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Research Article

EFFECT OF *DILLENIA INDICA* L. AGAINST OXIDATIVE STRESS-INDUCED CARDIOMYOPATHY ON ALLOXAN-INDUCED DIABETES MICE MODEL

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ABSTRACT

Objective: The aim of the present study is to investigate the antihyperglycemic and antioxidative properties of *Dillenia indica* fruits.

Methods: Aqueous fruit extract and methanolic fruit extract (MFE) were prepared, and preliminary phytochemical screening was carried out. Diabetic mice were prepared with alloxan (150 mg/kg) body weight (b.w.). Antihyperglycemic study of short duration was carried out with doses (150–550) mg/kg b.w. of MFE in diabetic mice. Antioxidant enzymes (superoxide dismutase, catalase, and glutathione reductase) activity assays and histopathological analysis were done in heart tissue of mice.

Results: Preliminary phytochemical screening showed that the phytoconstituents were strongly present in the MFE and therefore was considered for further studies. From the antihyperglycemic study, it was found that 350 mg/kg b.w. dose was the most effective in reduction of blood glucose level. A significant increase in the activities of the antioxidant enzymes was observed in the MFE-treated group. From the histopathological studies, it was observed that detrimental effects of oxidative stress were attenuated in the treated group.

Conclusion: Concluding the studies, it could be ascertained that *D. indica* fruits were found to be quite effective in proving its potential against hyperglycemia and oxidative stress, and therefore, the fruits could be considered to be of therapeutic value in diabetes.

Keywords: *Dillenia indica*, Alloxan, Antihyperglycemic, Antioxidative.

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INTRODUCTION

Diabetes mellitus (DM) is a group of metabolic alterations which is characterized by hyperglycemia either resulting from defects in insulin secretion, action, or both [1]. DM can be divided primarily into Type 1 or insulin-dependent DM (IDDM) and Type 2 or non-IDDM. In both cases, symptoms such as polyuria, polydipsia, and various complications including retinopathy, nephropathy, and neuropathy are caused due to prolonged hyperglycemia [2]. A total of 171 million cases of diabetes have been registered worldwide by the year 2000 as per the World Health Organization studies. According to estimations, 366 million people will suffer from this disease by 2030 [3]. Development of macrovascular and microvascular complications has been shown to be dependent on hyperglycemia mainly [4,5] or partly [6,7] resulting in tissue damage through different pathways: (1) Enhanced polyol activity, causing sorbitol, and fructose accumulation; (2) increased formation of advanced glycation end products; (3) activation of protein kinase C; and (4) increased hexosamine pathway flux [8]. According to evidence, these pathways activated by hyperglycemia are associated with reactive oxygen species (ROS), generation ultimately leading to increased oxidative stress [8,9]. Several therapies are available for diabetes which includes insulin and various oral antidiabetic agents such as sulfonylurea, biguanide, metformin, α-glucosidase inhibitors, and troglitazone, but these are known to have a number of serious adverse effects [10]. Recent molecular investigations all over the globe highlight the power of herbs [11]. Hence, there is a need to explore herbal medicine in the context of modern science and validate accordingly. Herbal formulation and plant drugs [12,13] have been used successfully as they are considered to be less toxic and free from side effects than synthetic medications.

Dillenia indica is an evergreen large shrub or small- to medium-sized tree growing to 15 m tall belonging to the Dilleniaceae family with a common name elephant apple. Conventionally, it was also found that, for the treatment of cancer and diarrhea in various parts of Northeast India, the juices of bark and leaves were mixed and given orally [14,15]. The leaves and bark are also used as a laxative and astringent [16]. The review of various literature showed that the leaves, bark, fruits, or the various parts of the D. indica have extensive medicinal values. *In vivo* antioxidative potentials of the fruits of *D. indica* are yet to be validated. Hence, this study was taken, to evaluate the potential of the fruits of *D. indica* against hyperglycemia and oxidative stress in alloxan-induced diabetic model of mice.

