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ORIGINAL ARTICLE

The Antioxidant Potential of *Azadirachta indica* Ameliorates Cardioprotection Following Diabetic Mellitus-Induced Microangiopathy

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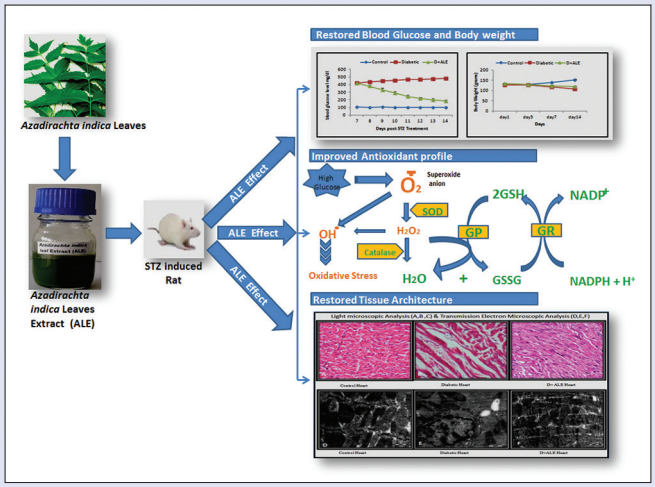
ABSTRACT

Background: Cardiac complications associated with diabetes mellitus have become major cause of concern. Antidiabetic drugs, with varied mode of action, are although available, apprehensions exist for their limited action or side effects upon prolonged use. Efforts are therefore inclined toward finding other alternatives. The present study was, thus, undertaken to evaluate the cardioprotective effect of *Azadirachta indica* (AI) on microangiopathic changes in rat model of diabetes. **Materials and Methods:** Diabetes was induced in male rats by single intraperitoneal injection of streptozotocin (60 mg/kg body weight). Seven days after glucose levels are stabilized, aqueous leaf extract of AI (ALE) (600 mg/kg[1] body weight) was administered orally to diabetic animals every day for 7 days. **Results:** High blood glucose characterizing diabetes in these animals was found to show increased lipid peroxidation (LPO), altered antioxidant biomarkers together with microangiopathic alterations. The treatment of diabetic rats with ALE reduced the levels of blood glucose, LPO, and restored the activities of antioxidant enzyme. Light and transmission electron microscopic analysis revealed reduced necrotic areas and inflammation in tissue architecture of ALE treated heart in comparison to untreated diabetic group. **Conclusion:** AI provides cardioprotection by ameliorating oxidative stress in rat model of diabetic mellitus.

Key words: Antioxidants, *Azadirachta indica,* diabetes mellitus, lipid peroxidation

SUMMARY

- The streptozotocin (STZ) treatment (60 mg/kg body weight) to animals induced diabetic changes such as elevated blood glucose levels, decreased body weight, altered lipid profiles together with development of proxidant state evidenced by elevated levels of lipid peroxidation (LPO), depletion in reduced glutathione (GSH) levels and altered antioxidant enzymes with consequent microangiopathic alterations in heart tissue evinced by localization of necrotic and inflamed areas in heart tissue
- The treatment of animals with *Azadirachta indica* leaf extract (ALE) (600 mg/kg body weight) post-STZ treatment significantly reversed the adverse effects witnessed by normalized blood glucose levels, improvement in reduced body weight and stabilized lipid profiles
- Further, ALE treatment also significantly reduced the LPO indices, improvement in GSH content and restoration of antioxidant enzyme activities, suggesting antioxidatant potential of ALE
- The microangiopathic changes in the heart tissue consequent to induction of diabetes and oxidative stress by STZ as reiterated through light microscopy and transmission electron microscopy were found to be reversed by ALE treatment. These observations pointed toward cardiopreventive effects of ALE following microangiopathic changes as seen following induction of diabetes mellitus.



Abbreviations used: AI: Azadirachta indica, ALE: Azadirachta indica Leaves Extract. STZ: Streptozotocin, LPO Lipid per oxidation, GSH: Glutathione, GSSG: Glutathione disulphide, SOD: Superoxide dismutase, GP: Glutathione peroxidase, GR: Glutathione reductase.

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INTRODUCTION

Diabetes mellitus (DM) is a predominant endocrine disorder and has emerged as an epidemic in both developed as well as in developing countries. According to a study conducted in 130 countries, 382 million diabetics cases were reported in 2013 and predicted ~592 million cases by 2035.[1] Diabetes affects people of all ages[2] resulting in functional compromise of all vital tissue viz., liver, kidney, heart, brain, and skeletal muscles. A tremendous increase in diabetic cases associated with cardiac

