CHAPTER 10

Mechanisms of Antidiabetic Effects of CGA Isolated from Aqueous Fraction of MEC

As stated earlier, disturbances in glucose metabolism are major factors leading to diabetes. Insulin, the glucose homeostatic hormone, stimulates hepatocytes, myocytes, and adipocytes to uptake glucose from the circulatory system (Garrett and Grisham, 1997). The inappropriate utilization of insulin leads to insulin resistance, which is characterized by the inability of cells to respond to normal levels of circulating insulin thus leading to the occurrence of the disease (Sesti, 2006). All currently available antidiabetic agents have a major shortcoming in that they are only designed to alleviate T2DM and not to cure it (Chang et al., 2013). To ensure patients’ welfare, there is still an obvious need to develop antidiabetic medicines with satisfactory efficacy and no side effects. There is a growing interest in herbal remedies because of their effectiveness, minimal side effects in clinical experience and relatively low costs (Gupta et al., 2005). Therefore, studies with plant extracts are useful to know their efficacy and mechanism of action and safety (Shukia et al., 2000).

There are several experiments which confirmed the benefits of medicinal plants with hypoglycemic effects in the management of diabetes mellitus (Chauhan et al., 2010). Numerous mechanisms of actions have been proposed for these plant extracts. Some hypotheses relate to their effects on the activity of pancreatic β-cells (synthesis, release, cell regeneration/revitalization) or the increase in the protective/inhibitory effect against insulinase and the increase of the insulin sensitivity or the insulin-like activity of the plant extracts (Patel et al., 2012). Other mechanisms may involve improved glucose homeostasis (increase of peripheral utilization of glucose, increase of synthesis of hepatic glycogen and/or decrease of glycogenolysis acting on enzymes), inhibition of intestinal glucose absorption, reduction of glycogenic index of carbohydrates, reduction of the effect of glutathione (Bnouham et al., 2006). All of these actions may be responsible for the reduction and or abolition of diabetic complications.
In the previous studies, we explored the effect of active component, CGA, present in aqueous fraction of MEC and found that CGA exhibited antidiabetic potential which were comparable to that of the aqueous fraction. Furthermore, we demonstrated that CGA beneficially modulated the mitochondrial oxidative phosphorylation and lysosomal marker enzymes in STZ induced diabetic rats. Hence, the current study was carried out to evaluate the mechanisms of action of CGA in mediating its antidiabetic effect.

**Materials and methods**

**Chemicals**

MTT, DMSO, collagenase, BSA, ficoll – type 400, HEPES, pepstatin, leupeptin, aprotinin and PMSF were purchased from Sigma-Aldrich Corporation, St. Louis, MO, USA. Kits for cDNA synthesis and RT-PCR were purchased from Fermentas - Thermo Fisher Scientific. Mumbai, India. Hank’s balanced salt solution, RPMI-1640, KRB buffer were purchased from HiMedia Laboratories, Mumbai, India. All other chemicals used were of the highest analytical grade.

**Animals and in vivo experimental design**

Male albino rats of Sprague Dawley strain weighing 150-200 g were used for the study. The animals were housed individually in polypropylene cages in a room maintained at 25±5°C with a 12 h light and 12 h dark cycle. Standard laboratory animal feed and water were given *ad libitum* to rats in all groups. Animals were divided into 5 groups of 6 rats each and treated as follows:

- **Group I** - Normal Control
- **Group II** - Diabetic Control
- **Group III** - Diabetic + AF (140 mg)
- **Group IV** - Diabetic + CGA (2.5 mg)
- **Group V** - Diabetic + metformin (500 mg)
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Group I rats were served as normal control, while rats in group II were served as diabetic control. Rats in group III were given aqueous fraction of MEC (AF) at a dose of 140 mg/kg body weight/day orally by intra gastric intubation. Group IV rats were orally administered with 2.5 mg/kg body weight of CGA per day. Group V rats were orally treated with 500 mg/kg body weight of metformin per day. AF, CGA and metformin were suspended in sterile water prior to intra gastric administration. Type 2 diabetes was induced in rats of groups II, III, IV and V by a single intraperitoneal injection of freshly prepared streptozotocin at a dose of 45 mg/kg body weight, dissolved in 0.1M citrate buffer pH 4.5, 15 minutes after the intraperitoneal injection of nicotinamide (110 mg/kg body weight). The rats were then kept on 5% glucose solution for the next 24 hour to prevent drug induced hypoglycemic phase. The blood glucose level was checked 72 h after STZ-NA injection. The animals were considered diabetic when the fasting blood glucose level was raised beyond 200 mg/dL.