METHODS

Chemicals required

Alloxan and metformin were procured from Sigma-Aldrich Co. (St. Louis, MO, USA.). Serum glutamic oxaloacetic transaminase, serum glutamate pyruvate transaminase, cholesterol, and triacylglycerols kits were purchased from coral. The other chemicals used were of analytical grade procured from Merck Co. (Mumbai, India) and Sisco Research laboratory.

Plant materials

Fruits of *D. indica* were collected from Beltola area of Guwahati, Assam on 15/04/2014 (Voucher No: 12056). The specimen submitted was identified by Dr. P. B. Gurung, Curator herbarium, Department of Botany, NEHU, Shillong, Meghalaya.

Experimental animals

Male Swiss albino (Balb/C strain) mice, weighing 25–30 g were used for the study. All the experiments were carried out in accordance with the Institutional Ethics Committee guidelines (Animal model), North Eastern Hill University, Shillong, India, dated 04/12/2014. Mice were housed in polypropylene cages and maintained at a temperature of 25±2°C with a 12-h day-night cycle. They were fed with mice feed obtained from Amrut Laboratory, Pune, India, and were provided with *ad libitum* water.

Preparation of extracts

Fruits of *D. indica* were cut into small pieces and air-dried at room temperature till it dried completely. Then, it is powdered in a blender and extracted by continuous stirring overnight with 10 volume of methanol:aqueous solution (4:1). The mixture was filtered and dried in a rotary evaporator at 38°C till it evaporates to become dry. The dried extract was used for further experiments. For aqueous extraction, the fruit powder was soaked in 250 ml (10× volumes) of distilled water overnight at 25–30°C with vigorous shaking. The mixture was filtered and the filtrate was lyophilized (lyophilization) for 1–2 days [17].

Experimental induction of DM

Overnight fasted mice only provided with water *ad libitum* were administered with a single high dose of alloxan (150 mg/kg) body weight (b.w.) intraperitoneally (i.p.) prepared in citrate buffer (0.1 M, PH 4.5). After 72 h, fasting blood glucose (FBG) level was checked with SD check glucometer. Mice with 200 mg/dl and above FBG level were selected for further studies.

Acute toxicity study

Acute toxicity study has been conducted in female mice divided into 6 groups which received methanolic fruit extract (MFE) at doses ranging from 400 to 2000 mg/kg b.w. The animals were observed for toxic symptoms and death continuously for 24 h after dosing. Finally, the number of survivors was noted [18]. The acute toxicity study was carried out according to the OECD guidelines 425.

Phytochemical screening

Phytochemical screening was carried out on both aqueous fruit extract (AFE) and MFE, examining the chemical secondary metabolites of alkaloids, flavonoids, glycosides, tannins, terpenes, and saponins [19-21].

Antihyperglycemic study

Short term study

The study was performed in six different groups of overnight fasted mice consisting of six mice in each group:

- Group A: Diabetic control
- Group B: Diabetic mice administered 150 mg/kg b.w. of MFE
- Group C: Diabetic mice administered 250 mg/kg b.w. of MFE
- Group D: Diabetic mice administered 350 mg/kg b.w. of MFE
- Group E: Diabetic mice administered 450 mg/kg b.w. of MFE
- Group F: Diabetic mice administered 550 mg/kg b.w. of MFE.

In short-term antihyperglycemic study, MFE doses ranging from 150 to550mg/kg b.w. were intraperitoneally injected to overnight fasted mice of respective groups for 24 h.

In vivo antioxidative assays

The study was performed in four different groups of overnight fasted mice consisting of six mice in each group:

- Group 1: Normal mice untreated
- Group 2: Diabetic mice untreated
- Group 3: Diabetic mice treated with 350 mg/kg b.w. of MFE
- Group 4: Diabetic mice treated with 50 mg/kg b.w. of Vitamin C.

