Academic Sciences International Journal of Pharmacy and Pharmaceutical Sciences

ISSN- 0975-1491 Vol 4, Issue 1, 2012

Research Article

PROTECTIVE ROLE OF *AZADIRACHTA INDICA* AGAINST OXIDATIVE DAMAGE IN SKELETAL AND CARDIAC MUSCLE OF ALLOXAN DIABETIC RATS

SWETA SHAILEY, SEEMI FARHAT BASIR*

Department of Biosciences, Jamia Millia Islamia, New Delhi, India 110025. E-mail: seemifb@gmail.com

Received: 21 Sep 2011, Revised and Accepted: 20 Nov 2011

ABSTRACT

The aqueous extract of *A. indica* leaf and bark have been evaluated for their antioxidant potential in alloxan diabetic rats and compared with insulin treatment. The oral effective dose of *A. indica* leaf extract (AILE) and bark extract (AIBE) was given once daily for 21days to diabetic rats. At the end of the experimental period, the effect was studied on the activity of antioxidant enzymes and membrane lipid peroxidation in different fractions of cardiac and skeletal muscle. Tissues were analyzed for oxidative stress induced DNA damage and change in PKC-β2 content. Quantitative HPLC analysis of azadirachtin was done in AILE and AIBE. Diabetic rats showed high blood glucose ($p<0.01$), increased level of MDA ($p<0.05$) and a significant alteration in the activity of antioxidant enzymes. Alloxan induced oxidative stress caused DNA fragmentation and alteration in PKC-β2 protein content. Treatment with insulin AILE and AIBE restored the above altered parameters close to the control ones. Both, AILE and AIBE were found significantly effective in reducing hyperglycemia induced oxidative stress in heart and skeletal muscle of alloxan induced diabetic rats.

Keywords: Alloxan diabetes, Oxidative stress, Antioxidant enzymes, PKC-β2 protein.

INTRODUCTION

Diabetes mellitus is a metabolic disorder characterized by hyperglycemia and insufficiency of secretion or action of endogenous insulin. Experimental and clinical evidence indicates that oxidative stress is involved in both the pathogenesis and the complications of diabetes mellitus.[1] Oxidative stress has been implicated in the destruction of pancreatic cells and could largely contribute to the oxidant tissue damage associated with chronic hyperglycemia. Diabetes is usually accompanied by increased production of free radicals or impaired antioxidant defenses. Under normal physiological conditions, there is a critical balance in the generation of oxygen free radicals and antioxidant defense systems used by organisms to deactivate and protect themselves against free radical toxicity.[2] Impairment in the oxidant/antioxidant equilibrium in favor of the former provokes a situation of oxidative stress and generally results from overproduction of reactive oxygen species (ROS).[3,4] Mechanisms by which increased oxidative stress is involved in the diabetic complications are partly known, including activation of transcription factors, advanced glycated end products (AGEs), and protein kinase C.[5]

Oxidative stress is known to be a component of molecular and cellular tissue damage mechanisms in a wide spectrum of human diseases.[6] While exogenous insulin and other medications can control many aspects of diabetes, numerous complications affecting the vascular system, kidney, retina, lens, peripheral nerves, and skin are common and are extremely costly in terms of longevity and quality of life. Various hypoglycemic drugs are also being used for the treatment but their use is restricted by their limited action and accompanying side effects. During the last five decades, considerable progress has been achieved regarding the biological activity and medicinal application of *Azadiracta indica* A Juss (Neem). Neem bark and leaf extracts have been therapeutically used in ayurveda as a folk medicine to control leprosy, intestinal helminthiasis, respiratory disorders and constipation and also as a general health promoter.[7] There are several reports which suggest the hypoglycemic potential of *A. indica*.[8,9] The present study explores the hypoglycemic and antioxidant potential of *A. indica* leaf extract and bark extract, evaluates their effect on altered level of antioxidant enzymes and other related parameters in the alloxan-diabetic rat muscles and compared with insulin treatment.

MATERIAL AND METHODS

Healthy leaves and bark of *A. indica* were collected from the university campus. Extracts were prepared by following the method of Bandyopadhyay et al.[10]

Leaf Extract: One kg of freshly collected, shade dried, powdered leaves of *A. indica* were ground in 4 liters of distilled water and allowed to soak overnight at room temperature. The suspension was then centrifuged at 5000 rpm for 20 min and filtered through Whatman No.1 filter paper. The filtrate was lyophilized to yield 12.9 gm of dry powder and stored at −20°C. A measured amount of the extract was dissolved in distilled water at a suitable concentration prior to experiment.

Bark Extract: Air dried bark from a full grown Neem tree, devoid of external hard wood was cut into small pieces and 100 gm was soaked overnight in one liter distilled water at room temperature with occasional shaking. After filtration, the brown red extract was lyophilized to yield 3.7 gm of dry powder and stored at −20 °C. A required concentration of powder was prepared in distilled water prior to experiment.

Treatment of Animals

Healthy female rats of Wistar strain (2-3 months old) weighing between 180-210 gm were selected for the study. All animals were kept at 25-30°C and 45-55% relative humidity, acclimatized with standard chow and water *ad libitum* throughout the study under 12 hr light – 12 hr dark cycle. The animals were carefully monitored and experimental protocols were in accordance with the recommendations of the Institutional Animal Ethical Committee.

