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Attenuation of diabetic hepatopathy in alloxan-induced diabetic mice by methanolic flower extract of *Phlogacanthus thyrsiflorus* Nees

Jutishna Bora, Plabita Sahariah, Abani Kumar Patar, Donkupar Syiem, Surya Bhan*
Department of Biochemistry, North Eastern Hill University, Shillong-793022, Meghalaya, India.

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ABSTRACT

The present investigation is aimed to determine the effect of methanolic flower extract (MFE) of *phlogacanthus thyrsiflorus* on hyperglycemia, hyperlipidemia and oxidative stress in alloxan-induced diabetic mice. Diabetic mice prepared with alloxan (150 mg/kg) body weight (bw) were treated with effective different doses (150-550 mg/kg bw) of MFE. Intraperitoneal glucose tolerance test (IPGTT) was performed. Activities of marker enzymes and lipid profile were determined. Similarly, superoxide dismutase (SOD) - cytosolic CuZn-SOD and mitochondrial Mn-SOD, catalase (CAT) and glutathione reductase (GR) activities were examined. After treatment with MFE, 250 mg/kg bw dose was found to be effective in significantly reducing fasting blood glucose (FBG) level. In IPGTT, there was a significant reduction of FBG level in treated groups. The MFE also exhibited a significant hypolipidemic effect as evident from a decrease in total cholesterol, triglycerides, low-density lipoproteins-cholesterol (LDL-C), very low-density lipoproteins-cholesterol (VLDL-C) and increased high-density lipoproteins-cholesterol (HDL-C) level. Activities of marker enzymes were significantly decreased in treated diabetic groups. However, the activities of antioxidant enzymes were significantly increased in treated diabetic groups. The histopathological studies of the liver tissue in MFE treated diabetic mice revealed almost normal appearance. This study supports the traditional proclamations and use of flowers of *Phlogacanthus thyrsiflorus* for the management of diabetes.

INTRODUCTION

Diabetes, termed as diabetes mellitus, is a major epidemic of this century (Shaw *et al.*, 2010). The incidence of diabetes is rapidly increasing with estimates that the number will almost be doubled by 2030 (Wild *et al.*, 2004). Diabetes is clinically characterized by hyperglycemia due to its chronic and/or relative insulin insufficiency (Mathis *et al.*, 2001). Diabetes mellitus is also associated with insulin resistance, hyperlipidemia, hypertension (Taylor *et al.*, 1994). Moreover, it is linked with increased free radicals leading to compromise defense system (Saxena *et al.*, 1993). The management of diabetes mellitus is considered to be a global problem and successful treatment has yet to be discovered. In India, indigenous remedies have been always used in the treatment of diabetes mellitus since the sixth century (Kashikar and Tejaswita, 2011). *Phlogacanthus thyrsiflorus* is a shrub from a family of Acanthaceae, which has several common names such as titaaphul, chuhai, dieng-soh etc. *Phlogacanthus thyrsiflorus* is one of a common herbal plant used by local people of Assam to manage diabetes. The generation of free radicals has been implicated in the causation of several diseases such as Rheumatoid arthritis, Cancer, Diabetes etc., and compound that can scavenge free radicals present in *Phlogacanthus thyrsiflorus* have great potential in ameliorating these disease processes. *Phlogacanthus thyrsiflorus* has prominent free radical scavenging property so it may prove as a very good medicinal herb (Jaiswal, 2010). Considering the above medicinal properties of *Phlogacanthus thyrsiflorus*, it is of interest to undertake a systematic study of such plant. Thus present study was aimed to investigate the antihyperglycemic, antihyperlipidemic and antioxidative properties of methanolic flower extract (MFE) from *Phlogacanthus thyrsiflorus*.

MATERIALS AND METHODS

Chemicals

Alloxan, metformin, and pyrogallol were purchased

*Corresponding Author
Dr. Surya Bhan, Assistant Professor, Department of Biochemistry, North Eastern Hill University, Shillong, 793022.
E-mail: sry_bhan @ yahoo.co.in

from Sigma-Aldrich Co. (St. Louis, MO, USA.). Glibenclamide was procured from Emcure Pharmaceuticals Ltd. (Pune, India). Insulin was procured from Gland Pharma Ltd. (Hyderabad, India). Total cholesterol, triglyceride, HDL and SGOT, SGPT kits were purchased from Coral Biosystems (Goa, India). The other chemicals used were of analytical grade procured from Merck Co. (Mumbai, India) and Sisco Research laboratory.