Preparation of cytosolic and mitochondrial fractions of tissues

After a 21-day study period, mice were sacrificed by cervical dislocation and dissected to remove the heart tissue. Tissue was homogenized to make 10% (w/v) homogenates in a homogenization medium containing 10 mM HEPES buffer, pH 7.4 containing 0.2 M mannitol, 50 mM sucrose, and 1 mM EDTA. Cytosolic and mitochondrial fractions were obtained by centrifugation [22]. Total protein concentrations of both the cytosolic and mitochondrial fractions were determined by the Bradford method [23] using bovine serum albumin as the standard.

Antioxidant activity determination in heart tissue

Superoxide dismutase (SOD), both (manganese SOD [MnSOD] and copper-zinc SOD [CuZnSOD]) activity assays were performed by the method of Marklund and Marklund [24]. The change in absorbance was read at 420 nm against a blank and activity was expressed as units/mg protein.

Catalase (CAT) activity assay was performed by the method of Aebi [25]. In this assay, the rate of decomposition of hydrogen peroxide (H_2O_2) was measured at 240 nm and activity was expressed as units/min/mg protein.

Glutathione reductase (GR) activity was assayed according to the method of Carlsberg and Mannervik [26]. The activity of the enzyme was measured at λ340 nm following the oxidation of NADPH. One unit of GR activity is defined as 1 μmol of NADPH oxidized per minute at 37°C, and the specific activity was expressed as units/mg protein.

Histopathological investigation

Heart tissue was removed after sacrifice by cervical dislocation. Hematoxylin-eosin (HE) staining technique was used for histopathological studies with slight modifications. Tissue was fixed in formalin solution (10%) for 24 h and later fixed in 70% ethanol. Tissue was dehydrated in ethanol gradients (30–100%) and later embedded in paraffin wax blocks. Using a rotary microtome, the embedded tissue was trimmed into 5 μm-sized sections. Further, sections were fixed on glass slides, stained with HE stains. Finally, sections were mounted with DPX and observed under light microscope.

Statistical analysis

Results were expressed as mean ± standard error of the mean for six mice in each group. One-way analysis of variance followed by Tukey's *post hoc* test was performed to compare differences between experimental groups using the statistical package "IBM SPSS Statistics 19.0 for Windows." Statistical significance was set at $p<0.05$.

RESULTS

Acute toxicity study

The MFE of *D. indica* was found to be safe up to 2000 mg/kg b.w. by the i.p route. After 24 h, animals were found to be well tolerated. The mortality rate was observed to be negligible. The extract was found to be safe.

Phytochemical screening

Screening results of AFE and MFE of *D. indica* showed different chemical constituents. As shown in Table 1, tannins, glycosides, flavonoids, terpenes, and saponins were strongly present in MFE as compared to AFE. Present strongly = (++), present slightly = (+), absent = (−).

Antihyperglycemic study

Short term antihyperglycemic study

The result of the short-term study is depicted in Fig. 1. There was no significant reduction of FBG level in Group B, whereas in Groups C and D, FBG level was reduced by 30.1% at 6 h, 41% at 4 h, and 47.6% at 6 h, respectively, compared to Group A. The FBG level was reduced significantly by 40.2% at 2 h, 49.9% at 4 h, and 66.4% at 6 h in Group E in comparison with Group A. Reduction of FBG level was seen

Table 1: Phytochemical screening results of MFE and AFE of *D. indica*

Sl.no.	Chemical constituents	MFE	AFE
1	Alkaloids	+	++
2	Tannins	+ +	+
3	Glycosides	+ +	−
4	Flavonoids	+ +	+
5	Terpenes	+ +	+
6	Saponins	+ +	+

D. indica: Dillenia indica, MFE: Methanolic fruit extract, AFE: Aqueous fruit extract

to be drastic by 44% at 1h, 51.8% at 2 h, 68.9% at 4 h, and 72.2% at 6 h in Group F as compared to the Group A in the study.

Antioxidant activity determination in heart tissue

SOD activity in heart

Fig. 2 shows a significant increase in the activity of CuZnSOD in Group 4 (4.60±0.11) and Group 3 (3.60±0.13) as compared to Group 2 (2.16±0.08) in heart. Similarly, the activity of MnSOD in Group 3 (9.39±0.18) and Group 4 (9.13±0.19) was observed to be higher than Group 2 (6.55±0.14) in the present study.