The treatment lasted for 45 days. After the treatment period, the rats were euthanized and skeletal muscle and small intestine tissues were taken out, transferred to cold containers and used for the isolation of total genomic RNA. Different sets of normal and diabetic rats were maintained separately for isolation of islets in culture, in situ intestinal perfusion and isolation of hemi diaphragm. The procedures for RT-PCR, isolation of islets of Langerhans, MTT bioassay, western blot analysis, intestinal perfusion and isolation of hemi diaphragm were described in chapter 2.

Results

10.1 Methylthiazoletetrazolium (MTT) bioassay for the cell viability

Results of MTT cell viability assay showed that AF did not exhibit much toxicity to β-cells below 0.4 mg/mL concentration. Maximum β-cell viability was observed at 0.2 mg/mL concentration. From 0.4 mg/mL, cell viability decreased in a concentration dependent manner up to 1.6 mg/mL (Figure 10.1a). In the case of CGA, the maximum cell viability observed at a concentration of 40 µg/mL. CGA did not show much cytotoxicity to islets below this concentration. As the concentration of CGA increased from 80 µg/mL, the cell viability decreased gradually and maximum cytotoxicity observed at 160 µg/mL (Figure 10.1b).
Figure 10.1a Effect of AF on pancreatic β-cell viability

Values are expressed as mean ± SD (n=6). Comparison is between concentrations of AF.
Different alphabets indicate significant difference at p<0.05.

Figure 10.1b Effect of CGA on pancreatic β-cell viability

Values are expressed as mean ± SD (n=6). Comparison is between concentrations of CGA.
Different alphabets indicate significant difference at p<0.05.
10.2 *In vitro* insulin secretion from isolated islets

It was observed that in islets isolated from normal rat pancreas, there was increase of insulin release from 56.5 to 62.10 pmol/L in 1 h of incubation with AF (200 µg/mL), while incubation of islets with CGA (40 µg/mL) enhanced insulin release from 56.80 to 63.50 pmol/L in 1 h. Incubation of islets with AF enhanced insulin release by 9.02%, while with CGA by 10.56% in normal rats. There was no enhanced insulin release in basal control incubation which has the same amount of buffer in place of AF or CGA (Figure 10.2a).

Islets isolated from STZ-induced diabetic rat pancreas and incubated with 200 µg/mL of AF enhanced insulin release from 26.5 to 40.2 pmol/L in 1 h compared with control incubation. Similarly, incubation with 40 µg/mL of CGA enhanced insulin release from 27.6 to 42.9 pmol/L in 1 h of incubation. It was found that incubation of islets with AF enhanced insulin release by 34.08% in 1 h, while with CGA by 35.67% in 1 h in diabetic rats (Figure 10.2b).

*Figure 10.2a In vitro insulin secretion from isolated islets in normal rats*
Figure 10.2b *In vitro* insulin secretion from isolated islets in diabetic rats

\[\text{Insulin concentration of 1 ng/mL} = 172.5 \text{ pM/L}\]

*Values are expressed as mean of triplicate determination

10.3 Intestinal glucose absorption by *in situ* intestinal perfusion

There was 54.5% absorption of glucose by the intestinal mucosa when AF or CGA was not added to the medium (served as control). In the presence of 140 mg of AF, the rate of glucose absorption by the intestine was 27.98% while with 2.5 mg of CGA, it was 23.82%. It was clear from the above results that AF and CGA inhibited the glucose absorption through intestine indicated by the reduced glucose absorption from the medium compared with control (Figure 10.3).
Figure 10.3 Changes in intestinal glucose absorption

Values are expressed as mean ± SD (n = 6). Comparison is between control, AF and CGA.

Different alphabets indicate significant difference at p<0.05.

