Antidiabetic Effect of Partially Purified Fraction E from the Stem Extract of Coscinium fenestratum

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Abstract: Our study was aimed at evaluating the effect of Partially Purified Fraction of dichloromethane extract from the stem of Coscinium fenestratum on plasma glucose level, liver, kidney, hematology parameters and antioxidant activity on streptozocin-induced Type 2 diabetic rat model. The major bioactive component present in partially purified fraction was also identified using high performance liquid chromatography. Sprague Dawley rats numbering 24 were divided into 4 groups (n=6); I- Normal, II- Saline (1ml/kg body weight) (Negative) control, III- Tolbutamide (Positive control (100mg/kg body weight) and IV- Fraction E (100mg/kg body weight). The rats were fasted overnight prior to Streptozocin diabetic induction. Each drug was administered via oral gauge once a day for 90 days. The weight and plasma glucose level of the rats was measured every week for 12 weeks. Diabetes mellitus was induced in the Sprague-Dawley rats by intraperitoneal injection of 50 mg/kg Streptozocin. A week later rats with plasma glucose level of 11mmol/L or 200mg/dL and above were confirmed diabetic. The rats were orally administered with Fraction E (100mg/kg) for 90 days; the plasma glucose level and body weight of the rats was measured weekly. After 90 days treatment, blood was withdrawn from the rats by cardiac puncture, they were sacrificed by cervical dislocation; the liver and pancreas were harvested for further studies. HPLC was used to identify the bioactive component present in the partially purified fraction. The plasma glucose level was significantly ($P<0.0001$) reduced in the rats treated with fraction E. It also decreased lipid peroxidation, normalized in vivo antioxidant defensive mechanism, lipid profile and hematological parameters. Palmatine was found to be present in the partially purified fraction. Due to its plasma glucose lowering activity, partially purified Fraction may be considered in the treatment of diabetes.

Key words: Plasma Lowering • STZ • Coscinium fenestratum • Antioxidant • Type 2 Diabetes

INTRODUCTION

Diabetes mellitus is a metabolic disorder of the endocrine system. The disease is found in all parts of the world and it is rapidly increasing in most parts of the world. People suffering from diabetes are not able to produce or properly use insulin in the body therefore they have a high content of blood glucose. The two most common type of diabetes is named Type 1 and Type 2. Type 1 diabetes is characterized by non-production of insulin, most often occurs in children and young adults. People with Type 1 diabetes must take daily insulin injections to stay alive. It accounts for 5-10% of people with diabetes. Type 2 diabetes is characterized by decreased production or improper use of insulin; this is the most common form of the disease accounting for 90 - 95% diabetes.
Type 2 is nearing epidemic proportions, due to an increased number of elderly people and high prevalence of obesity and sedentary lifestyles [1]. Diabetes is becoming the third killer of the health of mankind along with cancer and cardiovascular diseases due to its high prevalence and mortality. Worldwide projections suggest that more than 300 million people will have diabetes by the year 2025 [2]. The cause of diabetes is both genetic, lifestyle and environmental factors such as obesity and lack of exercise. Coscinium fenestratum is a medicinal plant, its stems is widely used in traditional Ayurveda and Siddah systems of medicine for the treatment of diabetes mellitus, dyspepsia and febrifuge among others. Scientifically it has been reported to have anti-ulcer, hypotensive, antioxidant, hepatoprotective activities among others [3].

MATERIALS AND METHODS

Plant Materials: The stem of the plant Coscinium fenestratum (locally known as Akar Kuning in Malay and tree turmeric, false calumba or columbo weed in English) was used for this experiment. The stems of the plant from Sungai Perak Malaysia were collected and identified by Dr. Shamsul Khamis, a plant taxonomist from the laboratory of Natural Products (NATPRO), Institute of Bioscience (IBS) University Putra Malaysia. The specimen voucher number IBS/UPM/CF 143 was assigned and deposited in the herbarium of laboratory of Natural Products (NATPRO), Institute of Bioscience (IBS) University Putra Malaysia.