CAT activity in heart

As shown in Fig. 3, the activity of CAT in the heart was observed to be higher in Group 4 (0.10±0.01) compared to Group 2 (0.07±0.01), whereas the activity of Group 3 (0.12±0.01) was observed to be higher than Group 2 and even Group 4.

GR activity in heart

As shown in Fig. 4, the activity of GR was observed to be higher in Group 3 (2.30±.55) and Group 4 (2.74±0.06) as compared to Group 2 (1.73±0.15) in the heart.

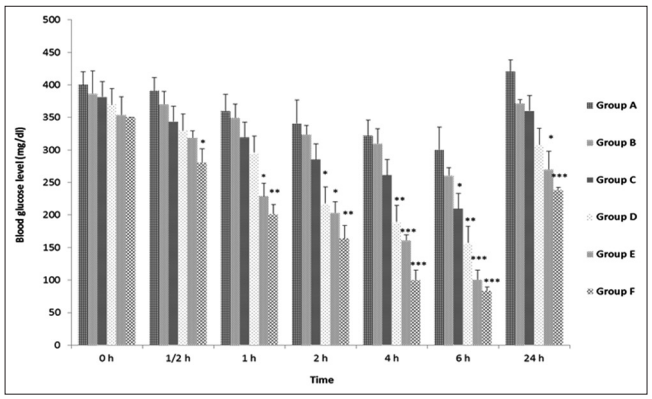

Fig. 1: Effect of different doses of methanolic fruit extract on fasting blood glucose level in different experimental groups of diabetic mice assayed at different time intervals. Values expressed as mean ± standard error of the mean, n=6. Statistically significant differences: *p<0.05, **p<0.01, and ***p<0.001 as compared with the control Group A

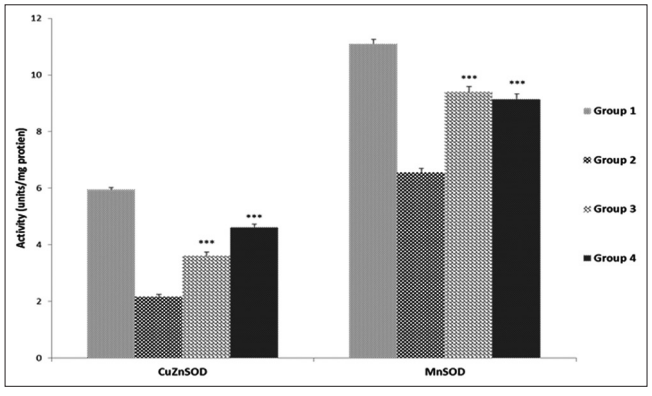

Fig. 2: Mean activities of copper-zinc superoxide dismutase (SOD) and manganese SOD in the heart. Values expressed as mean± standard error of the mean; n=6. The enzyme activities were expressed as units/mg protein. Group 1 - normal mice untreated, Group 2 - diabetic mice untreated, Group 3 - diabetic mice treated with methanolic fruit extract, and Group 4 - diabetic mice treated with Vitamin C. Statistically significant differences: ***p<0.001 as compared with Group 2

Histopathological investigation of heart tissue

As shown in Fig. 5, the heart tissue with enlarged interstitial spaces has been distorted in reference diabetic group as compared to the normal group. However, in the case of extract treated and Vitamin C-treated diabetic groups, heart tissue was less altered showing fewer enlargements in the interstitial space.

DISCUSSION

The pathogenesis of DM and its possible management by existing therapeutic agents having negligible side effects have drawn interest in recent years [28]. With the rise of this metabolic disease and associated healthcare costs, alternative or complementary therapies have grown interest [29]. Fruits of *D. indica* were selected for the present study. Phytochemical screening tests showed a strong presence of tannins, glycosides, flavonoid, terpenes, and saponins in the MFE as compared to the AFE (Table 1). Therefore, MFE was chosen for further studies. The aim of the present study is to investigate the effect of the fruits of *D. indica* on hyperglycemia and oxidative stress-induced cardiomyopathy.