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complications have seen an unprecedented surge in the recent years and have been shown to be the leading cause of death.[3] The risk of developing cardiac complications in diabetic population is 12 times higher than nondiabetic individuals.[4] Hyperglycemia, a primary symptom and hallmark of disease, has often been shown to be associated with overproduction of free radicals that play a major role in the pathogenesis and development of oxidative stress.[5] Unsaturated fatty acids present in cell membrane are highly susceptible to free radicals during diabetes and undergo peroxidation to enhance the progression and development of cardiac complications. Inappropriate scavenging of free radicals and depleted concentration of antioxidants leads to metabolic or structural changes in the tissue via glycation of proteins, increased formation of advance glycosylated end products (AGE), and accumulation of sorbitol. In the cardiac tissue, these products may interact with the extracellular proteins and cellular matrix leading to fibrosis, stiffness of myocardium. However, upon interaction of AGE with collagen, the microangiopathy ensues.[6] Besides, atherosclerosis is another commonly encountered complication in diabetic patients. Which at biochemical level is thought involve oxidation of cholesterol specially low-density lipoproteins (LDLs), causing increase in the very LDLs (VLDLs) and triglyceride with consequent arthrosclerosis, endothelial dysfunction, coronary artery disease, and stroke.[7,8] Thus, diabetes is an intricate metabolic disorder, requires multi factorial and long-term management. Owing to such a complexity, the need of hour is to develop a combined therapy, which besides controlling hyperglycemia, maintaining normal antioxidants level, also provides efficacy and safety. World Health Organization has also encouraged the use of traditional plants in combating the disease. The plant products are commonly known to be well tolerated through oral administration with seldom showing any potentially toxic side effects.[9]

According to ethnobotanical record, around 800 medicinal plants can be used in diabetes.

Azadirachta indica (AI, neem), a tropical plant under the family *Meliaceae*, is another such plant, widely known for its antimicrobial, antifungal and antiantipyretic activities.[10] At present, it is widely used in dentistry, soaps, and detergents. Various parts of AI have been explored to evaluate antidiabetic, antioxidative properties. According to a previous report crude ethanol extract (250 mg/kg body weight) of neem leaves when administered orally for 2 weeks partially lowered blood sugar levels in alloxan induced diabetic rats.[11] The petroleum ether extract of neem seed kernel (2 g/kg body weight) and seed husk (0.9 g/kg body weight) restricted the oxidative stress in heart and erythrocytes in streptozotocin (STZ) induced diabetic rats.[12] The hypoglycemic effect of AI[13-15] also shows antihypercholesterolic effect.[16] The role of AI in tissue microangiopathy level is not fully explored and need more elucidation. Since it is largely accepted that biomarkers and histopathological alterations play a significant role in the diabetic complications, in present study, we have investigated the effect of AI at ultrastructural level to mark out its anti microangiopathic and cardioprotective effect in STZ induced diabetes.

MATERIALS AND METHODS

Chemicals

The enzyme glutathione (GSH) reductase, thiobarbituric acid, was purchased from Sigma (St. Louis MO, USA). STZ, nicotinamide adenine dinucleotide phosphate (NADPH), 1-chloro 2, 4-dinitrobenzene was purchased from Hi-Media, Bombay, India. Potassium phosphate monobasic, sodium phosphate hydrogen dibasic, sodium phosphate hydrogen monobasic, Tris (hydroxymethyl) aminomethane (Tris) base, ethylenediaminetetraacetic acid, and all other chemical used in the study were procured from Sisco Research Laboratory (SRL, Bombay, India).

Preparations of *Azadirachta indica* leaf extract (aqueous)

Fresh matured leaves of AI were collected from botanical garden of Panjab University Chandigarh, India and duly certified by National Institute of Science Communications and Information Resources. The aqueous leaves extract was prepared by taking 200 g of leaves of AI and grounded in double distilled water using electric blender. Total volume of this extract was made up to 1 L. Well-mixed suspension was then filtered (Whatman filter paper no. 1) and lyophilized to obtain powdered extract which was kept in refrigerator at 4°C until further use. For the purpose of administration, a fresh dose (600 mg/kg body weight) was daily prepared by dissolving powder extract in double distilled water.

Animals model of diabetes

Healthy male Sprague-Dawley rats weighing 125–135 g were procured from central animal house Panjab University, Chandigarh. Animals were kept in the polypropylene cages at ambient temperature with 12 h dark and 12 h light cycle and were fed pellet diet (Hindustan Liver Ltd., Bombay, India) with free access to water. All procedures and treatment were carried out in accordance with guidelines issued by the committee for the purpose of control and supervision of experimentation on animals of Panjab University, Chandigarh.

One week after acclimatization, animals were divided into three groups designated as Group 1 (control), Group 2 (diabetic, D), and Group 3 (diabetic treated with ALE [D + ALE]).

The diabetes was induced in Group 2 and 3 animals by a single intraperitoneal injection of STZ (60 mg/kg body weight) in saline solution.[17] Post-STZ treatment (72 h) diabetes was established in rats showing fasting blood glucose level ≥ 250 mg/dl. These diabetic animals were kept as such for 7 days with free access to food and water. After 7 days, the animals in Group 3 received oral administration of ALE 600 mg/kg body weight daily, for next 7 days. The optimum concentration of ALE was selected (based on glucose lowering response curve starting from 200 mg to 800 mg/kg body weight). The animals in Group 1 and 2 received same volume (0.5 ml) of saline solution (0.9% NaCl) orally for same duration.