Diabetes was induced by single subcutaneous injection of alloxan monohydrate (15 mg /100 g body weight) dissolved in a freshly prepared 0.154M sodium acetate buffer (pH 4.5).[11] Each animal was then given 2U of insulin for next 7 days to reduce mortality. Control animals received only the vehicle. The rats with fasting glucose level above 360 mg/dl were considered diabetic and grouped as Normal Control (N), Diabetic untreated (D), Insulin treated diabetic group (D+I) (received intraperitoneal injection of 2 units of protamine-zinc insulin once daily), *A. indica* Leaf Extract treated diabetic group (D+AILE) (500 gm/Kg body weight), *A. indica* Bark Extract treated diabetic group (D+AIBE) (100 mg/Kg body weight).

Alloxan monohydrate, Insulin and glutathione reductase were purchased from Sigma and other chemicals and reagents used were of analytical grade.

Both control and experimental groups were kept separately in individual cages. The experimental groups, D+AILE and D+AIBE, received orally an aqueous solution of the lyophilized powder of AILE (500 gm/Kg body wt.) and AIBE (100 mg/Kg body wt.) respectively by intragastric tube. Insulin treated diabetic group received 2U of insulin once daily and the control group received

vehicle only. The treatment was given for 21 days. For each dose, the required amount of the lyophilized powder calculated from the body weight of the animal was weighed, dissolved in distilled water at suitable concentration so that the desired amount for each dose can be administered in 0.5 ml to each animal. Effective dosages were selected from the literature and after doing pilot studies.

Antioxidant enzymes

At the end of the experiment, rats were sacrificed by cervical dislocation. The tissues were rapidly excised and washed with chilled normal saline. The 10% (w/v) tissue homogenate was prepared in 0.25 M Sucrose, 0.02 M Triethanolamine hydrochloride buffer, pH 7.4 containing 0.12 M Dithiothreitol (DTT). Homogenates were then centrifuged at 1000×g for 10 min to remove nuclei and cell debris. The supernatant was again centrifuged at 12,000xg for 40 min to obtain cytosolic fraction of the tissue. All the procedures were carried out at 4°C. The tissue cytosolic fraction was used for determination of enzymes activities.

The activity of Superoxide dismutase (SOD) was measured by the method of Marklund et al with some modification, an assay based on the ability of SOD to inhibit the autoxidation of pyrogallol by 50%.[12] The change in absorbance of assay mixture was monitored at 420 nm for 3 min at 25°C against blank. One unit of enzyme activity is defined as the amount of enzyme that causes 50% maximal inhibition of pyrogallol autoxidation. The assay of catalase was performed by following the method of Aebi.[13] One unit of enzyme activity is defined as the amount of enzyme required to decompose 1µmol of H_2O_2. Change in absorbance was monitored at 240 nm at 25°C. The activity of Glutathione peroxidase (GPx) was measured by using a coupled enzyme assay as described by Lawrence and Burk.[14] The decrease in absorbance was monitored at 25°C at 340 nm. One unit of enzyme activity is defined as 1 µmol of NADPH oxidized/min/mg protein. The Glutathione reductase (GR) activity was measured in the soluble tissue extracts by the modified method of Erden et al.[15] One unit of enzyme activity is defined as 1 µmol of NADPH oxidized/min/mg protein. The decrease in absorbance was monitored at 25°C at 340 nm.

Lipid peroxidation

The level of lipid peroxidation was assessed by measuring the formed malondialdehyde (MDA), an end product of fatty acid peroxidation, by using thiobarbituric acid reactive substance (TBARS) method.[16] The 10% tissue homogenate was centrifuged at 1000xg for 10 min and deproteinized with half volume of 20% trichloroacetic acid (TCA). Precipitated proteins were removed by centrifugation. The supernatant in 10 mM Potassium phosphate buffer (pH 7.4) was incubated at 80°C for 15 min in water bath with 0.53% Thiobarbituric acid in glacial acetic acid and centrifuged. The concentration of MDA-TBA complex was determined spectrophotometrically at 532 nm against blank.

Glycemic Index

Blood glucose was determined by using Glucose-kit from Span Diagnostics India, which quantitatively estimates D-glucose, the form that is present in blood plasma. Glycosylated hemoglobin (GHbA1c) was estimated by Ion Exchange Resin method using kit purchased from Aristha Pharmaceuticals, India. Soluble protein was determined by method of Bradford using BSA as standard.[17]

Degradation of genomic DNA

For isolation of genomic DNA, liver tissues were rapidly excised and washed with chilled normal saline. The tissues were blotted dry and minced well with sterile scissors. Tissues were treated with 5ml of digestion buffer and 50µl of proteinase K. The phenol method was used to isolate the DNA samples.[18] Equal volume of Tris EDTA (TE)-saturated phenol was added to an aqueous DNA sample. The upper aqueous layer was carefully removed avoiding the phenol interface and subjected to two ether extractions to remove residual phenol. DNA was concentrated by ethanol precipitation.[18] The quantity of isolated DNA was checked by adding 5µl of DNA sample to 1ml of distilled water and the optical density was read at 260 nm against water as described by Ausubel.[19] Isolated DNA samples from control and different experimental groups were separated and analyzed by horizontal gel electrophoresis as described by Nagata.[20]