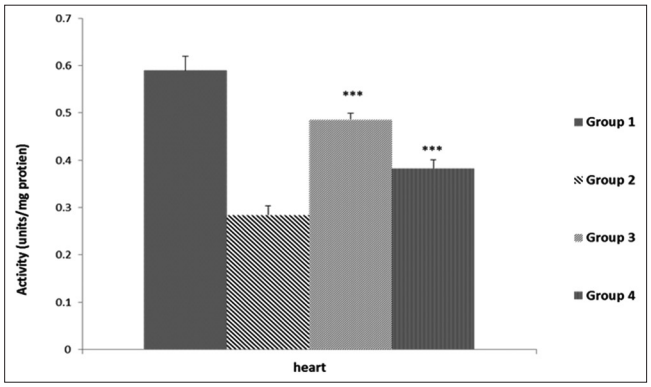

Fig. 3: Mean activities of catalase in the heart of mice. Values expressed as mean± standard error of the mean; n=6. The enzyme activities were expressed as units/mg protein. Group 1 - normal mice untreated, Group 2 - diabetic mice untreated, Group 3 - diabetic mice treated with methanolic fruit extract, and Group 4 - diabetic mice treated with Vitamin C. n=6. Statistically significant differences: **p<0.01 and ***p<0.001 as compared with Group 2

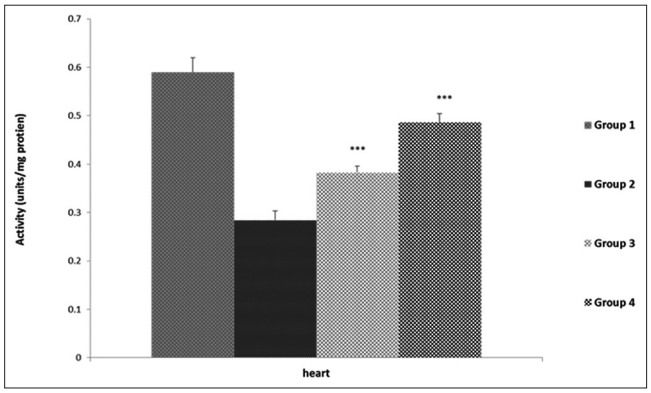

Fig. 4: Mean activities of glutathione reductase in the heart of mice. Values expressed as mean± standard error of the mean; n=6. The enzyme activities were expressed as units/mg protein. Group 1 - normal mice untreated, Group 2 - diabetic mice untreated, Group 3 - diabetic mice treated with methanolic fruit extract, and Group 4 - diabetic mice treated with Vitamin C statistically significant differences: ***p<0.001 as compared with Group 2

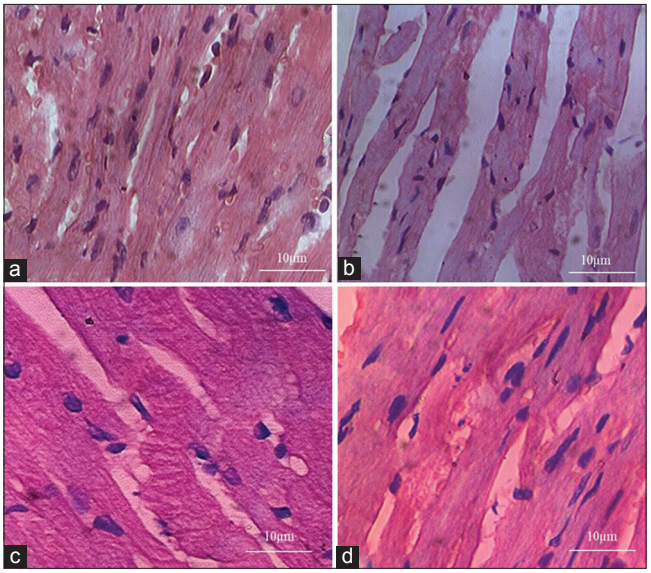

Fig. 5: Photomicrographs of histological changes in heart tissues. (a): Normal mice, (b): Diabetic mice, (c): Diabetic mice treated with methanolic fruit extract, (d): Diabetic mice treated Vitamin C. All images are under 40× magnifications.