10.4 mRNA expression of sodium-dependent glucose cotransporter 1 (SGLT-1) in small intestine

The gene expression of SGLT-1 in the small intestine of diabetic control rats were found to be up regulated on comparison with normal control animals. But, treatment of diabetic rats with either AF or CGA down regulated the mRNA expression of SGLT-1 in small intestine, which was comparable with the effect of metformin on SGLT-1 expression in this tissue (Figure 10.4).
Figure 10.4 mRNA expression of SGLT-1 in small intestine

Comparison is between groups
Different alphabets indicate significant difference at p<0.05.

10.5 Peripheral consumption of glucose in vitro by isolated hemi diaphragm

It was observed that, AF and CGA significantly enhanced the glucose uptake by hemi diaphragm compared to the basal control, in which there was only incubation solution. AF and CGA showed 1.6 and 1.83 fold increase in glucose uptake respectively over the control in the absence of insulin (Figure 10.5a). The present data also showed that the effect of both AF and CGA was potentiated in the presence of insulin and a 2.04 fold increase for AF and 2.2 fold increase for CGA was found in the uptake of glucose in the presence of insulin (Figure 10.5b). Moreover, CGA seemed to be more
effective in enhancing peripheral glucose uptake in the absence and presence of insulin compared to AF.

**Figure 10.5a Changes in glucose uptake by isolated hemi diaphragm in the absence of insulin**

Comparison is between AF and CGA with control
Different alphabets indicate significant difference at p<0.05.

*Glucose uptake was expressed in mg/10 mg dry diaphragm
10.6 mRNA expression of protein-tyrosine phosphatase 1B (PTP 1B) in skeletal muscle

The gene expression of PTP 1B in muscular tissue of diabetic control rats was found to be up regulated when compared with normal control animals. Treatment of diabetic rats with both AF and CGA significantly down regulated the mRNA expression of PTP 1B in the myocytes, which were comparable with the effect of metformin on PTP 1B gene expression in this tissue (Figure 10.6).

Figure 10.6 mRNA expression of PTP 1B in skeletal muscle

Comparison is between groups
Different alphabets indicate significant difference at p<0.05.
10.7 mRNA expression of insulin receptor substrate 1 (IRS 1) in skeletal muscle

The mRNA expression of IRS 1 in the muscle tissues of diabetic control rats were found to be down regulated when compared with the normal control animals. But, treatment of diabetic rats with either AF or CGA up regulated the mRNA expression of IRS 1 in muscle tissue, which was comparable with the effect of metformin on IRS 1 expression in this tissue (Figure 10.7).

Figure 10.7 mRNA expression of IRS 1 in skeletal muscle

Comparison is between groups
Different alphabets indicate significant difference at p<0.05.
10.8 mRNA expression of glycogen synthase kinase-3 (GSK-3) in skeletal muscle

The gene expression of GSK-3 in muscular tissue of diabetic control rats was found to be up regulated when compared with normal control rats. Treatment with both AF and CGA to diabetic rats significantly down regulated the mRNA expression of GSK-3 in the skeletal muscle tissue. There was no significant difference observed in the effect of AF, CGA and metformin on GSK-3 gene expression in muscular tissue (Figure 10.8).

Figure 10.8 mRNA expression of GSK-3 in skeletal muscle

Comparison is between groups
Different alphabets indicate significant difference at p<0.05.
10.9 Immunoblot of phosphorylated AMP-activated protein kinase (p-AMPK)

There was a significant decrease in the phosphorylation of AMPK found in the homogenates of skeletal muscle of diabetic control rats compared to the normal animals. Treatment with AF, CGA and metformin to diabetic rats caused a significant increase in the phosphorylation of AMPK in comparison with diabetic control rats (Figure 10.9).

Figure 10.9 Immunoblot of p-AMPK in skeletal muscle

Comparison is between groups
Different alphabets indicate significant difference at $p<0.05$. 

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10.10 Immunoblot of phospho-Akt in skeletal muscle

A significant decrease was observed in the phosphorylation of Akt in the skeletal muscle homogenates of diabetic control rats compared to the normal animals. Treatment with AF, CGA and metformin to diabetic rats caused a significant increase in the phosphorylation of Akt in comparison with diabetic control rats (Figure 10.10).