Western blot analysis

Membrane fractions from heart and skeletal muscle were prepared by following the method of Wakasaki et al.[21] 1g of frozen tissue was minced well and homogenized (1/10, w/v) in homogenization buffer (20mM Tris-HCl pH 7.5, 2mM EDTA, 1mM phenylmethylsulfonyl fluoride, 1mM DTT, 0.3M sucrose and 25µg/ml leupeptin) at 4°C. The homogenate was centrifuged at 1000xg for 10 min at 4°C and supernatant was ultracentrifuged at 190,000xg for 60 min at 4°C to obtain total membrane fraction. The membrane pellets were resuspended in homogenization buffer and the total membrane protein content were measured by following the method of Bradford.[17]

Western blot analysis was done by following the method described by Towbin and Gordon.[22] Total membrane fractions (30µg each lane) were subjected to SDS-PAGE on 7% polyacrylamide gel and resolved proteins were transferred to PVDF membrane. After transfer the membranes were blocked in 5% fat free milk and incubated with a 1:500 dilution of isoform β2 specific anti-PKC antibody. The antigen-antibody complexes were detected with HRP-conjugated secondary antibody using DAB as the coloring reaction. Quantification was performed by densitometery after scanning the blots. Isoform β2 specific anti-PKC antibody and HRP-conjugated anti-rabbit secondary antibody were purchased from Sigma Aldrich.

HPLC analysis

To compare the chemical profile of the extracts, quantitative HPLC analysis of azadirachtin was done.[23] An HPLC system (UV-2075 Plus, JASCO, Japan) with two gradient pump systems (PU-2080, JASCO) were used for the analysis. The separation was performed on a C-18 column (Thermoscientific, Dim (mm) 250x 4.6, ODS Hypersir). HPLC conditions were as follows: Solvent A (water) and Solvent B (acetonitrile), gradient elution was used as A: 0-10 min, 30-40%; B: 10-15 min, 40-45%; A: 20-25 min 50-60%. Flow rate of mobile phase solution was 1.0 ml/min and detection was at 217nm. 10µl of each sample was injected in to the HPLC machine.

Statistical analysis

All values were calculated as mean ± SEM. The ANOVA test followed by Dunnet Multiple comparison test was employed for statistical comparison between control and various groups. Significance was considered at $p<0.05$.

RESULTS AND DISCUSSION

The present study explored the effect of experimental diabetes on different parameters related to antioxidant defense system in skeletal and cardiac muscle, and whether the treatment with AILE and AIBE can restore the alterations occurred due to hyperglycemia induced oxidative stress.

All the diabetic animals showed characteristic symptoms of glycosuria, polydipsia, polyphagia and reduced rate of growth. The changes in body weight were observed in all experimental groups and results are presented in Table 1. After 21 days of diabetes induction, body weight was significantly reduced in the diabetic group ($p<0.01$). Insulin, AILE and AIBE treatment for 21 days resulted in significant increase in body weight when compared to the diabetic rats. Alloxan induced diabetic rats showed marked hyperglycemia with almost 3-fold higher blood glucose concentration when compared to the control values (Table 1). All the diabetic treated groups showed reduced hyperglycemia, AILE and AIBE were found significantly ($P<0.05$) effective in lowering the blood glucose levels in diabetic rats. Glycosylated hemoglobin, measured as % of HbA1c, was significantly increased ($p<0.05$) in untreated diabetic animals when compared with healthy controls (Table 1). Though, the decrease of HbA1c was observed in all the diabetic treated groups, treatment was more effective in AILE treated group.

Table 1: Changes in body weight and glycemic index of control, diabetic and diabetic treated rats

Parameters	C	D	D+I	D+AILE	D+AIBE
Body weight (g)	188.3 ± 11.0	91.6 ± 9.3[b]	165 ± 9.1	155 ± 15	136± 18.4
Fasting plasma glucose (mg/dl)	85.5 ± 5.6	296.0 ± 13.4[a]	101.7 ± 10.1	114.3 ± 11.9[b]	142.1 ± 8.2[b]
Glycated Hemoglobin (% GHb1Ac)	5.4 ± 0.27	10.1 ± 0.35[b]	7.3 ± 0.34	7.7 ± 0.24	8.4 ± 0.16

Each value is the mean ± SEM of five separate experiments. *P* values are shown as [b]*p*<0.05, [a]*p*<0.01 *vs.* control.

