Antidiabetic activity of crude stem extracts of Coscinium fenestratum on streptozotocin-induced type-2 diabetic rats

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Received: 23 July 2011, Revised and Accepted: 11 Aug 2011

Asian Journal of Pharmaceutical and Clinical Research
Vol. 4, Issue 4, 2011
ISSN - 0974-2441

ABSTRACT
Coscinium fenestratum (CF) (Gaertn.) Colebr traditionally has been used for the treatment of many diseases including diabetes Mellitus. In the present study, we investigated the effects of crude dichloromethane (DCM), Ethyl Acetate (EA), and Butanol (BuOH) extract of CF on streptozotocin-induced diabetic rat’s models. The crude stem extracts at concentration of 250mg/kg were administered for 4 weeks and the effects of extracts on blood glucose, body weight and carbohydrate metabolizing enzymes Hexokinase and Lactate Dehydrogenase (LDH) were determined. The total phenolic content, anti-oxidant activity and phytochemical screening and HPLC profiling of extracts were also investigated. DCM and EA extracts possesses a significant (p<0.001) hypoglycemic effect by lowering the blood glucose levels and increasing the body weight in STZ induced diabetic rats. The activities of hexokinase and LDH increases in the diabetic group treated with DCM and EA extract compared to the diabetic rats group. The total phenolic content (TPC) and antioxidant studies revealed the presence of phenolic and antioxidant activity in DCM and EA extracts. Phytochemical screening, TPC of DCM and EA extract confirmed the presence of phenols, alkaloids, flavonoids, terpenes, saponins, tannins, steroids and possess strong antioxidant properties. In conclusion, DCM and EA stem extract of CF showed strong plasma glucose lowering and antioxidant activity. These effects may be as a result of the presence of phytotoxins, alkaloids, flavonoids, terpenes, tannins, and steroids in DCM and EA stem extract.

Keywords: Coscinium fenestratum; Antidiabetic; Streptozotocin; Antioxidant; Diabetes.

INTRODUCTION
Diabetes and its complications have been a major cause of premature death all over the world and most of these are avoidable 1. In 2006 Malaysian Third National Health and Morbidity Survey showed prevalence of Type 2 Diabetes Mellitus for Adults aged 30 years and above to be 14.9% upped by almost 79.6% in the space of 10 years from 1996 to 2006. The prevalence of type 2 diabetes is the highest among Indian ethnic at 19.9% for those aged 30 and above 2.

The β-cells from the islet of Langerhans in the pancreas detect the post meal rise in blood glucose and begin to stimulate insulin release to enhance the diffusion of glucose into cells to produce energy to fuel body functions or store as glycogen. The release of insulin from the pancreas is not only stimulated by increase blood glucose, it is also stimulated by incretins, vagal nerve stimulation and other factors as well 3. Pancreatic β-cells of the islet of Langerhans are the only cells that produce insulin, but they have a limited capacity for regeneration, which is predisposing factor for the development of diabetes mellitus 4. Intercellular signalling cascade results in storage of glucose as glycogen, if the insulin receptors fail to initiate the intercellular signalling the body compensate by secreting greater amount of insulin hyperglycaemia occur. Also when the body cannot produce enough insulin or is unable to effectively use the insulin it makes (insulin resistance) glucose accumulate in the blood leading to hyperglycaemia. Hyperglycaemia is the most consistent sign of diabetes, but is not a sensitive indicator at the onset of the disease 5. In the past few years new evidence and studies has made a major impact on diabetes management. From new targets for control, emphasis and recognition of the cluster of cardiovascular risk factors that make up the metabolic syndrome in which type 2 diabetes is a principal player and new classes of pharmacological agents targeting novel pathways as well as major outcome studies, these have changed algorithms for the management of diabetes care. Coscinium fenestratum is a woody climber found in south east Asia and has traditionally been used as a medicinal plant 6. The infusion and tincture preparation of stem is widely used in the traditional Ayurvedic system for the treatment of diabetes mellitus 7. In the siddha system of medicine, the powdered stem is dissolved in milk and given to the diabetic patients 8. The rural people of Kanyakumari District, Tamilnadu, India use the decoction of the stem for treatment of diabetes 9. Alcoholic extracts of CF has been reported to possess anti-diabetic effect 10. In the present study, we investigated the effects of crude dichloromethane (DCM), Ethyl Acetate (EA), and Butanol (BuOH) extracts from the stem of CF on blood glucose, body weight and carbohydrate metabolizing enzymes Hexokinase (HK) and Lactate Dehydrogenase (LDH) in streptozotocin-induced diabetic rat's models. The total phenolic content, anti-oxidant activity and phytochemical screening of extracts were also investigated.