Animals: Healthy adult Sprague Dawley rats weighing 150-250 g were housed in UCSI University animal holding area. They were divided into 4 groups with 6 rats per group and 1 rat per cage made up of a standard polystyrene, false calumba or colombo weed in English) was used for this experiment. The stems of the plant from Sungai Perak Malaysia were collected and identified by Dr. Shamsul Khamis, a plant taxonomist from the laboratory of Natural Products (NATPRO), Institute of Bioscience (IBS) University Putra Malaysia. The specimen voucher number IBS/UPM/CF 143 was assigned and deposited in the herbarium of laboratory of Natural Products (NATPRO), Institute of Bioscience (IBS) University Putra Malaysia.

Drugs: Streptozotocin (STZ), tolbutamide and Palmatine, where bought from Sigma-Aldrich, USA while xylazil and Ketamine where bought from Troy, Australia.

Preparation of Plant Extract: Stems of Coscinium fenestratum (1.2 kg) were air dried in the fume hood for 7 days and ground into fine powder. About 170 g of stem powder in a 2500 mL conical flask filled with DCM as the extraction solvent at room temperature. The ratio of solvent to solid used was 1:15. After 48 hours of maceration, each flask of the mixtures was filtered four times by using Whatman No.1 filter paper to obtain clear filtrates. The clear filtrates were then rotary evaporated at 45°C for 12 hours. The total yield of the concentrated crude extract was recorded. The mixture was stirred till homogenized and left to dry in the fume hood for a night. 80 g of silica gel mixed with 200 mL of hexane in a beaker and the mixture was stirred into slurry form and packed in a pre-washed column with the height of 60 cm and diameter of 2.5 cm with the aid of a filter funnel and glass rod. The crude extracts obtained were mixed with silica gel in the ratio of 5:1. Ethyl acetate and hexane with the ratio of 9:1 was used to flush out fraction A to D. Then ethyl acetate and methanol at a ratio of 9:1 until 100% methanol was used to flush out Fraction E. TLC spotting(s) were done to confirm the identity of the targeted fraction, fraction E by judging from the color (bright yellow) under the UV wavelength of 365 nm and Rf values of the spot (0.35). The solvent system used in developing TLC was according to the mobile phase system of the column.

HPLC Analysis: HPLC analysis of the partially purified Fraction E extract was performed by using Perkin Elmer Flexar FX15 Ultra High Performance Liquid Chromatography (UHPLC) system. The column used was Agilent Zorbax XDB C18-150mm X 4.6mmX 5uM. The mobile phase consisted of buffer A: water with 0.1% formic acid and 5mM ammonium formate. B: acetonitrile with 0.1% formic acid and 5mM ammonium. Gradient run program were 10% B to 95% B from 0.01min to 5.0min, hold for 1 min and back to 10% B in 0.1 min and re-equilibrated for 1min. All samples were dissolved accordingly as per instructed (saline which is aqueous, methanol or 50:50 methanol: water). Samples were then filtered with 0.22uM nylon filter before injection and volume of injection 5ul.

Study Design: Sprague Dawley rats numbering 24 were divided into 4 groups (n=6);

Group 1: Normal rats received 1ml/kg of saline
Group 2: diabetic rats received 1ml/kg of saline
Group 3: Diabetic rats received 100 mg/kg of Tolbutamide
Group 4: Diabetic rats received 100mg/kg of Fraction E.
The rats were fasted overnight prior to Streptozocin (STZ) diabetic induction. Each drug was administered via oral gauge once a day for 90 days. The weight and plasma glucose level of the rats was measured every week for 12 weeks.

**Diabetic Induction and Treatment**: The induction of diabetes was performed according to the method of Bagheri et al. [4]. The rats were treated according the study design above.

**In vivo Antioxidant Assay**

- **Lipid Peroxidation Assay (LPO)**: Lipid peroxidation was done according to the method of Nasker et al. [5].
- **Superoxide Dismutase (SOD) Enzymatic Assay**: For superoxide dismutase assay was done according to the method of Kono [6].
- **Catalase (CAT) Assay**: Catalase activity was evaluated according to Beers and Sizer [7].
- **Glutathione Reductase (GSH)**: Glutathione reductase was carried out according to the method of Oyedemi and Afolayan [8].

**Hematology, Biochemical and Lipid Profile Assay**: Rats were anaesthetized with xylazil and ketamine (1:1) and blood was collected by cardio puncture. The plasma and serum were obtained and sent to the pathology lab (Gribbles Kuala Lumpur) for the respective tests.