In diabetes, the persistence of hyperglycemia has been reported to cause increased production of oxygen free radicals through auto-oxidation and non-enzymatic glycation. If the diabetic state is associated with a generalized increase in tissue oxidative stress it might be reflected in the changes in tissue antioxidant system. Therefore, the activities of some major antioxidant enzymes were measured in control and experimental rats. Diabetic rats showed altered levels of the antioxidant enzymes in skeletal and cardiac muscle (Fig. 1 and 2). Under normal physiological condition there is a critical balance in the generation of oxygen free radicals and its antioxidant defense systems used by organism to deactivate and protect themselves against free radical toxicity.[24]

Impairment in the oxidant /antioxidant equilibrium in favor of the former provokes a situation of oxidative stress which is known to be a component of molecular and cellular tissue damage mechanisms in wide spectrum of human diseases and contributes substantially to the pathogenesis of diabetic complications.[6]

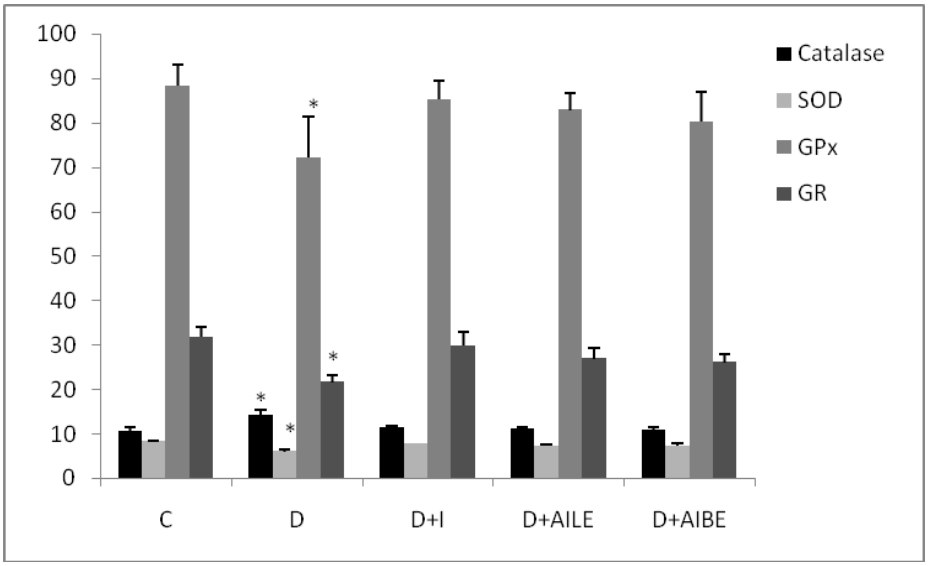

Fig. 1: Changes in Antioxidant enzyme activities in control, diabetic and diabetic treated rats in cytosolic fraction of skeletal muscle

Results are expressed as mean ± SEM of three separate experiments and compred with control values. *p<0.05. Enzyme activities are given as- SOD, Catalase: U/mg protein; GPx, GR : mU/mg protein

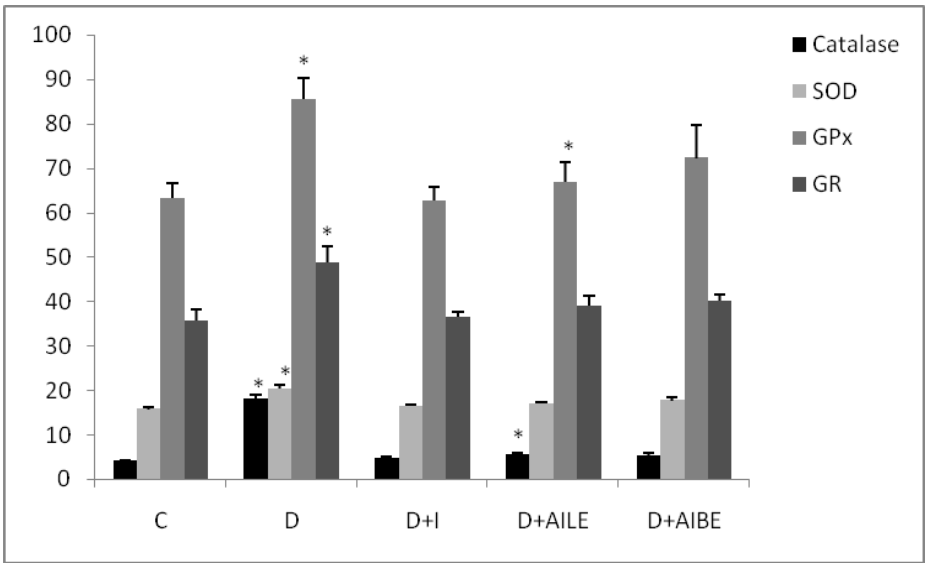

Fig. 2: Changes in Antioxidant enzymes in control, diabetic and diabetic treated rats in cytosolic fraction of cardiac muscle

Results are expressed as mean ± SEM of three separate experiments and compred with control values. *p<0.05. Enzyme activities are given as- SOD, Catalase: U/mg protein; GPx, GR : mU/mg protein.

In skeletal muscle SOD, catalase, GPx and GR activity significantly ($p<0.05$) decreased in diabetic rats (Fig. 1). However, the heart tissue exhibit different susceptibility to oxidative stress. The increased activity of SOD, catalase, GPx and GR were observed in diabetic rats which are in agreement with the earlier published data.[16] This increase may be a manifestation of and adaptive response to compensate for the increased oxidative stress. Treatment with insulin, AILE and AIBE restored the altered levels of SOD, catalase, GPx and GR close to their normal values in heart and skeletal muscle of diabetic rats. The efficacy of AILE and AIBE treatment was found comparable to the insulin treatment. All the enzyme activities are expressed as per milligram protein and therefore, represent true changes under these conditions.