Preparation of tissue homogenate

At the end of 2 weeks following overnight fasting, animals were sacrificed under mild ether anesthesia followed by cervical dislocation. Blood was collected in clean test tubes for serum preparation and heart tissues were excised, washed in cold saline solution and blotted dry. Heart tissue homogenate (10%, w/v) was prepared in cold 100 mM Tris buffer pH 7.4 using Teflon plunger and centrifuged the content at 12,000 rpm for 30 min to prepare postmitochondrial supernatant (PMS).

Biochemical investigations

Enzymatic and nonenzymatic estimations were carried out in PMS. Glutathione reductase (GR) activity was assayed at 340 nm by the method of Carlberg and Mannervik.[18] Activity of catalase (CAT) was measured in PMS by the method of luck at 240 nm.[19] The activity of glutathione peroxidase (GP) was measured at 340 nm by the method of Flohé and Günzler[20] in the presence of NADPH. Superoxide dismutase (SOD) activity was determined by method of Kono.[21] Protein content was estimated by the method of Lowry *et al.*[22] Reduced GSH content was measured by the method of Ellman[23] and lipid peroxidation (LPO) was estimated in terms of malondialdehyde (MDA) formed, by the method of Wills.[24] Blood glucose level was estimated using Glucometer (Abbott Labs Ltd.). Optical density of all biochemical estimations was read on Shimadzu UV/VIS spectrophotometer 1240.

Histological examination

Histological analysis was carried out using light microscopy and transmission electron microscopy (TEM). For light microscopic analysis of heart, small section from ventricular portion was incised and fixed in 10% formaldehyde solution. After complete fixation, washing and dehydration were carried out in ascending grades of alcohol (30%, 50%, 70%, 90%, and absolute alcohol). Embedding was done with paraffin wax, and thin sections were cut 3 µm using Rotary microtome. Sections were stained with hematoxyline and eosin (H and E) stain and examined under light microscope (Nikon) (×40) to evaluate the histopathological modifications.

For TEM, very small sections (0.1 mm) of tissue were cut and fixed in Karnowsky fixative. Dehydration and cleaning were done using acetone and toluene, respectively. Embedding was performed using liquid Araldite, which polymerizes on heating. Ultra-thin sections of 60–80 nm were cut, stained and finally viewed under TEM (Morgagni 268D) at the Department of Anatomy, AIIMS, New Delhi.

Statistical analysis

The data was expressed as mean ± standard error of mean of six animals and variation in the data was analyzed using one-way analysis of variance followed by least significant difference *post hoc* test. The $P < 0.05$ was considered statistically significant.

RESULTS

Dose response of *Azadirachta indica* leaf extract and toxicology analysis

The optimum concentration of ALE was selected on basis of antihyperglycemic response along with discerning its toxicological effect. To achieve this animal model of diabetes was established by administering a single intra peritoneal injection of STZ (60 mg/kg body weight). With respect to the control animals, the blood levels of glucose in the diabetic animals were found to be raised by single injection of STZ. The levels of blood glucose were monitored at different time intervals starting with 3 days post-STZ treatment and followed till 14th days [Figure 1]. As could be seen by 3rd day post-STZ treatment the blood glucose started to rise to 250 mg/dl and thereafter its levels reached to ~400 mg/dl by day 7th and continued to remain high at this level until 14th days post-STZ treatment in comparison to control animals. Hence, the ALE treatments were started post 7th days of STZ treatment when the blood glucose levels stabilized to ~400 mg/dl.

Having established the diabetic model, the effects of ALE were observed, throughout this study, on the diabetic animals 7 days post-STZ treatment. As shown in Figure 2, post 7th days of STZ treatment a rise in blood glucose levels to ~4-fold with respect to control was observed. Daily administration of various concentrations of ALE (200–1000 mg/kg body weight), administrated orally to different groups of diabetic rats (Group 3) for 7 days produced a dose-dependent decrease in the levels of blood glucose in these animals. As shown in Figure 2, the extent of glucose lowering by ALE at lower concentrations, i.e. 200–400 mg/kg remained low (~16%). However, increasing the ALE concentrations further, i.e. from 500 to 1000 mg/kg body weight, a significant reduction in the levels of blood glucose amounting to ~29% was observed at a dose of 500 mg ALE, reaching to ~50% at 600 mg with 1000 mg rendered it to be ~60% reduced in comparison to diabetic group (Group 2).