Plant material

Flowers of *Phlogacanthus thyrsiflorus* were collected during January from Assam, India (Voucher No: 12055). The specimen was submitted and authenticated by Dr. P.B. Gurung Curator herbarium, Department of Botany, NEHU, Shillong, Meghalaya.

Methanolic extracts preparation

The collected flowers were separated, weighed, washed and subjected to the shade-dried. It was then powered, homogenized and repeatedly extracted with 10X volume of aqueous-methanol solution (1:4) (Harborne, 1998). The dried mass was stored at −20°C and used for further studies.

Phytochemical screening

The MFE was subjected to qualitative phytochemical analysis for alkaloids, flavonoids, tannins, saponins, and phenols as per the standard methods (Wali *et al.*, 2012).

Test animals

Adult healthy Swiss male albino mice (Balb/C strain), 20-30 g in weight were used for all the studies. The procedure of all the experiments was reviewed and carried out in accordance with the Institutional Ethics Committee guidelines (Animal models), North-Eastern Hill University, Shillong, Meghalaya, 04-12-2014. Mice were housed under controlled temperature i.e. at 22°C on a 12 hr light/dark cycle and were fed with mice feed which were obtained from the laboratory of Amrut, Pune, India and water *ad libitum.*

Acute toxicity study

Acute toxicity test i.e. lethal dose 50 (LD50) for the MFE was carried out as per Organisation for Economic Cooperation and Development Guidelines 425 (OECD) guidelines. Six different test groups were considered containing six female mice in each group and increasing doses from 200 mg/kg body weight (bw) to 2000 mg/kg bw were administered intraperitoneally following the limit test procedure. Animals in all groups were observed till 24 hr. LD50 value was determined by the Arithmetic method described by Ghosh (Ghosh, 2015).

$$LD50 = LD100 - (\text{Product a} \times \text{b/number of animals in each group}),$$

where a = Dose difference (Dose in given interval – Dose in upper interval) and b = Mean mortality (No. of dead mice in given interval + No. of dead mice in upper interval)/2.

Induction of diabetes mellitus in test animals

Alloxan monohydrate (150 mg/kg bw) prepared in citrate buffer (0.1 M, pH 4.5) was administered intraperitoneally to overnight fasted test albino mice for induction of diabetes (Syiem *et al.*, 2002). After alloxan injection, mice had free access to food and water. The fasting blood glucose (FBG) levels were checked after 72 hr of alloxan injection and the mice showing blood glucose level more than 200 mg/dl were selected and used for further tests.

Antihyperglycemic study

Alloxan induced diabetic mice were divided into one control and six test groups comprising of six mice in each group. Control group consisting of diabetic mice administered with only distilled water and diabetic mice in test groups were administered with increasing doses (150 mg/kg bw–550 mg/kg bw) of MFE intraperitoneally to carry out the antihyperglycemic study. Doses were injected intraperitoneally every alternate day for 21 days (d) and FBG levels were monitored on 7 d, 14 d and 21 d respectively (Syiem and Khup, 2006).

Intraperitoneal glucose tolerance test (IPGTT)

Alloxan-induced diabetic mice were divided into one control (diabetic mice) and four test groups comprising of six mice in each group to perform intraperitoneal glucose tolerance test (IPGTT) test. The MFE (250 mg/kg bw), the drugs- metformin (200 mg/kg bw) and glibenclamide (10 mg/kg bw) dissolved in 10X volume of aqueous-methanol solution (1:4) and the hormone insulin (10 U/kg bw) were administered to the test groups intraperitoneally prior to the glucose load (2 g/kg bw) (Syiem *et al.*, 2002). The reason behind selecting three standard drugs in this study only to determine the possible mechanism of action of MFE since each drug has a different mechanism of action to lower the blood glucose level. The FBG levels were monitored before the administration and subsequently at 0.5 hr, 1 hr, 2 hr and 4 hr respectively after the glucose load.