MATERIALS AND METHODS
Collection of plant material
The stems of the plant CF (20kg) were collected from the jungles of Pahang, Mr. Shamsul Khamis a plant taxonomist from the Laboratory of Natural Products (NATPRO) in Institute of Bioscience, University Putra Malaysia (UPM), Selangor DarulEhsan specifically identified the plant.

Preparation of Plant Extract
The stem of CF was washed, cut, dried and ground into a powder with a miller in the Institute of Bioscience, UPM. Approx. 2kg of stem powder was macerated with 8L Methanol into four 2L conical flask for two days at room temperature. The extract was filtered using Whatman Filter papers and the filtrate concentrated by evaporation at 35-37°C using rotary evaporator to give a concentrated semi-solid crude methanol extract. The dried methanol extract about 10g was suspended in 300mL of water and then partitioned with DCM, EA and BuOH respectively

Animals
Healthy adult Male Wistar albino rats weighing 170-230g were used for the study. Rats were maintained under standard laboratory conditions (12h light/12hr dark cycle; 25°C; 35-60% humidity). The animals were fed with standard rat pellet diet and water ad libitum. Rats were allowed to acclimatize for 2 weeks prior to the experiment.

Acute toxicity studies
The acute toxicity study of the crude extracts from stems of C. F. was performed in rats (n=10). In this assay, increasing doses of crude extracts were orally administered to groups of animals for each dose after a 12h fast. Animals receiving the vehicle (saline) served as control. The signs and symptoms associated with the crude extracts
administration (5g/kg, p.o. volume of 10mL/kg of body weight) were observed at 0, 30, 60,120,180 and 240 min after administration and then once a day for the next 14 days. At the end of the period the number of survivor was recorded. The acute toxicological effect was estimated by the method described by 11 and the death, when occurred, was expressed as LD50 according to 12.

Induction of Diabetes

Diabetes was induced 13 in overnight fasted wistar rats were induced diabetes using Streptozotocin (STZ) (60mg, St Louis, MO). STZ was dissolved in ice-cold citrate buffer (0.1M. pH 4.5). STZ and administered in a dosage of 60mg/kg by intraperitoneal (i.p.) injection. Blood glucose level and the bodyweight of the rats were assessed 72 hours post STZ injection to confirm hyperglycaemia and only rats with elevated blood glucose levels above 11 mmol/L or 200mg/dL were used for this study.

Study Design

Total number of 30 rats were used and divided into 5 groups (n=6).

Group 1: Normal Treated with saline (1mL)

Group 2: Diabetic Positive control treated with Tolbutamide (100mg/kg)

Group 3: Diabetic treated with DCM crude extract (250mg/kg).

Group 4: Diabetic treated with EA crude extract (250mg/kg).

Group 5: Diabetic treated with BuOH crude extract (250mg/kg).

Animals were treated respectively for 4 weeks orally. Glucose measured by glucometer and body weight was measured using weight machine on week 0, 2 and 4. Blood samples were collected during the 14 days of observation after the administration of crude extracts. The results obtained indicated the absence of acute toxic effect of crude extract.

Antidiabetic activity

All the crude stem extracts of C. exhibited plasma glucose lowering activity (hypoglycaemic) in STZ-induced diabetic rats compared to the negative control as shown on Table 1. Group I did not show any effect compared to treated group. Group II-IV, showed significant reduction in sugar level when compared with negative control rats (P<0.001) 35.5%, 57.8%, and 43.5%, respectively. In contrast Group V did not show significant effect (P>0.001) when compared to control groups with percentage reduction of only 19%. Similarly, the body weight of the negative control group was decreased compared to the treated groups which continuously increase as shown in Table 2. Group III showed a significant (P=0.001) increase in weight 5.85%, Group IV 4.13%, Group II 1.81% while Group IV 0.9% when compared to control group.