Having observed the effectiveness of ALE as for the reduction in the levels of blood glucose was concerned, the concentrations of ALE reducing blood glucose to more than 50% (500, 600, 800, and 1000 mg/kg body weight) were screened for observing any toxic effects. These effects were based on the behavioral observations [Table 1] made every day after administering ALE to these animals. As per the reports of Dube *et al.* and Shit *et al.*, the parameters such as amount of food and water intake, changes in texture of skin/fur, behavior response and the mortality are commonly exploited to toxicological analysis.[25,26] Throughout the span of the experiment none of the concentrations of ALE administration produced any mortality, however, obvious behavior changes, such as decreased food intake, overall lethargy, less activity, together with reduction in body weight was observed in the animals which had received ALE 800 mg and 1000 mg/kg body weight. However, the rats which received 600 mg/kg of ALE concentration, their body weights increased in parallel to control animals and animals maintained normal activity with respect to food and water intake, activity [Table 1]. Thus, a dose of ALE, 600 mg/kg body weight, which besides being effective with no observable toxicity, was hence used for further studies.

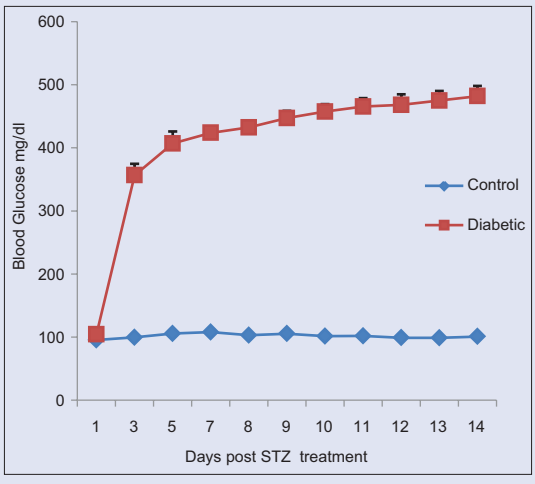

Figure 1: Blood glucose profile post-STZ treatment. $n = 6$ in all groups, values are mean ± Standard error mean. STZ: Streptozotocin

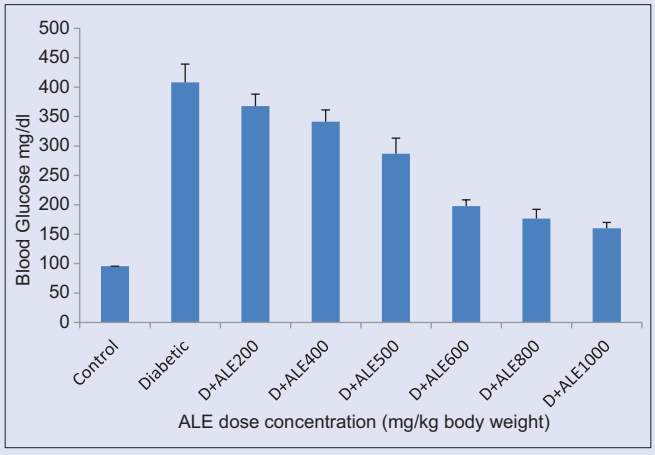

Figure 2: Blood glucose profile with various concentrations of ALE treatment. $n = 6$ in all groups, values are mean ± standard error mean. ALE: *Azadirachta indica* leaf extract; D: Diabetic

Time response effects

Having selected the optimum concentration of ALE, a time response effect ALE on blood glucose profile and body weight were measured. As shown in Figure 3, daily treatment of diabetic animals with ALE (600 mg/kg body weight) continues to decrease the blood glucose levels in comparison to diabetic animals with maximum reduction to almost 2-fold by day 7th. Besides lowering the blood glucose levels, ALE treatment, in parallel, also prevented the body weight reduction in comparison to diabetic animals, albeit at lower extent [Figure 4]. In addition, even though ALE caused reduction in the weight loss upon diabetes, it remained lower than the control animals, which continue to gain their weight in the whole span of experiment [Figure 4].

Biochemical analysis

The serum levels of various lipids are commonly analyzed to ascertain the cause and consequences during diabetes-induced changes in different organs specifically the cardiac complications. In this regards, the lipid profile among different groups of animals was analyzed and compared. As shown in Table 2, with respect to control animals, the diabetic animals showed significantly elevated levels ($P < 0.001$) of total cholesterol (TC), triglyceride, LDL, and VLDL cholesterol. While a significant reduction in the levels of high-density lipoprotein (HDL) was also observed. The ALE treatment to these animals significantly improved the lipid profile as witnessed by the LDL: HDL ratio of 6.15 in diabetes to 2.63 upon treatment of diabetic animals with ALE.

Cardiac proxidant state

Since the cardiac tissue is a direct target of altered lipid profile, therefore, the effects of ALE on proxoidant state of cardiac tissue were analyzed in diabetic animals. The data as shown in Table 3 represented the levels of the cardiac GSH and LPOs. As it is evident, induction of diabetes in these animals caused ~46% depletion of cardiac reduced GSH levels along with a parallel increase in the level of cardiac LPO to ~81% with respect to control animals. Exposing the animals orally to the leaf extracts of ALE significantly increased the cardiac content of reduced GSH by ~38% ($P < 0.001$) while the LPO, as measured by nmole of MDA formed $1min^{-1}mg^{-1}$ protein was found to be significantly reduced ($P < 0.001$, ~27%) and increased GSH content (37.6%) in diabetic treated animals.