Biochemical analysis

Serum and liver parameters

Four different test groups comprising of six mice in each group were considered and doses were injected intraperitoneally every alternate day for 21 d.

Normal untreated group: Normal mice administered only with distilled water.

Diabetic untreated group: Diabetic mice administered only with distilled water.

Ascorbic acid treated group: 50 mg/kg bw dose of ascorbic acid administered to diabetic mice.

MFE treated group: 250 mg/kg bw dose of MFE administered to diabetic mice.

Blood was collected from retro-orbital plexus of the animals and serum was isolated from all the blood samples for lipid profile tests and for hepatic marker enzyme assays. In lipid profile test, total cholesterol, high-density lipoprotein-cholesterol (HDL-C), and triglyceride levels were measured using CHOD/PAP kit method, PEG/CHOD-PAP kit method and GPO/PAP kit method respectively. The low-density lipoprotein-cholesterol (LDL-C) and very high-density lipoprotein-cholesterol (VLDL-C) levels were calculated according to the formula (Friedewald *et al.*, 1972) mentioned below

$$\text{VLDL-C} = \text{Triglycerides}/5,$$

$$\text{LDL-C} = \text{Total Cholesterol} - (\text{HDL-C} + \text{VLDL-C}).$$

Serum hepatic marker enzyme, serum glutamate oxalate - transaminase (SGOT) and serum glutamate pyruvate - transaminase (SGPT) activities were determined using the Coral diagnostic kit (Coral Clinical Systems, Goa, India) and mean activities were expressed in terms of units/liter (U/L).

Liver antioxidant enzyme assays

Swiss albino mice were divided into four groups comprising of six mice in each group for the study. Doses were administered intraperitoneally on alternate days for a total period of 21 d.

Normal untreated group: Normal mice administered only with distilled water.

Diabetic untreated group: Diabetic mice administered only with distilled water.

Ascorbic acid treated group: Ascorbic acid (50 mg/kg bw) treated diabetic mice.

MFE treated group: MFE (250 mg/kg bw) treated diabetic mice.

Preparation of homogenate

Liver tissue was excised from all the experimental groups by decapitation after 21 d. Tissue were homogenized to make 10% (w/v) homogenates in ice-cold 10 mM HEPES buffer, pH 7.4 containing 0.2 M mannitol, 50 mM sucrose and 1 mM EDTA. Nuclei and cell debris were sedimented by centrifuging tissue homogenate for 10 mins at 4°C at 1000 g. The supernatant collected was again centrifuged for 10 mins at 4°C at 7500 g. Mitochondrial fraction was obtained with the resulting mitochondrial pellet from above procedure being washed gently by suspending in homogenate buffer and then resedimented at 7500 g for 10 mins at 4°C. Cytosolic fraction was obtained by centrifuging the post-mitochondrial supernatant further for 10 mins at 4°C at 1500 g.

The protein concentration of cytosolic and mitochondrial fraction was determined by Bradford method (Bradford, 1976) using bovine serum albumin as a standard.

Superoxide dismutase (SOD) both Mn-SOD and Cu/Zn-SOD activity was determined with slight modifications according to the method of Marklund and Marklund (Marklund and Marklund, 1974). The Catalase (CAT) activity was estimated by the method of Aebi (Aebi, 1984). The glutathione reductase (GR) activity was assayed by the method of Carlberg and Mannervik (Carlberg and Mannervik, 1985).

Histopathological examination

Liver of normal, diabetic and diabetic mice treated with ascorbic acid and MFE were excised after 21 days by sacrificing the mice by cervical dislocation and the histological study was carried out using hematoxylin-eosin (HE) staining techniques (Kiernan, 1999).

Statistical analysis

Results of all the experiments are expressed as mean ± SEM for each group consisting of six mice in each group. Data were analyzed using one-way ANOVA followed by tukey's post hoc test which was performed to compare the differences between all the experimental groups using statistical IBM SPSS software package for Windows version 19.0. Statistical significance was put as $p < 0.05$.

RESULTS

Qualitative phytochemical analysis

Phenols, flavonoids, and tannins were strongly present in the MFE whereas alkaloids and saponins were slightly present.