Biochemical assay

Hexokinase

Hexokinase enzyme activity was increased in Groups II 0.893 u/g, compared to control group. The method of 15 was used for the determination of scavenging activity of DPPH free radical. One ml of 0.135 mM DPPH prepare in methanol was mixed with 1.0ml of DCM extract ranging from 0.2-8 mg. The reaction mixture was vortexed thoroughly and left in dark at room temperature for 30min. The absorbance was measured spectropho-meterically at 517nm. The scavenging ability of the plant extract was calculated using this equation;

\[
\text{DPPH scavenging activity} (\%) = \left(1 - \frac{\text{Abs}_{\text{sample}}}{\text{Abs}_{\text{control}}} \right) \times 100
\]

Acute toxicity studies

The oral toxicity of crude extracts was evaluated with dose five - fold higher than maximum tested antiscorbutic dose (1000mg/kg, p.o.). At this dose, no signs and symptoms of acute toxicity were observed in all treated rats. No significant difference was observed in the weight of heart, liver, kidney, or lungs when they were compared with those of control group (saline). None of the treated rats died during the 14 days of observation after the administration of crude extracts. The results obtained indicated the absence of acute toxic effect of crude extract.

RESULTS

Phytochemical analysis

A small portion of the dry DCM extract was used for the phytochemical tests for compounds which include tannins, flavonoids, alkaloids, saponins, and steroids in accordance with methods of 16,17, with little modifications. Exactly 1.0g of the DCM extract was dissolved in 10ml of distilled water and filtered (using Whatman No 1 filter paper) A blue colouration resulting from the addition of ferric chloride reagent to the filtrate indicated the presence of tannins in the extract. Exactly 0.5g of DCM extract was dissolved in 5ml of 1 % HCl on steam bath. A militre of the filtrate was treated with few drops of Dragendorff’s reagent. Turbidity or precipitation was taken as indicative of the presence of alkaloids. About 0.2g of DCM extract was dissolved in methanol and heated. A chip of magnesium metal was added to the mixture followed by the addition of few drops of concentrated HCl. The occurrence of a red or orange colouration was indicative of flavonoids. One gram of each extract was separately dissolved in 10 ml of chloroform and equal volume of concentrated sulphuric acid was added by sides of the test tube. The upper layer turn red and sulphuric acid layer showed yellow with green fluorescence. This indicated the presence of steroids.

Antioxidant activity

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Antioxidant assay

To a 0.1mL of Liver tissue homogenate 2.28mL of tris(200mmol L -1) / MgCl2 buffer (20mol L -1), pH 8, 0.5mL of 0.67M glucose, 0.1mL of 16 mM ATP, 0.1 MI OF 6.8 mM NAD and 0.01mL of 300 U mL -1 lactate dehydrogenase enzyme activity was added. The solution was mixed thoroughly and the absorbance was measured at 340 nm.

Lactate dehydrogenase enzyme activity

To 0.05mL of Liver tissue homogenate 2.5mL of Tris (81.3mmol, pH 7.2) NaCl (203.3 mmol) per NADH 0.244 mmol L -1 glucose-6-phosphate dehydrogenase were added. The solution was mixed thoroughly and the absorbance was measured at 340 nm.

Phytochemical analysis

The method of 15 was used for the determination of scavenging activity of DPPH free radical. One ml of 0.135 mM DPPH prepare in methanol was mixed with 1.0ml of DCM extract ranging from 0.2-8 mg. The reaction mixture was vortexed thoroughly and left in dark at room temperature for 30min. The absorbance was measured spectropho-meterically at 517nm. The scavenging ability of the plant extract was calculated using this equation;

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Biochemical assay

Hexokinase

Hexokinase enzyme activity was increased in Groups II 0.893 u/g, Group III 1.821 u/g, and Group IV 1.140 u/g compared to Group I 2.532 u/g Table 3.
Lactate Dehydrogenase
Similarly LDH enzyme activity was increased in Groups II 0.434u/g, Group III 1.413 u/g, and Group IV 0.822u/g compared to Group I 2.351u/g Table 4.

Antioxidant activity
DCM crude extract contain a total phenol content of 1092.33±34.89 mg GAE/100g, 47.13± 0.44 % Ferrous Ion Chelating activity and 66.30± 0.12 % DPPH free radical scavenging activity. Table 5

Phytochemical analysis
DCM crude stem extract was proved to have flavonoids, saponins, tannins, alkaloids, terpenes and steroid, cardiac glycosides were absent.