It has been well documented that alloxan is most commonly used to induce diabetes in animals. Alloxan is a toxic glucose analog, which selectively destroys insulin-producing cells in the pancreas. Alloxan is a potent diabetogen that reduces to dialuric acid which then further autoxidizes back to alloxan leading to the production of H_2O_2, O_2, $O^{2,-}$ and hydroxyl radicals and damages beta cells of islets of Langerhans [30]. In this study, significant hyperglycemia was achieved after alloxan (150mg/kg b.w.) injection. Alloxan-induced diabetic mice with more than 200mg/dl of blood glucose level were considered to be diabetic and used for the study. However, administration of the MFE at the dose of 150–550 mg/kg b.w. is done to carry out short-term antihyperglycemic study. In this study, it was observed that the FBG level for all the groups reduced in a dose-dependent manner showing 350 mg/kg b.w. dose and 450 mg/kg b.w. dose of MFE to be quite effective in reduction of blood glucose level in diabetic mice, whereas 550 mg/kg b.w. dose showed drastic reduction. Considering the entire doses, 350 mg/kg b.w. dose was considered as the optimum dose and used for further studies. Therefore, it could be indicating potent antihyperglycemic activity of MFE.

The etiology and pathogenesis of DM and its related complications have been the focus of several studies in which lipid peroxidation and ROS have played a role [31,32]. The darker side of oxygen often referred to as oxidative stress, which seems to be the underlying cause for all degenerative diseases [33]. When an imbalances occur between oxidants generation and antioxidants defence mechanism, where oxidants are in favour, leads to oxidative stress. SODs function as the body's first line of defense and are of the important classes of antioxidant enzymes that catalyze the dismutation of superoxide anions (O_2^-) to O_2 and H_2O_2 which are further reduced to water by CAT or peroxidase [34]. It has been seen in earlier studies that *in vitro* antioxidant activity of *D. indica* fruits in different extracts was assayed through some *in vitro* models such as phosphomolybdenum method, β-carotene-linoleate model system, and α,α-diphenyl-β-picrylhydrazyl method [35]. In this study, the *in vivo* antioxidant activities were assessed where it can be observed that the activity of SOD (CuZnSOD and MnSOD), CAT, and GR enzyme activities significantly decreased in diabetic mice as compared to normal mice. Further, in this study, it was observed that MFE-treated group had shown elevated activities of these antioxidant enzymes compared to diabetic control. As evidenced by histopathological studies, the present study demonstrates that tissues exhibited certain changes due to stress conditions under diabetes. After treatment with the extract, heart tissues displayed distinctly near normal architecture when compared to diabetic control under light microscopy (Fig. 5). Important finding of the study is that *D. indica* fruits have the ability to protect tissues from detrimental effect of diabetes; however, it is also clear from the study that the fruits were able to scavenge free radicals and enhance antioxidative enzyme activities, thus allowing tissues to be protected from further impairments in diabetes. The antioxidative activity of the fruits may be attributed due to the presence of tannins and flavonoids responsible for its strong antioxidative nature.

CONCLUSION

The study affirms that MFE of *D. indica* has potent antioxidative potential with results comparable to those of the standard compounds such as Vitamin C. It can also be concluded from the studies that the antioxidant activity of MFE may be due to the strong presence of the phytochemicals tannins and flavonoid. The study affirms that the extract lowers the blood glucose level showing its antihyperglycemic property. The study also demonstrated that treatment with MFE may provide beneficial effects against diabetes potentially by enhancing the levels of antioxidative enzymes and protecting the heart tissue. However, this study has not revealed the exact mechanism (s) of its action and further investigation is underway.

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AUTHORS' CONTRIBUTION

All authors contributed immensely and equally for this work and prepared the manuscript.

CONFLICTS OF INTEREST

All authors declare that there are no conflicts of interest.

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