**Histopathology**: This was carried out according to the method of Haihong et al. [9].

**Statistical Analysis**: All data were presented as mean (±) standard error mean (SEM) using graphpad prism 6.05. The data were statistically analyzed by two-way ANOVA followed by Dunnet’s test. Values were considered statistically significant when $p<0.05$.

**RESULTS AND DISCUSSIONS**

**HPLC Analysis**: The chromatograms from HPLC analysis clearly showed the presence of palmatine (palmiline) in the sample of fraction E (Figures 1 A and B). HPLC analysis detected Palmatine in Fraction E. Palmatine belongs to the protoberberine class of isoquinoline alkaloids. It is a close structural analog of berberine and has been used in the treatment of jaundice, dysentery, hypertension, inflammation and liver-related diseases [10]. Palmatine has been reported to have antioxidant effect [11].

**Diabetic Study**: The plasma glucose levels of treated group showed significant reduction (Figure 2) while the body weight of treated groups showed significant increase (Figure 3). Also our finding shows that Palmatine has a significant plasma glucose lowering property. Streptozotocin (STZ) induced diabetes in experimental animal model has been used to analyze the effect of antidiabetic agents. STZ’s action on β cells causes a distinctive decrease in blood insulin, body weight and increase in plasma glucose concentrations [12]. As was observed in this study, there was an increase in plasma glucose level from week 0 to week 5. Tolbutamide and fraction E were able to quench the effect of STZ and reduced the plasma glucose level from week 6 to week 12. Antidiabetic effect of sulfonylurea (tolbutamide) is thought to activate insulin release solely by inhibiting high – affinity ATP-sensitive potassium (K-ATP) channel receptors at the plasma membrane of β – cells [13]. It has also been reported that sulfonylurea (tolbutamide) potentiate insulin release through non-dependence on calcium. High-potassium stimulation evokes a sustained rise in β – cells calcium level but a transient rise in insulin secretion. With continued high-potassium depolarization addition of sulfonylurea (tolbutamide) dramatically enhance insulin release without affecting calcium [14]. Fraction E was also able to reduce the plasma glucose level as compared to tolbutamide. The mechanism action of fraction E may be partially acting in a similar mechanism to tolbutamide as discussed above.

**In vivo Antioxidant Assay**: For this study, the GSH, LPO, SOD and CAT activities were measured (Figure 4). Antioxidants are substances or nutrients in our food substances, which can slow down the oxidative stress/damage by quenching the free radicals in our body. Oxidative stress is increased in patients with diabetes mellitus and evidence suggests that oxidative cellular injury caused by free radicals contributes to the development of diabetes [15]. Antioxidant enzymes are used to scavenge the free radicals and protect the organs and membranes from oxidative damage. Diabetes has also been shown to induce changes in the tissue content and activity of the antioxidant enzymes [16].
Fig. 1: (A) Chromatogram of Fraction E. (B) chromatogram of Palmatine Fraction E and standard Palmatine were dissolved in methanol and filtered with 0.22uM nylon filter before injection. The injection volume was 5uL
Fig. 2: Mean plasma glucose level of rats per group. Rats were treated over a period of 90 days. All data were presented as mean (±) standard error mean (SEM) (n = 6) using SPSS. The data were statistically analyzed by two-way ANOVA followed by Dunnett’s test. Values were considered statistically significant when p< 0.05

Fig. 3: Mean body weight of rats per group. Rats were treated over a period of 90 days. All data were presented as mean (±) standard error mean (SEM) (n = 6) using SPSS. The data were statistically analyzed by two-way ANOVA followed by Dunnett’s test. Values were considered statistically significant when p< 0.05

