($p<0.05$ or $p<0.01$) than the inhibitory potential of standard drug acarbose. Among the three extracts CAME showed the highest inhibitory potential and CAAE the lowest ($p<0.01$significance when compared with acarbose). The inhibitory potency of the extracts range from fair to appreciable with significance difference $p<0.01$ to $p<0.05$, when compared with that of acarbose. In Fig.4.11, CAME, like that of acarbose showed more effectiveness

against α-glucosidase than α-amylase, but on the contrary CAEE and CAAE are more effective against α-amylase than α-glucosidase, suggesting different mode of inhibition of extracts on α-amylase and α-glucosidase activity which is in agreement with the findings of Kazeem *et al.* (2013). Varghese *et al.* (2013) reported a potent α-glucosidase inhibitory activity (IC_{50}, 63.75 ± 12.81 µg/ml) of kaempferol 3-O-gentiobioside, a flavonoid compound, isolated from CA leaf methanol extract. However, there might be other compounds that contribute to the antidiabetic activity of the plant, which can be further studied through phytochemical screening. Further survey of literature did not reveal any information about commercial utilisation of the compound as α-glucosidase inhibitory drugs. Apart from Varghese, so far as the literature is concerned, there is no other report of α-amylase and α-glucosidase inhibitory activity of CA. Polyphenolic compounds present in the extracts might be responsible for the inhibitory potential base on the fact that polyphenolic compounds in plants inhibit the activities of carbohydrate digestive enzymes because of their ability to bind to proteins (Bothon *et al.* (2013). The study further proves that there is a positive relationship among α-glucosidase/α-amylase inhibitory activities, antioxidant activities and polyphenol contents which is in accordance with findings of Mai *et al.* (2007), Bothon *et al.* (2013) and Kumar *et al.* (2012). The present result showed that CA is a source of inhibitory drugs of carbohydrate digesting enzymes which further gave a clue that *in vivo* antidiabetic activity of the extracts may be due to the inhibition of the above enzymes.

5. Acute oral toxicity of extracts in mice

Acute oral toxicity of the extracts was performed base on the Organization for Economic Co-operation and Development (OECD) guideline. Acute toxicity (LD50) test gives a clue on the range of doses that could be used in subsequent toxicity/bioactivity testing and estimating the therapeutic index of drugs and xenobiotics (Aniagu *et al.*, 2005). In the toxicity test performed, there was no sign of any lethality over the period of 14 days, even at the highest concentration (3000mg/kg body weight of mice). It is an indication that the extracts have no adverse effects. The median lethal dose (LD50) was indeterminable since there was no mortality. In an acute toxicity test, 3000mg/kg body weight is the limit dose and any sample nontoxic at this level is considered as safe (OECD guidelines, 2005). Decrease in body weight at high dose extract indicates its toxic potential (Singh *et al.*, 2013). No sign of

toxicity and lack of drastic change in body weight of mice at the end of the test (Fig.4.12) indicates that the extracts are nontoxic and can be used in further bioactivity test.

6. Oral Glucose Tolerance Test (OGTT)

The oral glucose tolerance test (OGTT) measures the body's ability to metabolize glucose (Islam *et al.,* 2009). In OGTT, both normal group and diabetic group, by comparing the bar groups of 0min with the bar groups of 30min [Fig.4.13.a) and Fig: 4.14.a)], it indicates that oral induction of glucose in mice resulted in 1 to 1.5 fold increase in blood glucose level. In the OGTT (normal group), after glucose load, the BGL reaches a peak and was eventually decreased to near normal indicating a normal glucose metabolism and further indicates that the extracts do not exhibit hypoglycaemic activity in normal mice based on Kaur *et al.* (2011). In diabetic group the most significant reduction was observed in methanol extract treated group with 16.43%, 15.81% and 9% reduction at 60min, 90 min and 120min respectively. However, it was not as effective as the standard drug glibenclamide which have 26.71%, 10% and 13.79% reduction potential at 60min, 90min and 129 min respectively. Among the extracts, CAME showed the maximum tolerance for glucose. The extracts showed a better inhibitory activity, compared with the diabetic control suggesting that the extracts could decrease the postprandial glucose level probably by inhibiting the activity of α-amylase and α-glucosidase which is in agreement with the work of Wan *et al.* (2013), or might be by enhancing the secretion of insulin in response to glucose load and increased peripheral utilization of glucose (Andrikopoulos *et al.,* 2008; Kaur *et al.,* 2011). In our study, it was observed that the methanol, ethanol and aqueous extracts of the said plant enhanced glucose utilization. The said extracts were able to reduce blood glucose level on STZ induced diabetic mice. The study reveals the positive effect of extracts in maintaining glucose homeostasis in mice.