Hyperglycemia has been shown to generate free radicals from auto-oxidation of glucose, formation of advanced glycated end products (AGEs) and increased polyol pathway with concomitant increase in cellular lipid peroxidation and damage of cellular membranes.[5] In the present study the formation of malonaldihyde (MDA), a product of lipid peroxidation, was significantly increased in diabetic heart and skeletal muscle tissues ($p<0.05$) (Table 2). The results are in agreement with earlier published data of Mohamad et al.[23] This increased lipid peroxides formation during diabetes disturbs the anatomical integrity of the membrane leading to the inhibition of several membrane bound enzymes.[26] The results revealed a sharp decreased level of MDA in diabetic rats treated with insulin, AILE and AIBE when compared to untreated diabetic rats indicating the efficacy of treatment.

Table 2: Changes in MDA levels in control, diabetic and diabetic treated rats in blood plasma and cytosolic fraction of liver and kidney

	C	D	D+I	D+AISE	D+AIBE
Heart cytosolic fraction (nmol MDA/mg protein)	0.79 ± 0.05	2.08 ± 0.24[b]	0.89 ± 0.12	1.07 ± 0.09	0.96 ± 0.07
Skeletal muscle cytosolic fraction (nmol MDA/mg protein)	0.68 ± 0.07	2.16 ± 0.51[b]	0.75 ± 0.03	1.12 ± 0.21	0.99 ± 0.08

Each value is the mean ± SEM of three separate experiments. *P* values are shown as [b]$p<0.05$ *vs*. control.

The decrease in antioxidant enzymes leads to the accumulation of ROS during diabetes. One of the important fallbacks of oxidative stress is increased H_2O_2 production. It has already been reported that during vascular conditions such as atherosclerosis, circulating macrophages release large amounts of H_2O_2. Apoptosis that occurs as a result of this increased H_2O_2 contributes towards these pathologies.[27] DNA fragmentation is one of the morphological changes during diabetes. Agarose gel electrophoresis of genomic DNA isolated from control animals showed no evidence of DNA fragmentation (Fig. 3). In contrast, genomic DNA isolated from diabetic rats showed smear pattern, which is characteristic of random DNA fragmentation.[20] Treatment with insulin, AILE and AIBE prevented genomic DNA degradation.

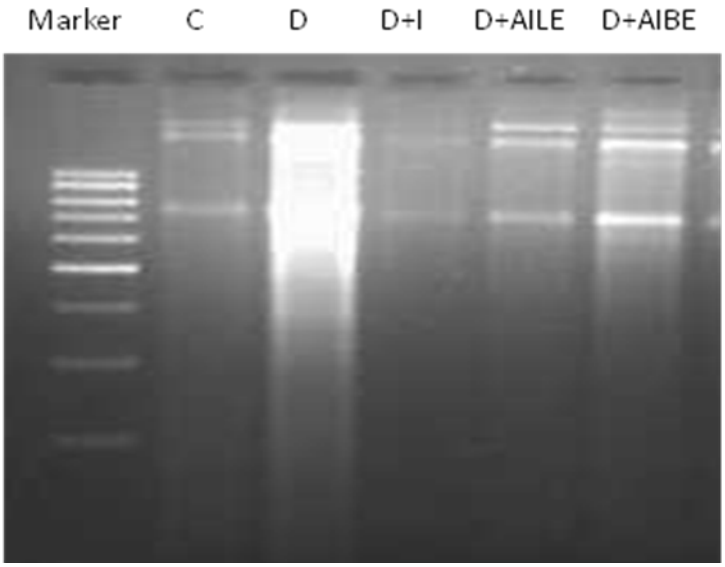

Fig. 3: Agarose gel electrophoresis of genomic DNA isolated from control, diabetic and diabetic rats treated with insulin, AILE and AIBE

Hyperglycemia may activate PKC isoforms indirectly through both ligation of AGE receptors and increased activity of polyol pathway by increasing ROS.[28] In diabetic state increased level of PKC-β2 protein was observed in cardiac and skeletal muscle tissues (Fig. 4 and 5 respectively). However exogenous supplement of insulin to diabetic rats considerably ameliorated the augmented levels of PKC-β2 in heart and skeletal muscle. Treatment with AILE and AIBE reverted the levels back to euglycemic condition as demonstrated by immunoblots of heart and skeletal muscle. This could primarily be due to subsequent lowering of blood glucose levels which reduces the oxidant levels.