Table 1: Evaluation of behavioral changes following *Azadirachta indica* leaf extract administration

Groups (*n*=6)	Food intake	Water intake	Body weight	Restlessness/behavior change	Skin/fur	Death
Control (saline solution)	Normal	Normal	Increased	Not observed	Normal	Nil
ALE 500 mg/kg body weight	Normal	Normal	Increased	Not observed	Normal	Nil
ALE 600 mg/kg body weight	Normal	Normal	Increased	Not observed	Normal	Nil
ALE 800 mg/kg body weight	Decreased	Decreased	Decreased	Not observed but less active	Normal	Nil
ALE 1000 mg/kg body weight	Decreased	Decreased	Decreased	Not observed but less active	Normal	Nil

n=6 in all groups. ALE: *Azadirachta indica* leaf extract; D: Diabetic

Table 2: Analysis of serum lipid profile in three groups of animals

Groups	Total cholesterol (mg/dl)	Triglycerides (mg/dl)	HDL cholesterol (mg/dl)	LDL cholesterol (mg/dl)	VLDL cholesterol (mg/dl)
C	89.86±1.76	78.22±2.50	31.04±0.96	43.17±5.73	15.64±0.50
D	139.64±4.83**a,*b	100.07±4.5**a,*b	16.3±0.81**a,*b	100.2±4.52**a,**b	20.01±0.91**a,*b
D + ALE	104.88±2.88**c	84.18±2.74*c,	24.23±0.75*a	63.82±2.46**c,*a	16.83±0.54*c

n=6 in all groups, values are mean±SEM. *Significant at 0.05 level; **Significant at 0.001 level; [a]Compared with control group; [b]Compared with D + ALE group; [c]Compared with diabetic group. All experiments were replicated thrice. HDL: High-density lipoprotein; LDL: Low-density lipoprotein; VLDL: Very low-density lipoprotein; SEM: Standard error of mean; ALE: *Azadirachta indica* leaf extract; D: Diabetic; C: Control; SEM: Standard error of mean

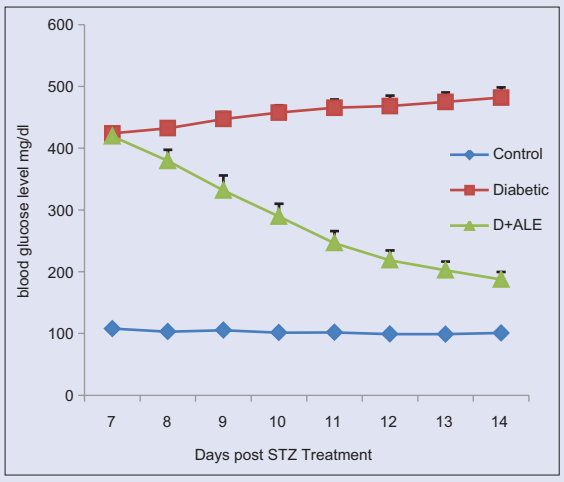

Figure 3: Blood glucose profile during ALE treatment. *n* = 6 in all groups, values are mean ± standard error mean. *Significant at 0.05 level; **Significant at 0.001 level; [a]Compared with control group; [c]Compared with diabetic group. All experiments were replicated thrice. ALE: *Azadirachta indica* leaf extract; D: Diabetic

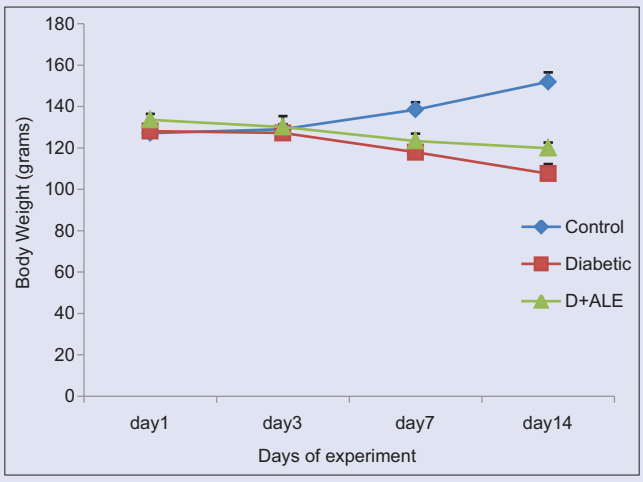

Figure 4: Body weight pre- and post-ALE treatment. *n* = 6 in all groups, values are mean ± standard error mean. *Significant at 0.05 level; **Significant at 0.001 level; [a]Compared with control group; [c]Compared with diabetic group. All experiments were replicated. ALE: *Azadirachta indica* leaf extract; D: Diabetic