Table 1: Effect of Coscinium Fenestratum crude DCM extract on blood glucose level in normal and diabetic rats

<table>
<thead>
<tr>
<th>Groups</th>
<th>Treatment</th>
<th>Blood Glucose Concentration (mg/dl)</th>
<th>Reduction (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Day 0</td>
<td>Day 3</td>
</tr>
<tr>
<td>I</td>
<td>Negative control</td>
<td>248.65± 1.85</td>
<td>292.65 +1.93</td>
</tr>
<tr>
<td>II</td>
<td>Positive Control (Tolbutamide 100mg/kg)</td>
<td>274.65 +1.48</td>
<td>221.75 + 1.46</td>
</tr>
<tr>
<td>III</td>
<td>DCM (250mg/kg)</td>
<td>271.47 ± 1.24</td>
<td>217.08 + 1.42</td>
</tr>
<tr>
<td>IV</td>
<td>EA (250mg/kg)</td>
<td>275.47 + 1.45</td>
<td>224.90 + 1.27</td>
</tr>
<tr>
<td>V</td>
<td>BuOH (250mg/Kg)</td>
<td>281.42 ± 1.50</td>
<td>264.30 ± 1.51</td>
</tr>
</tbody>
</table>

Each value represents Mean ± SEM; n = 6, analysis by one – way ANOVA followed by Dunnett’s test, significant at ***P<0.001, in comparison to negative control.

Table 2: Effect of Coscinium Fenestratum Crude DCM Extract on Body Weight in Normal and Diabetic Rats

<table>
<thead>
<tr>
<th>Groups</th>
<th>Treatment</th>
<th>Body weight (g)</th>
<th>% of Body weight Increase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Day 0</td>
<td>Day 3</td>
</tr>
<tr>
<td>I</td>
<td>Negative control</td>
<td>204.07 + 1.05</td>
<td>201.10 + 1.31</td>
</tr>
<tr>
<td>II</td>
<td>Tolbutamide (100mg/kg)</td>
<td>199.53 + 1.76</td>
<td>200.50 + 1.80</td>
</tr>
<tr>
<td>III</td>
<td>DCM (250mg/kg)</td>
<td>215.82 + 1.07</td>
<td>219.27 + 1.13</td>
</tr>
<tr>
<td>IV</td>
<td>EA (250mg/kg)</td>
<td>222.30 + 0.88</td>
<td>224.60 + 0.94</td>
</tr>
<tr>
<td>V</td>
<td>BuOH (250mg/Kg)</td>
<td>222.45 + 0.97</td>
<td>223.13 + 0.97</td>
</tr>
</tbody>
</table>

Each value represents Mean ± SEM; n = 6, analysis by one – way ANOVA followed by Dunnett’s test, significant at (***P< 0.001, NS –Not Significant) in comparison to negative control.

Table 3: Enzyme Activity of Hexokinase Enzyme

<table>
<thead>
<tr>
<th>Groups</th>
<th>Enzyme Activity (U/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I-Non- Diabetic Control</td>
<td>2.603 + 0.052</td>
</tr>
<tr>
<td>Group II-Diabetic Control</td>
<td>0.867 + 0.031***</td>
</tr>
<tr>
<td>Group III- DCM</td>
<td>1.854 + 0.019***</td>
</tr>
<tr>
<td>Group IV- EA</td>
<td>1.153 + 0.007***</td>
</tr>
</tbody>
</table>

Value expressed as Mean + SEM, n = 5, analysis by One-way ANOVA followed by Dunnett's test, significant at "***P<0.001, in comparing to Group I: U, u mol reduction of NAD+.

Table 4: Enzyme Activity of Enzyme Lactate Dehydrogenase

<table>
<thead>
<tr>
<th>Groups</th>
<th>Enzyme Activity (U/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I-Non- Diabetic Control</td>
<td>2.387 + 0.027</td>
</tr>
<tr>
<td>Group II-Diabetic Control</td>
<td>0.430 + 0.016**</td>
</tr>
<tr>
<td>Group III- DCM</td>
<td>1.441 + 0.019***</td>
</tr>
<tr>
<td>Group IV- EA</td>
<td>0.852 + 0.021***</td>
</tr>
</tbody>
</table>

Value expressed as Mean + SEM, n = 5, analysis by One-way ANOVA followed by Dunnett's test, significant at "****P<0.001, in comparing to Group I: U, u mol conversion of NAD to NADH.