Fig. 4: *In vivo* antioxidant activity. Values were considered statistically significant when p< 0.05. Rats were treated over a period of 90 days
Lipid peroxidation is a characteristic of diabetes mellitus. The increase of free radicals in diabetic condition is suggested to be due to the increased lipid peroxidation and the damage of antioxidant defense system [17]. Reactive oxygen metabolites have been implicated in the damage brought by ionizing radiation, as well as in the effects of several cytostatic compounds. The decreased activity of antioxidant molecules along with elevated lipid peroxide levels in diabetic rats could probably be associated with oxidative stress and decreased antioxidant defense potential [18]. SOD protects tissues against oxygen free radicals by catalyzing the removal of superoxide radical, converting it into H₂O₂ and molecular oxygen, which both damage the cell membrane and other biological structures [19]. Catalase is a haem-protein, which is responsible for the detoxification of significant amounts of H₂O₂. Reduced glutathione is one of the most abundant non-enzymatic antioxidant bio-molecules present in tissues whose functions are removal of reactive oxygen species and provision of a substrate for GPx and glutathione S-transferase (GST) [20]. Our results showed that in negative control the levels of lipid peroxidation were high in pancreas and were reduced after the treatment with Fraction E. There was a decline in the activities of SOD, GSH and CAT in negative control animals and it became near to normal levels in Fraction E and tolbutamide treated rats. This indicates that oxidative stress elicited by STZ may have been nullified due to the effect of fraction E [21]. The action of fraction E may be due to its strong antioxidant property, which has been previously reported by Dillard, Kunert and Tappel [22]. to possess a very strong in vitro antioxidant and free radical scavenging activity. Administration of fraction E may have assisted in protecting; stimulating the antioxidant enzymes in STZ induced diabetes rats and inhibiting lipid peroxidation processes in vivo.

**Hematology, Biochemical and Lipid Profile Assay**

**Hematology (Hemoglobin, Red Blood Cell Count and White Blood Cell):** Hemoglobin level of the negative control was 237gL⁻¹, which is higher as compared to the other groups: normal control 139gL⁻¹; tolbutamide 166gL⁻¹ and Fraction E 146gL⁻¹ which were within the standard range. The red blood cell count negative control was 12.04 x 10¹² gL⁻¹ which was also higher compared to normal control 7.52 x 10¹² gL⁻¹; tolbutamide 9.13 x 10¹² gL⁻¹ and Fraction E 7.59 x 10¹² gL⁻¹.

The white blood cell count of the negative control was 1.8 x 10³μL which was lower than the standard white blood cell count; Tolbutamide 9.1 x10³μL and Fraction E 8.3 x 10³μL which were within the standard range (Figure 5). The significant increase in hemoglobin and red blood cells level in the negative control maybe due to polyuria which is always present in poorly controlled diabetes and reflects poor control of plasma glucose levels in diabetic patients [23]. Hemoglobin rise may also have been in response to the damage of antioxidant defense system. Reports by Luo et al. [24] have shown that hemoglobin that is located outside the red blood cell has been shown to possess an antioxidant property and a regulator of iron metabolism. Contrary to other studies where increase in blood glucose level is associated with increase in white blood cell, this study showed a decrease rather than an increase in the white blood cell of the negative control. The reason for the low white blood cell count in this study may be because of the liver damage caused by STZ induction. Because liver damage has been associated with decreased white blood cell count [25].

**Biochemical Test (Renal and Liver Function Test)**

**Renal Function Test:** In this study, Urea levels were reported as 15.4 mmolL⁻¹ for negative control; 8.8 mmolL⁻¹ for normal control; 10 mmolL⁻¹ for tolbutamide and 8.1 mmolL⁻¹ for Fraction E. Creatinine measured was 30 umolL⁻¹ for negative control; 37 umolL⁻¹ normal control; 59 umolL⁻¹ for tolbutamide and 52 umolL⁻¹ for Fraction E (Figure 6). For the renal function test, the levels of urea were elevated and creatinine where decreased in the negative control compared fraction E, tolbutamide and normal group. The elevation of urea and reduction of creatinine in negative control, maybe due to an impaired filtering capacity of the nephron of the kidney. This condition, which is a sign of progressive renal damage, may cause an accumulation of waste products in the system and it may have been caused by STZ induction [26].