7. In-vivo antidiabetic activity assessment of the extracts

The screening of antidiabetic activity of natural products and synthetic compounds is performed in experimental animal models after induction of diabetes by several methods. STZ enters B cell via glucose transporter (GLUT 2) and activates poly ADP-ribosylation which in turn leads to depletion of cellular NAD^+ and ATP. Enhanced ATP dephosphorylation increases reactive oxygen species concentration

and thus causes pancreatic Beta-cell necrosis (Szkudelski, 2001), and subsequently the complications of diabetes (Arunachalam *et al.*, 2013; Anderson *et al.*, 1974; Szkudelski, 2001). Based on the above perspective, CAEE, CAME and CAAE were evaluated for its potential in controlling blood glucose levels in STZ induced diabetic mice keeping proper controls (normal, diabetic and glibenclamide treated mice). Glibenclamide is a commonly used standard antidiabetic drug in STZ-induced diabetes to compare the efficacy of variety of antidiabetic compounds (Arunachalam and Parimelazhagan, 2013). After 7 days of intraperitoneal injection of STZ in mice, the fasting BGL were above 200mg/dL ($p<0.001$, when compared with the normal mice), indicating a diabetic condition. Oral administration of CAEE, CAME and CAAE to diabetic mice for 21 days continuously caused a significant decreased in blood glucose level, with maximum fall observed on 28^{th} day of treatment suggesting a time dependent hypoglycaemic effect of the extracts (Table.4.8). The groups treated with 400mg/kg body wt. dose of extracts was found to be more effective than the groups treated with 200mg/kg body wt. dose of extracts revealing a dose dependent hypoglycaemic effect.

In both the doses, the percentage of decrease of BGL was highest in CAME followed by CAEE and then CAAE (Fig.4.15) revealing a better hypoglycaemic activity in CAME. The glucose lowering potential of the extracts was compared with that of the standard glibenclamide. Oral administration of CAME, CAEE and CAAE to diabetic mice can bring the elevated blood glucose level to normoglycaemia, though the effective nature of the extracts was less intensive and sudden than the standard drug glibenclamide. The hypoglycaemic activity of CAME is higher than CAEE and CAAE, and it correlates with the above discussed results i.e. CAME with higher phenolic contents, higher antioxidant activity and better inhibitory action of carbohydrate digesting enzymes than the remaining extracts.

Lipoprotein abnormalities, which is commonly observed in diabetic patients is cause by alteration of lipid metabolism due to insulin resistance. It increases cardiovascular disease associated with atherogenic dyslipidaemia (Ramachandran *et al.*, 2013). Dyslipidemia is one of the major risk factors for diabetes mellitus complications. The characteristic features of diabetic dyslipidemia are a high plasma triglyceride concentration, low HDL cholesterol concentration and increased concentration of small dense LDL-cholesterol particles (Mooradian, 2009). In the present long term

treatment study, a significant increased TG and TC and decreased HDL levels were observed in the diabetic mice, but in diabetic mice treated with both the doses of CAME, CAEE and CAAE, the levels of TG and TC were decreased slightly while HDL levels were increased considerably. Given this result, it appears that the extracts have a transitory lowering activity on lipid levels in serum. The extracts have the ability to reduce the coronary heart disease (diabetes complication) as it can lower the serum lipid level, based on the evidence provided by Holme *et al.* (1980). This observed result suggest favourable effects of CA on hyperlipidemia. It might be by enhancing a regular activity of insulin that normalized the lipid metabolism justifying the fact that insulin resistance has a central role in the pathogenesis of diabetic dyslipidemia which causes increased free fatty-acid release from insulin resistant fat cells and promotes more TG production (Mooradian, 2009; Olefsky *et al.,* 1974). The results suggest the beneficiary effects of the CA extracts in improving lipid metabolism which is comparable with that of the standard glibenclamide. Decrease in total protein content was observed in diabetic mice which might be due to excessive catabolism of protein to afford amino acids for gluconeogenesis. CAEE and CAAE treatment normalized the total protein in diabetic mice, suggesting its medicinal role on kidney function based on the study of Karuppusamy and Thangaraj, (2013).