Acute toxicity studies

LD50 value of the extract was 1867 mg/kg bw which indicates that MFE is safe and is not toxic to mice.

Antihyperglycemic study

The MFE administered to diabetic mice elicited marked and prolonged antihyperglycemic action in time and dose-dependent manner (figure 1). The antihyperglycemic effect was found to be more pronounced at the 21st d for all the doses used. There was a minimal reduction (18.3%) of FBG level at the dose of 150 mg/kg bw on 21 d. However, a significant reduction (23%) was observed at 250 mg/kg bw on 7 d, (34%) on 14 d and (45.9%) on 21 d from that of the control group. However, administration of MFE at the higher doses resulted in marked antihyperglycemic effect. At 350 mg/kg bw, the FBG levels were (28.7%) on 7 d, (46.6%) on 14 d, (58.8%) on 21 d when compared with the diabetic control group in the study. At the doses of 450 mg/kg, bw and 550 mg/kg bw showed a drastic reduction of FBG level. At 450 mg/kg bw, the FBG levels were 55% on 7 d, 63.2% on 14 d, 64.6% on 21 d while at 550 mg/kg bw the FBG levels were 67% on 7 d, 74% on 14 d and 76.6% on 21 d.

Intraperitoneal glucose tolerance test (IPGTT)

The result of IPGTT is shown in figure 2. Following intraperitoneal administration of glucose load (2 gm/kg bw), MFE treated group showed significant reduction of FBG level (44%) at 2 hr, (46.6%) at 4 hr and metformin-treated group showed significant reduction of FBG level (56.8%) at 2 hr and (60%) at 4 hr when compared to the diabetic control group. Glibenclamide treated group showed very low reduction of FBG level (15%) at 2 hr and (10%) at 4 hr whereas insulin-treated group showed more pronounced glucose tolerance than all other groups with significant reduction of FBG level (42.2%) at 5 hr, (60.9%) at 1 hr, (84%) at 2 hr and (89.4%) at 4 hr when compared with diabetic control group in the study.

Biochemical analysis

Serum and liver parameters

The result of lipid profile in different experimental groups is shown in table 1. There were significant differences in the level of total cholesterol, triglyceride, HDL-C, LDL-C, and VLDL-C between the diabetic untreated group and normal untreated group in the study. Further, the total cholesterol, triglyceride, HDL-C, LDL-C and VLDL-C levels were found to be different in ascorbic acid treated group, as well as MFE, treated group when compared to the diabetic untreated group in the study.

As shown in Figure 3, the level of SGOT and SGPT were found to be significantly higher in the diabetic untreated group (104.2 ± 3.2 and 58.7 ± 2.1) in comparison to the normal

untreated group (50 ± 4.2 and 34.1 ± 3.1). However, the level of SGOT and SGPT of ascorbic acid treated group (69.07 ± 3.2 and 40.6 ± 1.9) and MFE treated group (70.7 ± 1.2 and 38.2 ± 0.9) were significantly lower ($p < 0.001$) than untreated diabetic group in the study.

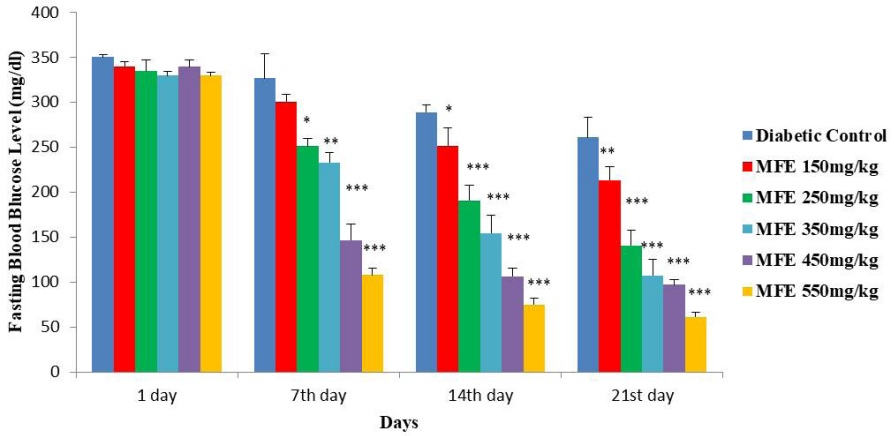

Fig. 1: Effects of MFE on diabetic mice showing changes of FBG level at the different time interval for different experimental groups (150–550 mg/kg bw dose). Values are expressed in Mean ± SEM; n = 6. *p < 0.05, **p < 0.01, ***p < 0.001 versus diabetic control. (One-way ANOVA followed by tukey's post hoc test).