Comparison is between groups
Different alphabets indicate significant difference at $p<0.05$. 
Many research efforts are currently directed toward development of novel antidiabetic compounds. It is necessary to establish a safer compound that shows antidiabetic activity, as the currently available oral antidiabetic drugs exert side effects on chronic administration (Wadkar et al., 2008). Intense research is being focused on the study of dietary phytochemicals found in plants (Krisanapun et al., 2011). Previously, we found that MEC possess potent antidiabetic effect. The present study evaluated the mechanisms of antidiabetic actions of aqueous fraction of MEC and its active component CGA in experimental diabetes. Different in vitro and in vivo physiological techniques and molecular tools were used for this purpose.

Plant parts or compounds isolated from them exhibited a number of mechanisms of action to reduce and/or abolish diabetes and diabetic complications (Rout et al., 2009). Some plant bioactive components act by stimulation of insulin secretion from islets β-cells (Chucla et al., 1988; Noreen et al., 1988). Several other phytochemicals enhance peripheral glucose utilization with insulin mimetic action both in vivo (Bailey et al., 1985; Ng and Yeung, 1985) and in vitro (Almeida et al., 1985; Welihinda and Karunanayake, 1986). Some other compounds act by reducing intestinal glucose transport (Monserecnusorn, 1980; Frati-Munari et al., 1988). Certain plants exert antidiabetic property by alteration of activity of some enzymes involved in glucose utilization and diminishing the release of some hormones like glucagon, that counteract insulin action (Shanmugasundaram et al., 1983; Ribes et al., 1984).

In the previous study, it was found that CGA is the pharmacologically active component present in the aqueous fraction of MEC (AF). To study the roles of AF and CGA on pancreatic β-cells, islets were isolated from the rat pancreas in culture and in vitro insulin release was assessed. The cytotoxic effect of AF and CGA on islets β-cells was evaluated using MTT bioassay and found that maximum cell viability obtained at 200 µg/mL for AF and 40 µg/mL for CGA. These non-toxic concentrations of AF and CGA were used to study the mechanisms underlying stimulation of insulin secretion. The results showed that AF and CGA caused significant release of insulin from the pancreatic islets of normal and diabetic rats. This observation is in close agreements with a previous report where Aegle marmelos leaf extract stimulated insulin release.
from isolated islets in rodents (Sharma et al., 2007). Even though the release of insulin from islets was moderate, it was indicative of one of the mechanism of action of AF and CGA. This observation from the present study supported the findings of our previous studies where increase in serum levels of insulin and more number of immunoreactions for insulin producing cells were found in MEC treated diabetic rats. Furthermore, there are several reports that CGA possesses strong antioxidant potential (Pari et al., 2010; Nishi et al., 2013). STZ selectively destructs β-cells and produces diabetes through a well-established free radical mediated mechanism (Gutierrez et al., 2011). Thus it was evident that, by its ability to scavenge free radicals and reactive oxygen species, CGA prevented STZ-induced oxidative stress, protected β-cells and resulted in increased insulin secretion. In this context, a previous study by Vessal et al., 2003 showed that quercetin, a flavonoid, decreased elevated blood glucose concentration and increased insulin release in STZ-induced diabetic rats.

One of the therapeutic approaches for decreasing postprandial hyperglycemia is to retard the absorption of glucose from intestine (Kim. 2012). Thus it was of great importance to investigate whether the antihyperglycemic effect of AF and its active compound, CGA was related to the inhibition of glucose absorption from the small intestinal lumen. This aspect was investigated using an in situ perfusion of small intestine. The inhibition of intestinal glucose absorption might have a role in the mechanism of action CGA to augment its antihyperglycemic activity. Because, one of the known pharmacological effect of chlorogenic acid is delaying the absorption of glucose from the gut and decreases post prandial hyperglycemia. Several reports agree to this observation (McCarty, 2005; Bassoli et al., 2008). This finding was supported by the results from the present study, which indicated that both the aqueous fraction and chlorogenic acid successfully decreased the intestinal absorption of glucose in situ. This is in line with a previous report where aqueous extract of Abutilon indicum Sweet inhibited glucose absorption from normal rat intestine (Krisanapun et al., 2009).