**Liver Function Test:** The Alkaline Phosphatase (AP) measured was 1224 UL⁻¹ for negative control, 80 UL⁻¹ for normal control, 380 UL⁻¹ for tolbutamide and 150 UL⁻¹ for Fraction E. Aspartate Transaminase (AST) measured was 947 gL⁻¹ for negative control, 144 gL⁻¹ for normal control, 1304 gL⁻¹ for tolbutamide and 311 gL⁻¹ for Fraction E. Alanine Aminotransferase (ALT) measured was 421 UL⁻¹ for negative control; 88 UL⁻¹ for normal control; 738 UL⁻¹ for tolbutamide and 73 UL⁻¹ for Fraction E (Figure 7). The elevation of AP, AST and ALP reflects liver synthetic function, measure of the concentration of intracellular hepatic enzymes that may have leaked into the circulation which serve as a marker of hepatocyte injury [27].
Lipid Profile Test: Triglycerides measured were 6.41 mmol/L for negative control, 0.78 mmol/L for normal control, 1.99 mmol/L for tolbutamide and 1.28 mmol/L for Fraction E. High density lipoprotein (HDL) measured was 0.63 mmol/L for negative control, 1.62 mmol/L for normal control, 1.01 mmol/L for tolbutamide and 1.26 mmol/L for Fraction E. The low density lipoprotein (LDL) content was 4.24 mmol/L for negative control, 0.05 mmol/L, normal control, 0.82-mmol/L tolbutamide and 0.75 mmol/L Fraction E (Figure 8). Lipid function test showed decreased levels of HDL and elevated levels of triglycerides and LDL. A study by Arshag [28] revealed that the distinguishing features of abnormal lipid levels in diabetes are high plasma triglyceride concentration,
Fig. 8: Lipid profile. The data were statistically analyzed by two-way ANOVA followed by Dunnet’s test. Values were considered statistically significant when $p < 0.05$

Fig. 9: H&E stained pancreatic sections of (A.) Non Diabetic - Normal, (B-D.) Diabetic rats; Negative, Tolbutamide and Fraction E, respectively. Rats were treated over a period of 90 days.

The changes in lipid function associated with diabetes mellitus may be due to increased free fatty acid flux secondary to insulin resistance [29]. Fraction E was able to maintain hemoglobin, red blood cells, AP, AST, ALP, LDL, HDL more than tolbutamide. This may be because of the protective effect of fraction E because of its strong antioxidant and free radical scavenging activity. This quality of fraction E enables it to restore the antioxidant defense system this may be the difference in the mechanism of action fraction E compared to tolbutamide.

**Histology:** The normal group showed normal lobular architecture of the pancreas. The pancreas had abundant islet of Langerhans interspersed among the pancreatic exocrine acini. The islets appeared lightly stained than the surrounding acinar cells, with intact Interlobular connective tissue and interlobular duct. The pancreatic sections of the negative group showed marked morphological changes. The border between the endocrine and exocrine region became indistinct. There were appearances of infiltration of inflammatory cells through the connective tissue septa and around the periphery of the islets. Blood vessels were seen congested and dilated and some islets were completely destroyed leaving empty spaces. Fraction E and tolbutamide groups showed improvement in the previous morphological changes in most of the rats. The border between exocrine and endocrine portions became distinct, more islets were shown with an increase in the cellular density and there was also a sign of reduction in the migration of inflammatory cells inside the islet (Figure 9).
The presence of intact β–cells based on the histological image of the fraction E treated group showed that it’s able to protect the β–cells and delay deterioration of β–cells. It has been reported that once hyperglycemia becomes apparent, beta cells function progressively deteriorates; glucose-induced insulin secretion becomes further impaired and degranulation of beta cells become evident, often accompanied by decrease in the number of β–cells. Fraction E as a strong antioxidant may be exerting beneficial effects on preservation of pancreatic β–cells, function in diabetes. It reduces blood glucose levels and glucose toxicity and contributed in part to the prevention of a decrease of β–cells mass and insulin content. Fraction E may also have rejuvenated β–cells, increasing the sensitivity of insulin receptors to insulin thereby allowing glucose to be taken up by the cells leading to a decrease in blood glucose level [30].

CONCLUSION

The present study showed that there is a proportional relationship between elevated blood glucose level and hemoglobin, red blood cells, urea, Creatinine, AP, AST, ALP, Triglyceride and LDL cholesterol and an inverse relationship between elevated blood glucose level and white blood cell and HDL cholesterol. This study also showed that Fraction E reduced the lipid peroxidation level and increased the antioxidant level in experimental rats, protected the pancreas by suppressing the oxidative stress associated with diabetes and decreased the blood glucose. HPLC analysis shows the presence of Palmatine.

Conflict of Interest: There are no conflicts of interests to report. This work was supported by funds from Centre of Excellence for Research, Value, Innovation and Entrepreneurship Research Grant Scheme UCSI University (UCSI-CERVIE-RGS Proj-in-FAS 017).

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