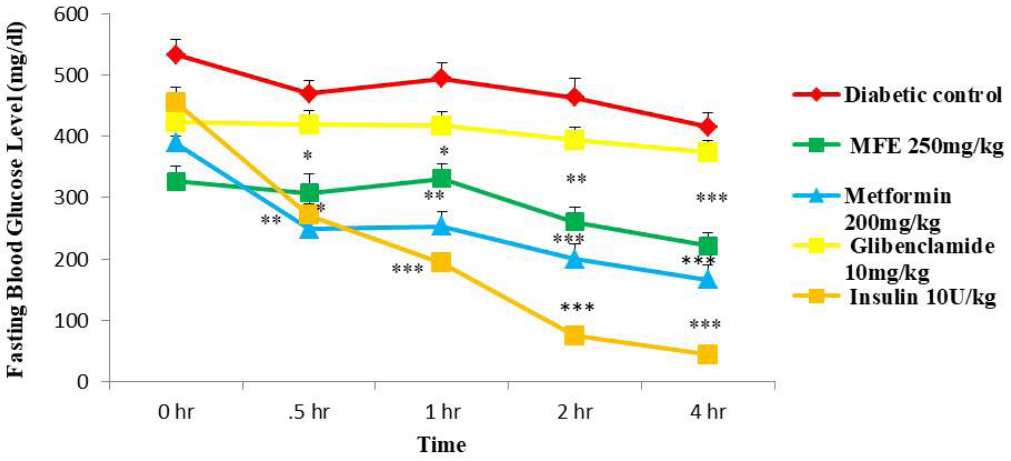

Fig. 2: IPGTT in different experimental groups administered with MFE and standard drugs (metformin, Glibenclamide, and insulin) in diabetic mice assayed at different time intervals. Values are expressed in Mean ± SEM; n = 6. *p < 0.05, **p < 0.01, ***p < 0.001 versus diabetic control. (One-way ANOVA followed by tukey's post hoc test).

Table 1: Effect of MFE on serum lipid profile in alloxan induced diabetic mice.

Groups	Total cholesterol (mg/dl)	Triglyceride (mg/dl)	HDL (mg/dl)	LDL (mg/dl)	VLDL (mg/dl)
Normal untreated group	118.0 ± 0.53	97.8 ± 3.11	75.5 ± 1.1	22.9 ± 1.1	19.5 ± 0.62
Diabetic untreated group	176.7 ± 2.9	159.5 ± 12.2	49.9 ± 1.9	94.8 ± 2.5	31.9 ± 3.3
Ascorbic acid treated group	115.5 ± 3.59***	101.8 ± 0.38**	72.5 ± 1.1**	22.5 ± 1.3***	20.3 ± 0.07**
MFE treated group	123 ± 5.2***	112.4 ± 2.1*	68.2 ± 0.91***	32.7 ± 5.2***	22.4 ± 0.42*

Values are expressed in Mean ± SEM; n = 6. *p < 0.05, **p < 0.01, ***p < 0.001 versus diabetic control. (One-way ANOVA followed by tukey's post hoc test).

Antioxidant enzyme assays

SOD (CuZn-SOD and Mn-SOD), CAT and GR activities in liver tissue of the experimental group are shown in table 2. The SOD, CAT and GR activities were significantly reduced in the diabetic untreated group. However, ascorbic acid treated diabetic group and MFE treated diabetic group showed a significant increase in antioxidant activity with increased in SOD, CAT and GR activities in comparison to the diabetic control group in the study.