Besides reduced GSH, enzymatic biomarkers are very sensitive component of cardiomyocytes and help in scavenging free radicals. Changes in their levels represent accordingly effect the normal tissue homeostasis. The activities of various antioxidant enzymes were thus compared among different groups of animals. The results as shown in Table 4 demonstrated activities of some of the key antioxidant enzymes. The results indicated that with respect to control, the activities of these antioxidant enzymes were significantly elevated upon induction of diabetes the increases in the respective activities were found to be ~139.7%, 36.2%, ~11.7% and 60% in CAT, GR, GST, and SOD, respectively. Upon treating these diabetic animals with ALE, the activities of SOD and GP were found to be completely restored to that of control whereas significant increases were also noted in the activities of CAT and GSH-S-transferase. The activity of GR, however, underwent an augmentation over the value seen in diabetes following treatment of these animals with ALE.

Histological analysis

These biochemical observations were also correlated with microarchitectural changes in the tissue of these animals. For this H and E stained sections [Figure 5a-c] were viewed under light microscope. The cardiac muscles from the control animals [Figure 5a] exhibited normal architecture with oval nuclei in microscopically distinct cardiomyocytes showing smooth lining with no fibrosis and inflammation. The histological analysis of the diabetic heart [Figure 5b], on the other hand, showed microangiopathic alterations such as distorted cardiac muscles (black arrow head), cardiomyocytes with decentralized nuclei, inflammation and mild fibrosis (white arrow head). Treating the diabetic rats with ALE [Figure 5c] led to significant improvement in the cardiac tissue architecture with remarkable absence of inflammation and fibrosis (white star). It was observed that majority of the cardiomyocytes, although, were able to retain normocytic structure and arrangement by addition of ALE yet there was mild distortion which remained unaffected [Figure 5c]. Further analysis of the cardiac architecture at ultrastructural level by TEM revealed changes at the organelle level [Figures 6a-c,]. As shown in photomicrograph [Figure 6a], the cardiac tissue of control animals indicated arrangements of the heart muscles depicting parallel array of myofibrils and single rows of mitochondria. The heart from the diabetic animals [Figure 6b], on the other hand, showed swollen mitochondria, disordered myofibrils, and deposition of glycogen. Treating the diabetic animals with ALE [Figure 6c] 1caused recovery of the cardiac changes as seen during diabetes with significant compaction and organized array of myofibrils with symmetrically placed mitochondria and absence of dense bodies.

DISCUSSION

DM is a state of metabolic dysfunction putting a whole lot of stress in the target organs. The debilitating effects of diabetes are often associated with multi-organ involvement including the major organs like heart. Cardiac complications namely cardiac microangiopathy, coronary artery disease, and stroke besides many others are largely considered as important implications of diabetes often resulting in mortality.[27] Moreover, factors affecting blood levels of insulin, lipid as well as the proxidant state besides causative are also seen to be the consequence of the disease.[28,29] The results of the present study further reiterated such observations. STZ, a potent diabetogen that also caused alkylation and fragmentation of beta cell DNA has been exploited in the present study for diabetes development. This lead to selective destruction of beta cells resulting in elevation of blood glucose level as seen in the type-1 diabetes.[30] Similar effects were seen the present study and it was observed that within 3 days the glucose levels were found to be higher in the treated rats which reached the peak by 7th day and remained at this level till 14th days. Our unpublished observations demonstrated that this effect of STZ was irreversible as the hyperglycemia remained persistent till animals die. Besides hyperglycemia, a constant maintenance of hyperglycemic status in diabetic animals is often linked to a parallel increase in the supply and breakdown of metabolites. The metabolic demand under such situations is met by the breakdown of stored carbohydrate and fats. Breakdown of fat of adipose tissue and stored glycogen of the body (liver/muscular tissue) causes decrease in the overall body weight.[31] These observations strongly lend credibility to the results obtained in the present study. Treatment of diabetic animals with ALE protected the effect of diabetes on these animals by decreasing the blood glucose level as well as preventing any further weight loss

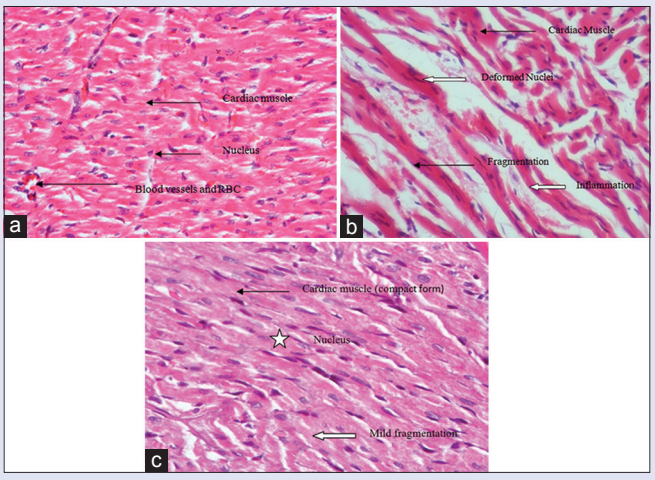

Figure 5: (a) Normal control showing cardiac muscles with compact arrangement and oval nuclei (b) diabetic group (streptozotocin) showing distorted cardiac muscles, deformed cardiomyocytes, decentralized nuclei, inflammation and mild fibrosis. (c) D + ALE group showing less distorted and parallel array of cardiac muscles, centralized nuclei. ALE: *Azadirachta indica* leaf extract, D: Diabetic (H and E, ×40)