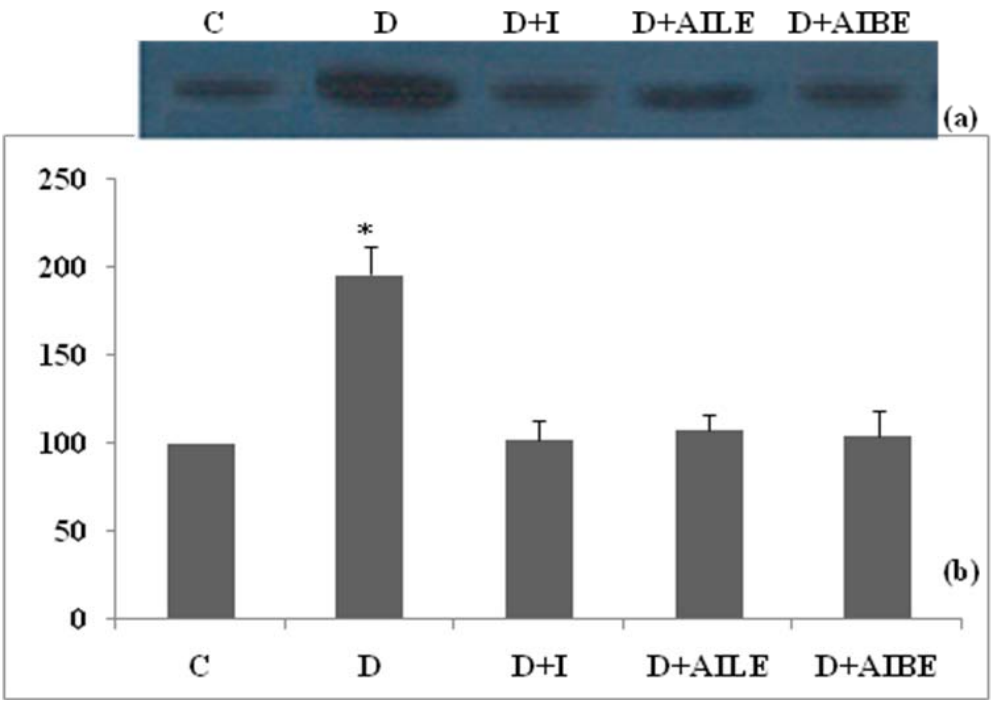

Fig. 4: Changes in PKC β2 protein levels in the cardiac muscle membrane fraction of control, diabetic and diabetic treated rats. (a) Representative picture of three separate experiment. *p<0.05.

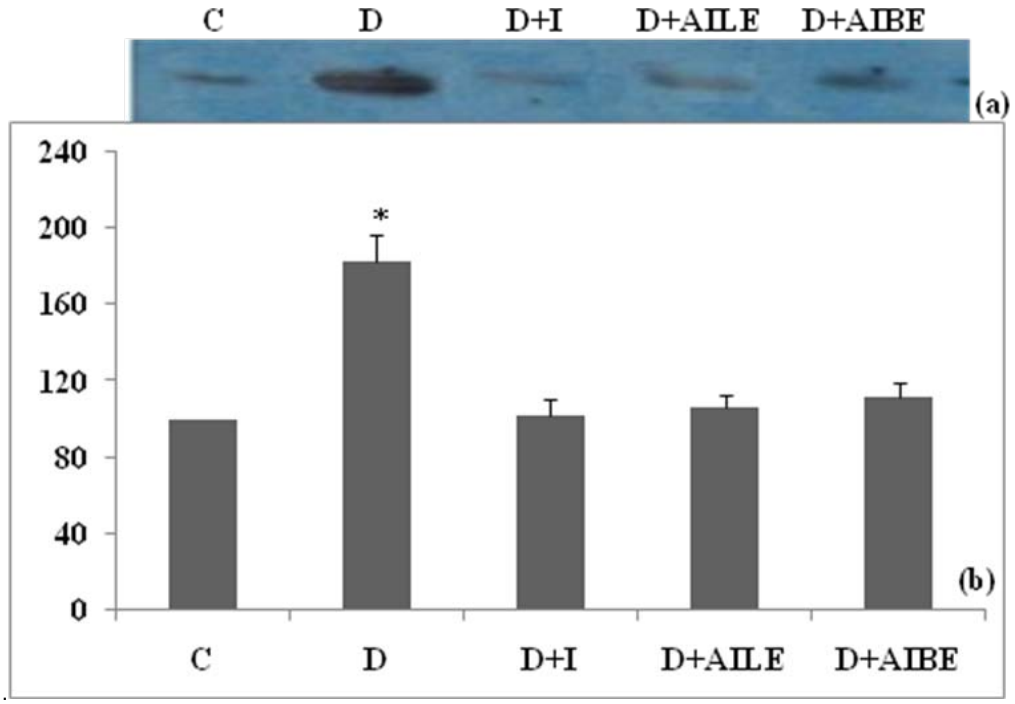

Fig. 5: Changes in PKC β2 protein levels in the skeletal muscle membrane fraction of control, diabetic and diabetic treated rats. (a) Representative picture of three separate experiment. *p<0.05.

In the study done by Priyadarsini et al,[29] azadirachtin (a limonoid) exhibited concentration-dependent anti-radical scavenging activity. The quantitative analysis of azadirachtin content in AILE and AIBE were performed using HPLC and representative chromatograms are shown in Figure 6. Azadirachtin in solvent was identified by matching retention time and spectra with standard and quantitative data was calculated on the basis of peak area of the compound. The aqueous extract of leaf (AILE) and bark (AIBE) showed 41µg/ml and 57µg/ml of azadirachtin content, respectively. Previous studies also reported the presence of azadirachtin in *A. indica* leaf and bark.[23,30] However, little variation may be due to the origin, genetic or geographical factors.[30]