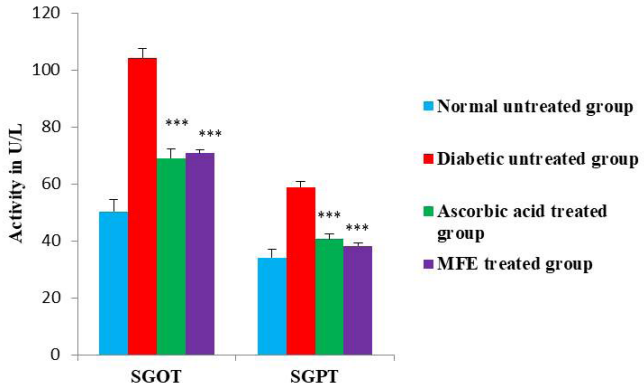

Fig. 3: Mean activity of hepatic marker enzymes (SGOT and SGPT) in the serum of Swiss albino mice. Values are expressed in Mean ± SEM; n = 6. ***p < 0.001 versus diabetic control. (One-way ANOVA followed by tukey's post hoc test).

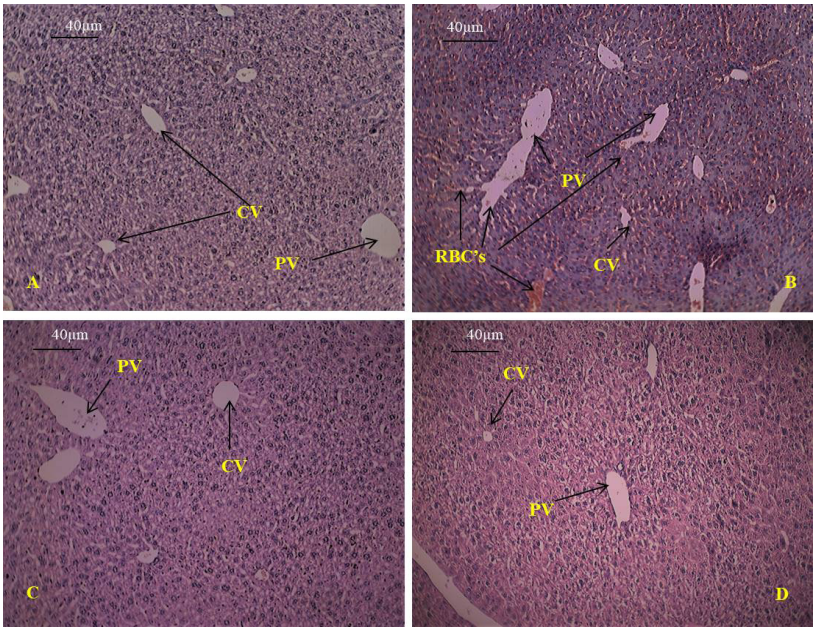

Fig. 4: Histological study of the liver (representative H&E-stained). A1: normal mice, B: diabetic mice, C: ascorbic acid (50 mg/kg bw) treated diabetic mice, D: MFE (250 mg/kg bw) treated diabetic mice. CV: central vein, PV: portal vein and RBCs: Red blood cells. All images are under 10X magnification.

Histopathological examination

As shown in figure 4, there was a loss of normal histological structure in the liver of diabetic mice with enlarged and distorted central and portal vein, sinusoidal dilation with red blood cells congestion was seen around central and portal vein when compared with normal mice. However, diabetic mice treated with ascorbic acid and MFE showed near normal histological structure with no central and portal vein enlargement or distortion. Moreover, sinusoidal dilation and red blood cells congestion around central and portal vein were also not seen.