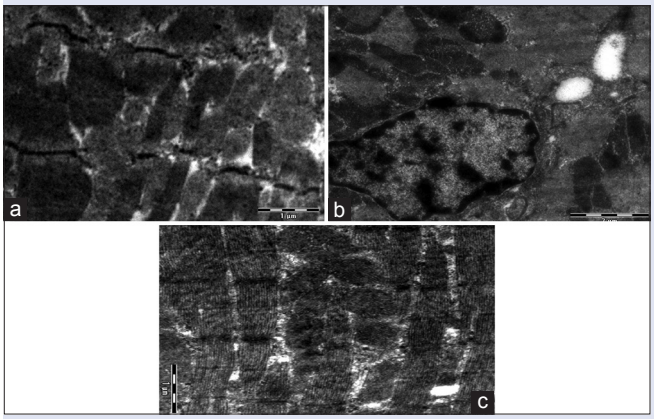

Figure 6: Transmission electron micrograph showing mitochondria and myofibrils of the cardiac muscle tissues (×5000). (a) Normal control group showing normal architecture of myofibrils with uniformly arranged mitochondria. (b) Diabetic group (streptozotocin) showing swollen mitochondria, disarrangement of myofibrils, and dense areas in cytoplasmic spaces. (c) D + ALE treated group showing a normal architecture of cardiac tissue with less cytoplasmic spaces and normal mitochondria. ALE; *Azadirachta indica* leaf extract, D: Diabetic

owing to diabetes. The exact mechanism involved in the reduction in blood glucose level by ALE treatment although remains largely unknown, the presence of terpenoid, flavanoid, and glycoside in ALE possibly render it antidiabetic.[32] These terpenoid, flavanoid, and glycoside in ALE could have increased the peripheral utilization of glucose and decreased the glucose level in D + ALE animals. Chattopadhyay (1996) also considered the role of ALE in peripheral utilization of glucose.[33] Further, it is presumed that the glucose lowering effect of ALE in diabetic rats may also be involved in preventing the breakdown of structural proteins of muscular tissue, consequently, the rate of weight loss was also decreased in D + ALE treated animals.[34,35] This has potentially improved the overall metabolic state of the organism which indeed gets corroborated through the observations of the present study. The analysis of the tissue function is an important paradigm to understand the function of organ in question. In addition, the fact that diabetes and dyslipidemia (disturbed lipid levels) is interconnected the observed effect of STZ treatment indeed supported this relation besides pointing toward alteration in the cardiac tissue function. Dyslipidemia affects cardiac function by causing atherosclerosis and blood pressure.[36] In our study, besides hyperglycemia and weight loss, dyslipidemia was also observed in diabetic animals. The elevated levels of cholesterols (TC, LDL, and VLDL) could be due to decline in lipid absorption and increased biosynthesis of cholesterol under diabetic conditions.[37] Thus, the obvious outcome of the excessive breakdown of lipids leading to observed increased levels of TC, LDL VLDL and decreased levels of HDL thus severely affecting the LDL/HDL ratio, which has also been well substantiated.[38] In addition, diminished activity of lipoprotein lipase[39,40] could also be an important reason for observed dyslipidemia. Collectively, this effect of diabetes seems to be responsible for putting an additional load onto the cardiac tissue leading to microangiopathic changes in the heart. This is actually has been reflected through architectural changes which were observed following induction of diabetes. Both light microscopic and ultramicroscopic examination of cardiac tissue of diabetic rat revealed microangiopathic alteration such as reduction in the size of cardiac cell, disruption of myofibrils and altered organelle arrangement. Previous reports provide sufficient support of such observations as during hyperglycemia the consequent hyperosmolarity leads to shrinkage of cardiomyocytes and change both shape and structure of nuclei.[41] Such constant changes at structural levels are also simultaneously reflected at the molecular levels leading to breakdown of structural proteins, protein, lipid degradation, and defect in mitochondrial function.[42] Shen *et al*. reported distorted cardiomyocytes, swollen mitochondria, and inflammation in the diabetic heart.[43] A strong association between the observed microangiopathic changes and oxidative stress following diabetes has reflected this study. The generation of proxidant state during the normal metabolic activity is routinely scavenged by the cellular antioxidant system. However, in hyperglycemic condition, multiple factors collectively crosstalk including auto-oxidation of glucose, glycation of proteins, and deficiency of GSH ultimately leading to overproduction of free radicals.[44] Depletion of cardiac GSH in the present study following diabetes whereas reassure generation of free radical also suggested the role of other factors as well. The free radicals through LPO cascade initiate chain of events severely affecting the membranous component of the tissue,[45] specifically the cardiomyocytes as observed in the present study. Moreover, the role of the antioxidant enzymes is also pertinent in affecting the cardiomyoangipathic effects. As these enzymes form the most crucial part of the tissue architecture and also gets affected in the diabetes. The observed microangiopathic effects thus seem to be akin to alterations in their activities. It seems that majority of these enzymes have responded as an adaptation to the proxidant state owing to diabetes. This is revealed by observed increases seen in the activities of SOD and CAT. Thus, it may be that during onset of diabetes, cardiomyocytes might predispose themselves with overexpression of enzyme SOD to dismutase superoxide radicals into hydrogen peroxide and oxygen. Pronounced increased in the activity of CAT in diabetic rats is possibly an adaptive response of a cell to neutralize hydrogen peroxide which was produced by hyper activity of SOD.[46] Besides, deregulation in the activities of GP and GR leading to low level of GSH/GSSG ratio together with increased activity of GST owing to limited GSH levels further augmented the effects leading ultimately to proxidant state and observed changes in the cardiac tissue architecture. Similar changes owing to proxidant generation following hyperglycemia, further substantiated our observations.[47-50] Treatment of these diabetic animals with ALE lends strong protective effects suggesting multifactorial effects of ALE. This even seems pertinent owing to the fact that ALE extract is a concoction of multiple factors such as polyphenols and flavanoids which include azadirachtin (A, B, D, H, and I) azadiradione, nimbin, nimbolin nimbolide, nimbinene, desacetylnimbin, azadirone, and salanin.[51,52] Majority of these phenolic compounds strongly influence the activity of antioxidant enzymes leading to re-maintenance of cardiac GSH levels and protection of cardiac tissue to diabetic effects.[53] Thus, it seems that the pro oxidant alleviating effect s of ALE is ascribed to the cumulative and concerted effects of its constituent components. Delineating these components, which warrants additional study, would certainly help to identify the