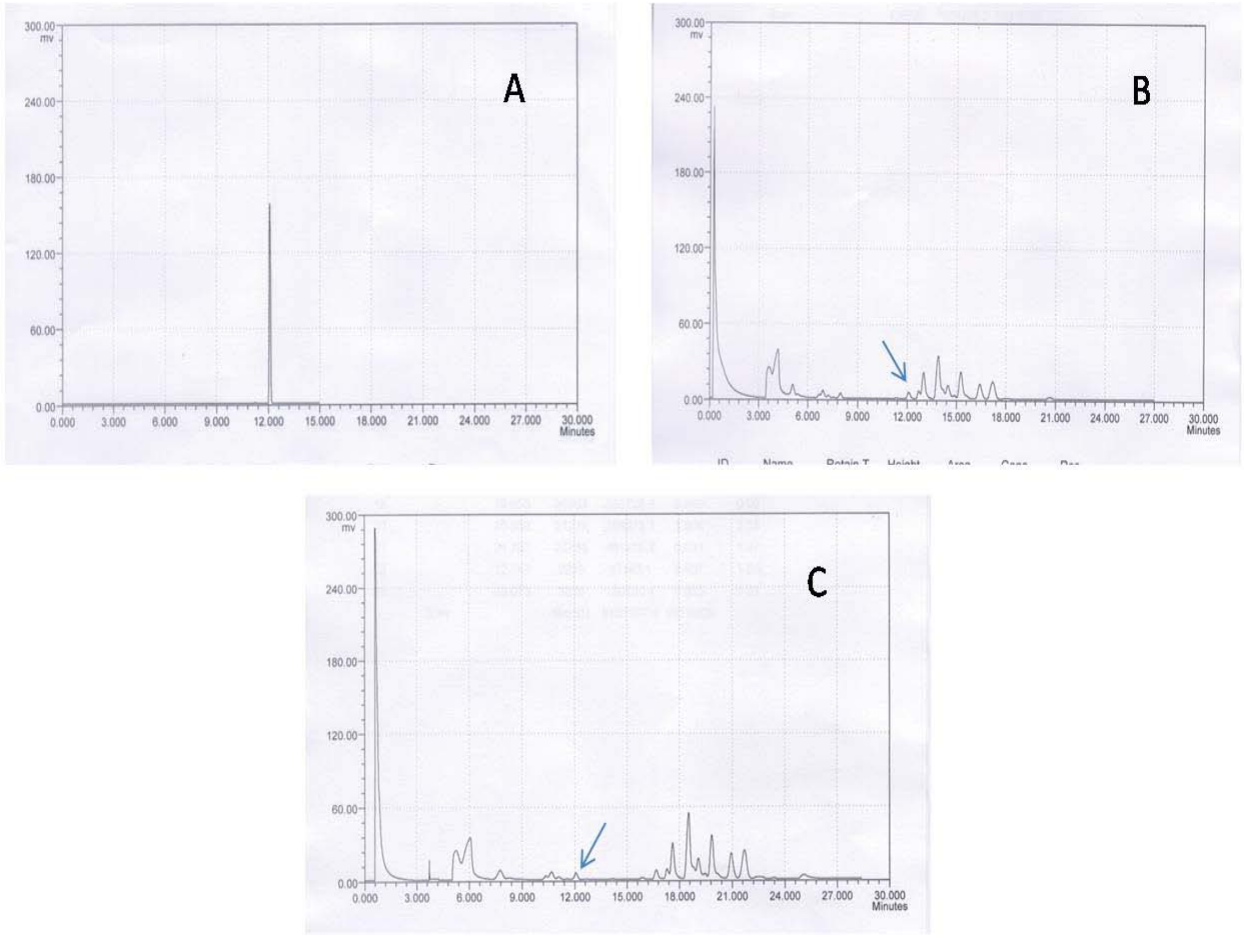

Fig. 6: HPLC result of *A. indica* extracts showing azadirachtin. (A) Standard azadirachtin with retention time 12.09 min. Arrows denotes the chromatogram of azadirachtin in AILE (B) and AIBE (C).

CONCLUSION

The present study concludes the antioxidant potential of *A. indica* leaf and bark extract that can be exploited for a potential antidiabetic drug. In diabetic condition, the function of insulin system is suppressed which leads to hyperglycemia, and this sustained increase in blood glucose caused antioxidant damage to tissues. Treatment with *A. indica* was found effective in improving the antioxidant status in cardiac and skeletal muscle. Further studies are needed to ascertain which photochemical fraction is the most efficacious in the treatment of diabetes and, development of potent antidiabetic formulation from the same.

ACKNOWLEDGEMENT

The first author acknowledges the receipt of research fellowship from Council of Scientific and Industrial Research (CSIR), India during this work.