DISCUSSION

The bioactivity of this crude extract is because of the presence of phenols, flavonoids, and tannins. Flavonoids are highly effective scavengers to oxidized molecules, including singlet oxygen, and various other free radicals implicated in several diseases (Bravo, 1998). Flavonoids suppress formation of reactive oxygen, chelate trace elements involved in the free-radical production, scavenge reactive species and up-regulate and protect antioxidant. Similarly, phenolic confer oxidative stress tolerance on plants (Agati, 2012). Acute toxicity study (LD50) was performed to examine the toxic effects when the extract is absorbed into the body via oral or intraperitoneal administration. The smaller the LD50 value, the more toxic would be the chemical (Turner, 1965). Induction of Type 1 diabetes by alloxan administration resulted in hyperglycemia in experimental groups. The doses below 150 mg/kg bw were found not to be effective to reduce the considerable blood glucose level and the doses above the 550 mg/kg bw showed severe hypoglycemic effect. The MFE exhibited a significant antihyperglycemic effect at the dose of 250 mg/kg bw. Hence, 250 mg/kg bw dose of MFE was considered to be the optimum dose for the study. In IPGTT, the MFE and other standard drugs showed a remarkable effect

on blood glucose tolerance in diabetic mice however the most notable effect was observed in the case of insulin. The pattern of glucose clearance from blood in MFE administered group was quite similar to the drug metformin, which action is to enhance insulin action and absorption of glucose in peripheral tissues. The functional mechanism of drug glibenclamide used in the study is to stimulate pancreatic beta cells to release more insulin and inhibit glucagon secretion. Therefore, from the present study, the functional mechanism of MFE in lowering blood glucose level can be assumed as similar to the mechanism of metformin action.

Table 2: Mean activities of SOD, CAT, GR in MFE treated diabetic mice.

Groups	Superoxide dismutase (Units/mg protein)		Catalase (Units/mg protein)	Glutathione reductase (Units/mg protein)
	CuZn-SOD	Mn-SOD	CAT	GR
Normal untreated group	5.6 ± 0.06	12.70 ± 0.64	2.7 ± 0.16	2.4 ± 0.13
Diabetic untreated group	2.33 ± 0.12	8.19 ± 0.17	1.500 ± 0.02	1.463 ± 0.11
Ascorbic acid treated group	3.99 ± 0.22***	10.45 ± 0.13***	2.205 ± 0.13**	2.237 ± 0.039***
MFE treated group	3.90 ± 0.12***	10.64 ± 0.21***	2.378 ± 0.12***	2.121 ± 0.12**

Values are expressed as Mean ± SEM; n = 6. **p < 0.01, ***p < 0.001 versus diabetic control (One-way ANOVA followed by tukey's post hoc test).

Total cholesterol, LDL-C, VLDL-C and triglyceride levels are known to increase during diabetes and triggers the risk of coronary heart disease and atherosclerosis in diabetic patients (Murray, 1993). In this study, treatment with MFE for 21 d remarkably reduced total cholesterol, LDL-C, VLDL-C and triglyceride followed by an increase in HDL-C levels which indicates that MFE has potent antihyperlipidemic activity. Serum aminotransferases levels are elevated when there is damaged to the liver, which can be considered to follow the progress during a diabetic condition in mice (Vigneri and Goldfine, 1987). MFE treated diabetic mice showed lower serum SGOT and SGPT levels than untreated diabetic mice.

SOD (Mn SOD and CuZn-SOD) present in mitochondria and cytosol is a first-line antioxidant enzyme that defends against reactive oxygen species. They eradicate superoxide anion to hydrogen peroxide (Evans, 2003). The hydrogen peroxide is further neutralized to water and oxygen by CAT enzyme (Johansen, 2005) which is also an antioxidant enzyme. GR antioxidant enzyme in cytoplasm functions through converting oxidized glutathione (GSSG) to reduce form (GSH) and helps in maintaining a reduced intracellular environment. In the present study, it was observed that MFE treatment restore and increase the activities of antioxidants enzymes when compared with untreated diabetic mice. Therefore, it could be said that MFE protects liver tissue from further oxidative stress under diabetic condition. From the histological study, it is evident that MFE has the ability to protect and restore tissues from the injury of oxidative stress induced by diabetes. It is also clear from the study that MFE is able to suppress oxidative stress by enhancing the antioxidative enzyme activities, thus allowing tissues to recover from further damage in diabetic mice.

CONCLUSION

The findings of the present study signify that MFE has both antihyperglycemic and antihyperlipidemic effects in alloxan-induced diabetic mice and treatment with MFE may provide beneficial effects against oxidative stress. However, the exact mechanism is yet to be elucidated and further investigation is underway.

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CONFLICT OF INTERESTS

There are no conflicts of interest.

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