Liver has the ability to store glucose as glycogen promoted by insulin. Glycogen synthesis in the liver is impaired in diabetes due to defective activation of glucogen synthase (Levinthal and Tavill, 1999). The glycogen content of the liver in both CAEE and CAAE treated diabetic mice were increased significantly as compared to the diabetic control mice. Treatment with the extracts might be responsible for the maintenance of the glycogen content. SGOT and SGPT are the reliable markers of liver function as the values of SGOT and SGPT tend to rise with increasing liver cell necrosis (Waes and Lieber, 1977). An increase in the activities of SGOT and SGPT in plasma is mainly due to the leakage of these enzymes from the liver cytosol into the blood stream which gives an indication of liver necrosis (Mooradian, 2009; Kasetti *et al.,* 2010). Treatment of the diabetic mice with CAEE and CAAE reduced the concentration of these enzymes in serum compared to the diabetic control which indicates the recovery of liver tissue in treated mice.

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). The regenerative effect of the pancreatic cells due to extract administration may enlighten the positive effects of this agent on the production of insulin (Kaneto *et al.,* 1999). Histopathological study of treated mice liver revealed the ability of CA to protect the mice from liver damage caused by elevated blood glucose level. The liver function is sure to increase as the activity of SGOT and SGPT are decreased in the serum of treated mice based on Vannaphan *et al.,* (2010) who described the association of SGOT and SGPT level and renal failure. Regaining of the kidney cellular structure further indicates the beneficiary effect of the extracts and might be responsible in preventing development of diabetic complications. Taken all together, it appears that CAME, CAEE and CAAE treatment to diabetic mice preserved the cellular function by its beneficial effects.

8. Effects of CAME on secretory function of pancreatic MIN6 β-cells

The mouse insulinoma MIN6 β-cell line was the cellular model to examine the *in vitro* effects of CAME on insulin secretion. MIN6 is a well-characterised insulin-secreting cell line with a higher insulin content than other β-cell lines which retains the physiological regulation of insulin secretion and so it is a good experimental model of insulin secretion studies and mechanisms of action of insulin secretagogues (Kasabri *et al.,* 2012a). Pancreatic β-cells secrete insulin in response to elevated glucose to maintain blood glucose homeostasis. The function of the secretory machinery is mainly regulated by changes in the electrical activity of β-cell ion channels (Kasabri, *et al.,* 2012b). In the absence of glucose, the cytoplasmic ATP concentration is too low to keep the ATP-sensitive K-channels (KATP) closed. When the extracellular glucose concentration is elevated, glucose enters the cells through GLUT2 transporters, and metabolic degradation of glucose increases production of ATP that is responsible for closing KATP in the plasma membrane, thereby depolarising the plasma membrane, which results in opening of voltage-gated Ca2+channels and increased influx of extracellular Ca2+ electrical activity together with Ca2+influx occurs in bursts and results in insulin release (Skelin *et al.,* 2010).

CAME which have shown the best result in bioactivity test was selected for insulin secretion test. CAME increased insulin release from MIN6 β-cells in a dose dependent manner (Fig.4.19). The insulinotropic effects of CAME was demonstrated in mouse pancreatic MIN6 cells. In the absence of Ca^{2+} free KRB medium, at the