To ascertain this finding, we examined the gene expression of Na\(^+\)- dependent glucose transporter 1 (SGLT-1). Intestinal glucose absorption is thought to be modulated by SGLT-1 at the apical membrane of the intestinal epithelia (Hediger and Rhoads, 1994). Regulation of SGLT activities (namely, inhibition of SGLT-1 and SGLT-2 activity and stimulation of SGLT-3 activity) represents a potential means of
managing hyperglycemia, thus preventing complications of diabetes (Castaneda-Sceppa and Castaneda, 2011). It has been shown in diabetic animals and in humans that the capacity of the small intestine to absorb glucose increases at the brush border membrane vesicles (BBMVs) due to the enhanced activity and abundance of SGLT-1 (Fedorak et al., 1991; Dyer et al., 2002). STZ-induced diabetic rats exhibited severe hyperglycemia with an up regulation of SGLT-1 expression in intestinal mucosa compared with normal rats. Treatment of diabetic rats with AF and CGA caused down regulation of SGLT-1 compared to diabetic control animals. This is in accordance with the previous reports by Welsch et al., 1989 and Oliveira et al., 2008 where dietary phenolic compounds and *Ilex paraguariensis* aqueous fraction decreased intestinal SGLT-1 expression in diabetic rats. Thus, as evidenced in this study, the antihyperglycemic activity of both AF and CGA treatments may also be mediated, at least in part, via the inhibition of intestinal Na\(^+\)-dependent glucose absorption mediated by down regulation of the gene expression of SGLT-1, an important co-transporter of glucose in the intestinal mucosa.

In type 2 diabetes, more often the etiology is the lack of insulin sensitivity or resistance to insulin action at the receptor or post-receptor level rather than lack of insulin (Chaiken et al., 1993, 1995). New therapeutic agents are required for treatment of type 2 diabetes, which increase insulin sensitivity or decrease insulin resistance in the peripheral tissues. To gain an insight into this aspect whether AF and CGA might involve, we studied the peripheral uptake of glucose by isolated hemi diaphragm, which represented muscle cells that are the major site of insulin-stimulated glucose disposal (Shepherd et al., 1999). The data obtained in the present study indicated that AF and CGA produced marked increase of peripheral glucose uptake in the absence and presence of insulin as compared with the corresponding controls. The observed increase in the peripheral uptake of glucose by AF and CGA in the absence of insulin suggests that they have insulin like action. Furthermore, the effect of AF and CGA on peripheral glucose uptake was more in the presence of insulin. This addictive effect of CGA in insulin-mediated glucose transport suggests that CGA may act as an insulin sensitizer that potentiates insulin action similar to the therapeutic action of metformin.

In the follow-up studies, we investigated the potential mechanisms underlying glucose transport and improvement in glucose uptake by AF and CGA using skeletal muscle of Sprague Dawley rats. The defective glucose transport system may play an
important role in the pathogenesis of peripheral insulin resistance; and glucose uptake in target tissues is a critical step in maintaining glucose homeostasis and in clearing the postprandial glucose load (Shulman, 2000). As stated before, GLUT 4 is classically referred as insulin responsive transporter and increased GLUT 4 expression may result in higher sensitivity to insulin which subsequently potentiates the influx of glucose into muscle and fat cells. Earlier studies with chlorogenic acid revealed that it stimulated glucose transport in myotubes via increased expression of GLUT 4 (Prabhakar and Doble, 2009). However, mere increase in GLUT 4 gene expression cannot explain the increase in glucose transport and lacks clarification on the mechanisms involved in enhanced glucose transport in skeletal muscle. Previous literature has documented that the protein kinase B (Akt) pathway plays an important role in the insulin signaling cascade leading to glucose transport and translocation of GLUT 4 (Lizcano and Alessi, 2002). Besides this, there are reports that compounds like caffeic acid phenethyl ester, which has structural similarities with CGA, stimulates glucose uptake through AMP-activated protein kinase activation in skeletal muscle cells (Lee et al., 2007). Taken together all these, we investigated the effect of CGA on AMPK and Akt in mediating insulin signaling cascade leading to the translocation of GLUT 4 and cellular uptake of glucose.