Table 3: Cardiac reduced glutathione content and lipid peroxidation indices in control and treated animals

Groups	GSH (nmoles/ mg protein)	LPO (nmoles MDA formed/mg protein)
C	5.58±0.23	0.693±0.16
D	3.0±0.21**a,**b	1.26±0.06**a,**b
D +ALE	4.13±0.17**a,**c	0.920±0.05**a,**c

n=6 in all groups, values are mean±SEM. *Significant at 0.05 level; **Significant at 0.001 level; [a]Compared with control group; [b]Compared with D + ALE group; [c]Compared with diabetic group. All experiments were replicated thrice. GSH: Glutathione; LPO: Lipid peroxidation; ALE: *Azadirachta indica* leaf extract; MDA: Malondialdehyde; D: Diabetic; C: Control; SEM: Standard error of mean

Table 4: Activities of cardiac antioxidant enzymes in control and treated animals

Groups	Catalase (µmoles hydrogen H_2O_2 decomposed/min/mg protein)	GP (nmoles NADPH oxidized/ min/mg protein)	GR (nmoles NADPH oxidized/ min/mg protein)	GST (nmoles CDNB conjugated/ min/mg protein)	SOD (enzyme units/mg protein)
C	14.63±1.15	70.61±2.90	17.90±0.66	132.82±1.78	2.5±0.12
D	35.08±1.36**a,**b	75.22±3.89	29.81±2.93**a	148.46±2.26**a,*b	4.0±0.42**a,*b
D + ALE	23.15±1.43**a,*c	64.0±5.78	24.38±0.84*a,*c	141.97±2.55*a,*c	2.1±0.23**c

n=6 in all groups, values are mean±SEM. *Significant at 0.05 level; **Significant at 0.001 level; [a]Compared with control group; [b]Compared with D + ALE group; [c]Compared with diabetic group. The enzyme activity of SOD was expressed as units/mg protein, where one unit of enzyme is the amount of enzyme inhibiting the rate of reaction (NBT reduction) by 50%. All experiments were repeated thrice. SOD: Superoxide dismutase; NADPH: Nicotinamide adenine dinucleotide phosphate; GST: Glutathione-S-transferase; GP: Glutathione peroxidase; GR: Glutathione reductase; ALE: *Azadirachta indica* leaf extract; D: Diabetic; C: Control; SEM: Standard error of mean; NBT: Nitro blue tetrazolium

active molecule(s) with strong therapeutic intervention. However, under present conditions, it is concluded that ALE has a potential to attenuate cardiac complications through modulation of antioxidant system and alleviation of diabetic symptoms.

CONCLUSION

The blood glucose lowering efficacy and attenuating pathological changes in diabetic heart concludes that Azadirachta indica provide cardio protection by ameliorating oxidative stress in rat model of diabetic mellitus.

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Conflicts of interest

There are no conflicts of interest.

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