Table 5: Total Phenolic Content

<table>
<thead>
<tr>
<th>TPC ( mg GAE / 100g )</th>
</tr>
</thead>
<tbody>
<tr>
<td>1092.33 ± 34.8918</td>
</tr>
</tbody>
</table>

Results are means +SEM ( n = 3)

Table 6: Ferrous ion Chelating (Fic) Activity

<table>
<thead>
<tr>
<th>Chelating Effect (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>47.13 + 0.44</td>
</tr>
</tbody>
</table>

Results are means + SEM (n = 3)

Table 6: DPPH Free Radical Scavenging Activity (%)

<table>
<thead>
<tr>
<th>Scavenging Effect (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>66.30 + 0.12</td>
</tr>
</tbody>
</table>

Results are means + SEM (n = 3)
DISCUSSION
Diabetes mellitus is a worldwide problem, and type 2 diabetes is found to be more prevalent. Patients in this group range from insulin deficiency and insulin resistance to a predominantly secretory defect with some insulin resistance. The oral toxicity of crude extracts was evaluated with dose five – fold higher than maximum tested antidiabetic dose (1000mg/kg, p. o.). At this dose, no signs and symptoms of acute toxicity were observed in all treated rats. No significant difference was observed in the weight of heart, liver, kidney, or lungs when they were compared with those of control group (saline). None of the treated rats died during the 14 days of observation after the administration of crude extracts. The results obtained indicated the absence of acute toxic effect of crude extracts.

STZ is well known for its selective pancreatic islet β-cells cytoxicity and has been extensively used to induce diabetes mellitus in animals. It interferes with cellular metabolic oxidative mechanisms. Intraportal administration of STZ effectively induced diabetes in normal rats, as observed by hyperglycemia, when compared with normal rats. Persistent hyperglycemia, the common characteristic of diabetes can cause most diabetic complications and it is normalized by the action of insulin. In this study significant hyperglycemia was achieved within 48 hours after STZ (60mg/kg body wt. ip.) injection. STZ induced diabetic rats with more than levels above 11 mmol/L or 200mg/dL of blood glucose were considered to be diabetic and used for the study. In this study it was observed that the oral administration of the extract (250mg/kg body wt) could reverse the above mentioned diabetic effect, possibly due to an insulin-like effect of the extract on peripheral tissues, either by promoting glucose uptake and metabolism, or by inhibiting hepatic gluconeogenesis. This result was in-line with traditional claim and the previous reports by and . A number of compounds have also been shown to exert hypoglycaemic activity through stimulation of insulin release. The hypoglycaemic potency of the extract was comparable with tolbutamide, a standard hypoglycaemic drug. Tolbutamide has long been used to treat diabetes and is known to act by stimulating insulin secretion through action on the pancreatic β-cells.

Hexokinase is an insulin-dependent and insulin-sensitive enzyme and are almost completely inhibited or inactivated in diabetic rat liver in the absence of insulin. Decrease enzymatic activity of hexokinase has also been reported in diabetic animals, resulting in depletion of liver and muscle glycogen . Administration of extract and tolbutamide to STZ treated rats resulted in an increased activity of hexokinase in liver. Increased hexokinase activity was observed in the extract-treated diabetic rats treated with extract which would have resulted in the activation of glycolysis, which, in turn, increased the utilization of glucose by restored insulin secretion in treated rats. Lactate dehydrogenase (LDH) activity increase in extracts treated groups compared to diabetic control group. The decreased activity of this enzyme in the diabetic condition diminishes the reducing equivalent of oxidative stress leading to diabetic complications. The mechanism of action of this extracts is not yet known however from its effect on LDH the extract seems to increase flux of glucose into the glycolytic pathway and pentose monophosphate shunt in an attempt to reduce high blood glucose levels and may have increased the production of the reducing agent, NADPH with concomitant decrease in oxidative stress.

Accumulating evidence indicates that ROS not only are strongly associated with lipid peroxidation resulting in deterioration of food materials, but are also involved in the development of pathology of many clinical disorders, such as cancer, gastric ulcers, Alzheimer, diabetes, arthritis and ischaemic reperfusion . The removal of free radicals and ROS is probably one of the most effective defences of the body to maintain the oxidative –antioxidant balance. The extract showed very high content of polyphenols and free radical scavenging (DPPH) and chelating (FIC) effects (Antioxidant) and phytochemical screening confirmed the presence of flavonoids, saponins, tannins, alkaloids, terpenes. There is an increasing interest in phytochemicals such as polyphenols, saponins, tannins, alkaloids, terpenes due to their potentially positive effect against certain diseases including diabetes. They can act as free radical scavengers, neutralising dangerous reactive oxygen species and metal ion chelators, which are responsible for antioxidant properties.

CONCLUSION
In conclusion our result indicates that DCM crude extract from the stem of F. bengalensis has antidiabetic action. The mechanism of action may be by stimulation of the pancreatic β-cells and the enzymes that regulate glycolysis. The effect maybe as a result of the presence of phytochemicals which provided antioxidant properties and have been reported to possess anti-inflammatory effect.

ACKNOWLEDGEMENT
UCSI University for the research grant provided for this experiment.

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