REFERENCE

1. Ceriello A. Oxidative stress and glycemic regulation. Metabolism 2000; 49 (2, Suppl 1):27–29.
2. Sies H: Oxidative Stress: Oxidants and Antioxidants .New York, Academic, 1991. pp2-8.
3. Naskar S, Mazumdar UK, Pramnik G, Bala A. Comparative *In-Vitro* Antioxidant activity of different parts of *Cocos nucifera* Linn. On reactive oxygen and nitrogen species. Int J of pharm pharm sci 2011; 3 suppl 3:104-107.
4. N. Subhashini, A Thangathirupathi, N Lavanya, Antioxidant activity of *Trigonella foenum graecum* using various *in-vitro* and *ex-vivo* models, Int J of pharm pharm sci 2011; 3 suppl 2:96-102.
5. Saxena AK, Srivastava P, Kale RK, Baquer NZ. Impaired antioxidant status in diabetic rat liver.Effect of vanadate. Biochem Pharmacol 1993; 45(3):539–542.
6. Halliwell B. Free radicals, antioxidants, and human disease: cause or consequence? Lancet 1994; 344:721–724.
7. Biswas K, Chattopadhyay I, Banerjee RK, Bandyopadhyay V. Biological and medicinal properties of Neem (*Azadirachta indica*). Curr Sci 2002; 82(11):1136-1145.
8. El- Hawary ZM, Kholeif TS. Biochemical studies on hypoglycemic agents (1) Effect of *Azadirachta indica* leaf extract. Arch Phytother Res 1990; 13: 108-112.
9. Chattopadhyaya RR. Possible mechanism of antihyperglycemic effect of Azadirachta Indica leaf extract. J Ethnopharmacol 1990; 67:373-376.
10. Bandyopadhyay U, Biswas K, Chatterjee R, Bandyopadhyay D, Chattopadhyay I, Ganguly CK *et al.* Gastroprotective effects of Neem (*Azadirachta Indica*) bark extract: Possible involvement of H^+-K^+ ATPase Inhibition and scavenging of hydroxyl radical. Life Sci. 2002; 71:2845-2865.
11. Sochor M, Baquer NZ, McLean P. Glucose over and under utilization in diabetes: Comparative studies on changes in activities of enzymes of glucose metabolism in rat kidney and liver. Mol Physiol 1985; 7:51-68.
12. Marklund S, Marklund G. Involvement of superoxide anion radical in the autoxidation of pyrogallol and a convenient assay for superoxide dismutase. Eur J Biochem 1974; 47:469-474.
13. Aebi H. Catalase; in Methods in enzymatic analysis (ed.) HV Bergmeyer, New York: Academic press. 1974; 673-684.
14. Lawrence RA, Burk RF. Gutathione peroxidase activity in selenium deficient rat liver. Biochem Biophys Res Commun 1976; 71:1161-1165.

15. Erden M, Bor NM. Changes in reduced glutathione, glutathione reductase and glutathione peroxidase after radiation in guinea pigs. Biochem Med 1984; 31:217-227.
16. Genet S, Kale RK, Baquer NZ. Alteration in antioxidant enzymes and oxidative damage in experimental diabetic rat tissues: effect of vanadate and fenugreek (*Trigonella foenum graecum*). Mol Cell Biochem 2002; 236:7-12.
17. Bradford MM. A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976; 72:248-254.
18. Gupta RC. Nonrandom binding of the carcinogen N-hydroxy-2-acetylaminofluorene to repetitive sequence of rat liver DNA in vivo. Proc Natl Acad Sci 1984; 81:6943-6947.
19. Ausubel F. Short protocols in molecular biology. Wiley & Sons Inc, New York, 1995.
20. Nagata. Apoptotic DNA fragmentation. Exp Cell Res 2000; 10 (256, Suppl 1):12-18.
21. Wakasaki H, Koya D, Schoen FJ, Jirousek MR, Hoit BD, Walsh RA, King GL. Targeted overexpression of protein kinase C beta-2 isoform in myocardium causes cardiomyopathy. Proc Natl Acad Sci 1997; 94(17):9320-9325.
22. Towbin H, Gordon J. immunoblotting and dot immunoblotting – current status and outlook. J Immunological Methods 1984; 72(2):313-340.
23. Ghimeray AK, Jin CW, Ghimire BK, Cho DH. Antioxidant activity and quantitative estimation of azadirachtin and nimbidin in *Azadirachta indica* A. Juss grown in foothills of Nepal. Af J Biotech 2009; 8(13):3084-3091.
24. Sies H. Oxidative stress: oxidants and antioxidants. New York: Academic Press; 1991.p. 2-8.
25. Mohamad S, Taha A, Bamezai RN, Basir SF, Baquer NZ. Lower doses of vanadate in combination with *Trigonella* restore altered carbohydrate metabolism and antioxidant status in alloxan-diabetic rats. Clin Chim Acta 2004; 342:105-114.
26. Kakkar R, Kalra J, Mantha SV, Prasad K. Lipid peroxidation and activity of antioxidant enzymes in diabetic rats. Mol Cell Biochem 1995; 151:113-119.
27. Nishikawa T, Edelstein D, Brownlee M. The missing link: a single unifying mechanism for diabetic complications. Kidney Int Suppl 2000; 77: S26-30.
28. Geng YJ, Libby P. Evidence for apoptosis in advanced human atheroma. Colocalization with interleukin-1 beta-converting enzyme. Am J Pathol 1995; 147(2): 251-266.
29. Priyadarsini RV, Manikandan P, Kumar GH, Nagini S. The neem limonoids azadirachtin and nimbolide inhibit hamster cheek pouch carcinogenesis by modulating xenobiotic-metabolizing enzymes, DNA damage, antioxidants, invasion and angiogenesis. Free radical res 2009; 43 (5): 492-504.
30. Kaushik N, Gurudevsingh B, Tomar UK, Naik SN, Satya V, Bisla SS *et al.* Regional and habitat variability in azadirachtin content of Indian Neem (*Azadirachta indica*). Curr Sci 2007; 92(10):1400-1406.