highest concentration of CAME (25mg/ml) the insulin stimulating effect of CAME was 2.25 fold greater than the control and the effect of gliclazide was 1.35 fold greater than the control. Indicating that the effective insulin secreting potential of CAME is greater than that of the positive control gliclazide. However, in a medium supplemented with 2.5mM Ca^{2+}, the effective insulin secreting potential of CAME (2.32 fold increase when compared with the control) was lower than the effective insulin secreting activity of gliclazide (5.16 fold increased when compared with the control), indicating a high Ca^{2+} dependent activity of gliclazide base on the study of Zhuo *et al.* (2013). The Ca^{2+} dependency of CAME is less as compared to gliclazide, suggesting a possibility of involvement of other mechanisms in insulin secreting potential, which is similar with the studies reported by some authors (Al-Romaiyan *et al.,* 2010; Brigand *et al.,* 1999). The possible underlying mechanism of action of CAME in insulin secretion might be by increasing the intracellular Ca^{2+} concentration which is the primary insulin secretory signal as described by Zhuo *et al.* (2013), and/or through cAMP signalling dependent mechanism (Seino *et al.,* 2009) and/or K^{+}ATP channel independent mechanism (Olofsson *et al.,* 2004) and/or stimulation of insulin release by saturated fatty acids (Warnotte *et al.,* 1994), etc.

9. GC-MS profile study of CAME

As a unique and powerful technology the GC-MS provides a rare opportunity to perform the analysis of new compounds for characterization and identification of synthesized or derivatized compound. It yields useful information that can be used in research publication internationally (Ziegenhals *et al.,* 2008). GC-MS chromatogram analysis of CAME have revealed the presence of 16 compounds (Fig.4.21). It was observed that CAME extract contain some of the important fatty acids required in the body for proper functioning. Fatty acids with fair antioxidant activity were described by Ismail *et al.,* (2010). Out of the 16 compounds revealed six are fatty acid esters (0.97% concentration), two are saturated fatty acids (2.09% concentration), one is diterpene and one is a glucose analogue. Most of the unsaturated and some saturated fatty acids were in the form of esters as fatty acids in plants generally react with alcohols in an esterification reaction to form esters (William *et al.,* 2000). Fatty acid ester formation may control the cellular concentrations of fatty acids, and acyl-ester formation may play a role in the control of metabolic pathways and the activation of the PPAR (Schmidt *et al.,* 1996). The compound which is found in highest

concentration (84.36%) was 3-O-Methyl-D-glucopyranoside, a glucose analogue. 3-O-Methyl-D-glucose (3-OMG), a nontoxic nonmetabolizable derivative of glucose, is effective in reducing the toxicity of streptozotocin (SZ). The protective effect of 3-OMG against STZ toxicity appears to be partially mediated through conservation of the nicotinamide adenine dinucleotide content in the tissue (Wick *et al.,* 1997). 3-OMG also can protect β-cells against alloxan and inhibit the *in vivo* diabetogenic action of alloxan (Malaisse-Lagae *et al.,* 1983; Sener *et al.,* 1982). The above view was apparently supported by the findings that the non-metabolized analogue of D-glucose, 3-OMG, mimics the effect of D-glucose in preventing the chemically-induced inhibition of insulin release (Tomita *et al.,* 1974). Considering the literature of 3-OMG and relating its activity with bioactivity test result (Table.4.8), it appears that the toxicity of STZ was greatly reduced in CAME treated mice. Thus the reason behind significant antihyperglycaemic activity of CAME can be partially attributed to 3-OMG. Saturated fats do have important bioactivity. Hays *et al.,* (2002) described the idea that addition of saturated fat and removal of starch from a high-monounsaturated fat and starch-restricted diet improved glycemic control and were associated with weight loss without detectable adverse effects on serum lipids. The fifth compound identified was palmitic acid, a saturated fatty acid, which comprises 1.64% of the volatile oil. Palmitic acid acutely stimulates insulin-induced glucose uptake via activation of Akt and ERK1/2 in skeletal muscle cells probably by enhancing the sensitivity of muscle cells to insulin (Pu *et al.,* 2011). Being supported by the above study, it appears that palmitic acid present in CAME at lower concentration might have positive influence on diabetes treatment. The sixth compound is the Ethyl ester of decanoic acid or Capric acid, ethyl ester. Capric acid is a medium chain fatty acid (MCFA). It has been proved that MCFAs reduce adiposity and preserve insulin action in muscle and adipose, despite inducing steatosis and insulin resistance in the liver. Dietary supplementation with MCFAs may therefore be beneficial for preventing obesity and peripheral insulin resistance (Turner *et al.,* 2009). The seventh compound identified was Oleic acid, methyl ester. The ethyl esters of palmitic and oleic acids are identified as naturally occurring transcriptional regulators of the members of the peroxisome proliferator-activated receptor family (Schmidt *et al.,* 1996). It was suggested that, in type 2 diabetes, an oleic acid-rich Mediterranean-type diet versus a linoleic acid-enriched diet may reduce the risk of atherosclerosis by decreasing the number of chylomicron remnant particles (Madigan