Insulin signaling cascade leading to the GLUT 4 translocation include positive and negative regulators, of which insulin receptor substrate (IRS) is one of the positive regulator and phosphotyrosine phosphatase (PTP) and glycogen synthase kinase (GSK) are negative regulators (Goldstein et al., 1998; Galic et al., 2005). Insulin receptor substrate-1 (IRS 1) is a substrate of the insulin receptor tyrosine kinase and appears to have a central role in the insulin-stimulated signal transduction pathway. Therefore, the IRS 1 gene has been studied extensively as a candidate gene for type 2 diabetes (Kovacs et al., 2003). The mRNA expression of IRS 1 is down regulated in type 2 diabetes and insulin resistance (Carvalho et al., 1999). In this study, the gene expression of IRS 1 was down regulated in the skeletal muscle of diabetic control rats. AF and CGA administration significantly increased the mRNA expression of IRS 1 indicating alleviation of insulin resistance and improved insulin signaling to target tissues.

Among several proteins in the insulin signaling pathway that are targeted for diabetes therapy, protein tyrosine phosphatase 1B (PTP 1B) is a typical non-receptor
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type that belongs to the PTP family (Rebecca et al., 2003). PTP 1B has been implicated as a negative regulator of tyrosine kinases, including those associated with insulin signaling, and the expression of PTP 1B is elevated in various experimental models of insulin resistance that are associated with diabetes (Yuan et al., 2013). On knocking down the PTP 1B gene in wild type mice, it was found that insulin sensitivity was greatly increased and the blood glucose levels were normalized (Jill et al., 2003). In the present study, STZ-NA diabetic rats showed increased expression of PTP 1B compared to the normal animals. Treatment of STZ-NA induced diabetic rats with AF and CGA resulted in the down regulation of gene expression of PTP 1B indicating the inhibition of PTP1B expression in vivo, which in turn markedly increased insulin signaling, with a marked stimulation of tyrosine phosphorylation of the insulin receptor and IRS 1 protein. These results confirmed that PTP 1B is indeed a potent negative regulator of insulin signaling in myocytes.

Glycogen synthase kinase - 3 (GSK-3), a serine/threonine kinase plays an important role in the regulation of glycogen synthesis and it suppresses the function of two key targets of insulin action namely, glycogen synthase and IRS 1 and its activity is higher in diabetic tissues (Kaidanovich et al., 2002). Oxidative stress is known to induce insulin resistance through reduced insulin-mediated suppression of the active form of GSK-3 in type 2 diabetic rats (Dokken et al., 2008). The inhibition of GSK-3 leads to an increase in glycogen synthesis, which promotes insulin sensitivity. Activation of GSK-3 leads to reduction in insulin mediated glycogen synthesis (Henriksen et al., 2006). Hence, we studied the effect of AF and CGA in modulating this insulin signaling molecule. Increased expression of GSK-3 seen in diabetic control animals compared to normal ones. Treatment with AF and CGA to diabetic rats caused the down regulation of gene expression of GSK-3 which were comparable with the effect of metformin. The decrease in the mRNA expression of GSK-3 in the present study was correlated with our previous findings, where an increase in glycogen content in both liver and skeletal muscle was observed up on administration of MEC to STZ-NA diabetic rats. The GSK-3 inhibition by AF and CGA in insulin resistant skeletal muscle causes improvement in insulin stimulated glucose transport by enhanced insulin signaling. This finding is in line with a previous report where kaempferol-3-neohesperidoside inhibited GSK-3 in rat soleus muscle (Cazarolli et al., 2009).
To characterize the molecular mechanisms of CGA, we evaluated its effects on the adenosine monophosphate-activated protein kinase (AMPK). AMPK is a master sensor and regulator of cellular energy balance (Kahn et al., 2005). Several upstream signaling were known to activate AMPK that include various pathological and metabolic stressors such as hypoxia, cellular stress that causes a fall in ATP: AMP ratio, calmodulin dependent protein kinase kinase-β (CaMKKβ) and exercise (Hardie and Hawley, 2001; Hawley et al., 2005). Two classes of oral antihyperglycemic drugs (metformin and thiazolidinediones) have been shown to exert some of their therapeutic effects by directly or indirectly activating AMPK (Gruzman et al., 2009). In skeletal muscles, AMPK activation leads to translocation of GLUT 4 from intracellular membranes to plasma membranes, thus increasing glucose transport (Kurth-Kraczek et al., 1999). Consistent with this, our results showed an increase in phosphorylation of AMPK as evidenced from the up regulation of protein expression of phosphorylated AMPK in diabetic rat skeletal muscle after treatment with CGA. There are reports that the activation of muscle AMPK by exogenous compounds or by contraction recruits GLUT 4 to the plasma membrane and augments the rate of glucose transport in a non-insulin-dependent manner (Merrill et al., 1997; Mu et al., 2003). In addition, AMPK-induced translocation of GLUT 4 containing vesicles to the plasma membrane is preceded by the phosphorylation of the protein AS-160 at Thr(642). This phosphorylated form of AS-160 releases the vesicle from intracellular storages and allows their recruitment to the plasma membrane (Eguez et al., 2005; Miinea et al., 2005). It has been reported that AS-160 is the substrate for Akt (Kane et al., 2002). Thus, the upstream nature of AMPK, by which Akt is activated by the phosphorylation of AMPK at Thr(172), was evidenced.

To corroborate the roles of CGA in insulin and AMPK mediated signaling pathways, we assessed the in vivo effects of CGA on protein kinase B (Akt) activation through phosphorylation using western blotting analysis. Akt, also known as protein kinase B (PKB), is a serine/threonine-specific protein kinase that plays a key role in multiple cellular processes such as glucose metabolism, apoptosis, cell proliferation, transcription and cell migration. Activation of Akt is essential for the downstream signaling of insulin to bring about GLUT 4 translocation, regulation of glycogen synthesis in muscles, and in liver and for the inhibition of gluconeogenesis (Tremblay et
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al., 2001; Farese et al., 2005). In this study, results of immunoblotting analysis showed that expression of phosphorylated Akt protein was down regulated in diabetic control rats. Administration of the aqueous fraction of MEC and its active compound, CGA to diabetic rats stimulated the protein expression of the phosphorylated Akt, which were comparable with the effect of metformin. The results suggest that CGA increases insulin sensitivity through the phosphorylation of Akt. Thus it was apparent that the phosphorylation of Akt had a key role in the translocation of GLUT 4 to plasma membrane and for improvement of insulin sensitivity for glucose uptake in diabetic rat skeletal muscle. This observation was supported by our previous findings in which MEC treatment to diabetic rats up regulated the gene expression of GLUT 4 in skeletal muscle.

Thus, it is highly notable that both insulin-dependent and AMPK-dependent pathways converge to activate Akt through its substrate, AS-160 and Akt may be the point of convergence instead of AS-160. Although, the outcome of insulin action and AMPK activation of the glucose transport system in skeletal muscles is similar, the transduction mechanism employed by insulin to recruit GLUT 4 to the plasma membrane is entirely different and independent of that recruited by AMPK. However, there are reports that when co-stimulated, both the AMPK and the insulin-dependent pathways increase the rate of glucose transport and uptake in an additive manner (Krook et al., 2004). This report supported one of our findings in this study, in which CGA potentiated the uptake of glucose by isolated hemidiaphragm in the presence of insulin.

From the above results, it can be inferred that CGA, the active constituent present in the aqueous fraction of MEC exerted its antidiabetic effect mainly by extra-pancreatic mechanisms in which it acted on two target tissues; i.e., skeletal muscle and small intestine. In the skeletal muscle, CGA activated AMPK by phosphorylation of its Thr(172) residue and modulated insulin signaling pathway by down regulating PTP 1B and GSK-3 and up regulating IRS 1 genes. Both these pathways operated in a parallel manner and activated protein kinase B (Akt) by phosphorylating its Ser(473) residue, which ultimately increased the translocation of GLUT 4 to the plasma membrane and increased the cellular uptake of glucose. This effect of CGA was comparable with the antidiabetic drug, metformin. In the small intestine, CGA inhibited the absorption of
glucose from intestinal mucosa by down regulating SGLT-1 gene. In addition, CGA exhibited its antidiabetic action partly by an intra-pancreatic mechanism in which it stimulated the insulin release from residual islet β-cells.

Concluding, the major finding of the present study was that CGA normalized the altered glucose metabolic function in the skeletal muscle during diabetes and that AMPK and Akt were instrumental in CGA mediated glucose homeostasis. Thus, as an active component present in CnI, the present data suggests that CGA may contribute to the beneficial effect of CnI in modulating type 2 diabetes. However, further experiments are needed to evaluate its efficacy and safety in clinical use.