et al., 2000). Ryan *et al.,* (2000), have proved the beneficial effect of oleic acid on insulin sensitivity, adipocyte glucose transport and endothelium-dependent vasoreactivity. A diet high in oleic acid, which can be easily achieved through consumption of peanuts and olive oil, can have a beneficial effect in type II diabetes and ultimately reverse the negative effects of inflammatory cytokines observed in obesity and non insulin dependent diabetes mellitus (Vassiliou *et al.,* 2009). Phytol is another important compound identified, which contain many beneficial effect on health. Phytol of natural or synthetic origin is used as active ingredient in formulations to lower serum levels of triglycerides and/or cholesterol (Yokoyama *et al.,* 2004) and the presence of phytol in CAME explained the probable mechanism behind the TG, TC lowering activity of CAME (Table.4.9). Phytol can be administered to patients with disease conditions related to increased levels of cholesterol or triglycerides such as type II diabetes, obesity or other patients in risk of cardiovascular diseases due to elevated cholesterol levels. Phytol can also be administered to healthy individuals to maintain normal levels of serum cholesterol (Yokoyama *et al.,* 2004). It was proved through Docking Simulation and Modulation of Biochemical Alterations that insulin sensitizing/anti-diabetic effect of phytol is mediated by partly from activation of nuclear receptors and heterodimerization of RXR with PPARc by phytanic acid (Elmazar *et al.,* 2013), thus exploring the importance of phytol as possible future antidiabetic drug. Phytol is important in the processing of glucose and can activate enzymes within the body that have strong positive effects on insulin level. This means that phytol in the human diet could possibly help restore the metabolic functions of those with type-2 diabetes (Raman *et al.,* 2012). The presence of phytol in CAME probably justified the antidiabetic activity of CAME (Table.4.9). Phytol has a potent antioxidant activity and was proved by many researchers (Santos *et al.,* 2013; Pejin *et al.,* 2014) and the presence of it in CAME might be one of the causes of the significant antioxidant activity of CAME (Fig.4.3, 4.5, and 4.7). Jananie *et al.,* (2011), identified phytol in an antidiabetic plant *Cynodon dactylon*. Stearic acid can protect cortical neurons against oxidative stress by boosting the internal antioxidant enzymes. Its neuroprotective effect may be mainly mediated by the activation of PPARγ and new protein synthesis in cortical neurons (Wang *et al.,* 2007). A diet enriched in stearic acid protects against the progression of type 2 diabetes in leptin receptor deficient mice (DB/DB). In the pancreatic islets, fatty acids, such as stearic acid, may bind to caveolin-1 and move

the fatty acid into the cell while simultaneously releasing the inhibition from insulin granules. This would increase pancreatic islet fatty acid content and increase insulin exocytosis (Reeves *et al.*, 2012; Warnotte *et al.*, 1994). The presence of strearic acid (0.45% concentration) in CAME justified the effective insulin secretion potential (Fig.4.19) of CAME which further adds to the intensity of antihyperglycemic activity of CA.

10. Molecular Characterization of the plant

In molecular characterization of the plant, RAPD analysis was carried out using fluorescent dyed RAPD markers. RAPD fingerprints/patterns of the plants using the three markers were developed. The dendrogram derived from RAPD profiles (Fig.4.25) shows that the samples F1 and F3 are most closely related with a similarity level of 0.12. However the sample F2 showed clear divergence and showed no similarity with the other two plant samples and appeared separately in the phylogenetic tree. The RAPD profiling data and the phylogenetic tree is in accordance with the phenotypic data. The sample F1 and F3 belong to same species *Cassia alata* Linn. F1 and F3 were the same plant species collected from different places with varied climatic conditions. F2 which showed some degree of variation from F1 and F3, belongs to a different species *Cassia tora* Linn. but of the same genus. The RAPD fingerprint of F1 and F3 developed, though of same species showed different pattern.