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EFFECT OF *AZADIRACHTA INDICA* ON HYPERGLYCEMIA INDUCED OXIDATIVE STRESS IN ALLOXAN DIABETIC RAT BRAIN

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Keywords:

Azadirachta indica, Oxidative stress, Superoxide dismutase, Catalase, Lipid peroxidation

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ABSTRACT: The present study evaluates antidiabetic effect of *A. indica* leaf extract (AILE) and *A. indica* bark extract (AIBE) on antioxidant enzymes in the brain of alloxan diabetic rats and compared with insulin treatment. The oral effective dose of *A. indica* leaf (500mg/Kg body weight) and *A. indica* bark (100mg/Kg body weight) was given once daily for 21days to separate groups of diabetic rats. At the end of the experimental period, blood glucose levels and activities of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR) and glucose-6-phosphate dehydrogenase (G-6-PD) were measured in cytosolic fraction of whole brain. Lipid peroxidation levels were measured in the blood plasma and brain of all the treated diabetic groups. Diabetic rats showed high blood glucose ($p<0.01$). Decreased activities of SOD, CAT, G-6-PD ($p<0.05$) and increased activity of GPx, GR were observed in diabetic rats brain. Diabetic controls showed significant increase in MDA level in brain tissue ($p<0.05$). Treatment with insulin, AILE and AIBE restored the above altered parameters close to the control ones. AILE and AIBE were found significantly effective in correcting these alterations

INTRODUCTION: Diabetes is characterized by hyperglycemia and metabolic abnormalities due to decreased insulin levels, causing metabolic and physiological changes in various organs including brain. [1, 2] Oxidative stress is suggested to be a potential contributor to the development of complications in diabetes. [3-6] High oxidative stress can lead to microvascular cerebral diseases, including stroke, cerebral hemorrhage and brain infarction. [7, 8]

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The reason for high risk of microvascular cerebral diseases, despite the fact that brain consumes 20% of the oxygen in body, is that it has a low content of unsaturated fatty acids and catecholamines that are easily oxidized, making the brain more vulnerable to oxidative damage than any other organ in the body. [9] It has been reported that free radicals generated during diabetes deteriorate membrane structure and decrease membrane fluidity.[10]

Various hypoglycemic drugs, e.g. sulfonylurea, metformin etc., are being used for the treatment of diabetes but their use is restricted by their limited action and accompanying side effects. Insulin treatment also fails to prevent the long term complications. Thus there is a need to seek newer and alternative approaches for effective therapy in

diabetes management. Considerable progress has been achieved in the last five decades regarding the pharmacological potential 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. [11-13] There are several reports which suggest the hypoglycemic potential of *A. indica*. [14, 15] The present study explores the antidiabetic potential of *A. indica* leaf extract and bark extract, evaluates their effect on altered level of antioxidant enzymes in the alloxan-diabetic rat brains and compared with insulin treatment.

MATERIAL AND METHODS:

Preparation of *Azadirachta indica* Extracts:

Healthy leaves and bark of A. indica were collected from the university campus. Extracts were prepared by following the method of Bandyopadhyay et al. [16]

Leaf Extract (aqueous):

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^{o}C. A measured amount of the extract was dissolved in distilled water at a suitable concentration prior to experiment.

Bark Extract (aqueous):

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^{o}C. A required concentration of powder was prepared in distilled water prior to experiment.

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^{o}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 ethical permission was obtained. Experimental protocols were in accordance with the recommendations of the Institutional Animal Ethical Committee.

To induce diabetes, animals were starved for 24 h and a single subcutaneous injection of alloxan monohydrate dissolved in a freshly prepared 0.154M sodium acetate buffer (pH 4.5) was given at a dose of 15 mg /100 g body weight. [17] Each animal was then given 2U of insulin for next 7 days to reduce mortality. Control animals received only the vehicle. The rats were monitored for plasma glucose level. The rats with fasting glucose level above 360 mg/dl were considered diabetic and divided into following 5 groups of 6 animals each:

Group 1- Normal Control (NC)

Group 2 - Diabetic Control (DC)

Group 3 - Insulin treated diabetic group (D+I)

Group 4- *A. indica* Leaf Extract treated diabetic group (D+AILE) and

Group 5- *A. indica* Bark Extract treated diabetic group (D+AIBE).

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

Treatment of Animals:

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 and 60 days separately to different set of groups. 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.

Estimation of enzyme activities:

At the end of the experiment, rats were sacrificed by cervical dislocation. The brains were rapidly excised and washed with chilled normal saline. The brains were then blotted dry and weighed. 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^{o}C. The tissue cytisolic fraction was used for determination of enzymes activities.

Superoxide dismutase:

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%. [18] The assay mixture of 1 ml contained in final concentration, 50 mM Sodium phosphate buffer (pH 7.4), 0.1 mM EDTA, 0.48 mM Pyrogallol and appropriate amount of tissue extract containing 7-10 µg of protein. The change in absorbance of assay mixture was monitored at 420 nm for 3 min at 25^{o}C against blank. One unit of enzyme activity is defined as the amount of enzyme that causes 50% maximal inhibition of pyrogallol autoxidation.

Catalase:

The assay of Catalase (CAT) was performed by following the method of Aebi. [19] The assay mixture of 1 ml in final concentration contained 50 mM Sodium phosphate buffer (pH 7.0) and 10 mM H_2O_2.The reaction was started by addition of cytosolic fraction containing 2-3 µg protein. 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^{o}C.

Glutathione peroxidase:

The activity of Glutathione peroxidase (GPx) was measured by using a coupled enzyme assay as described by Lawrence and Burk. [20] The assay mixture of 1 ml contained in final concentration, 10 mM Potassium phosphate buffer (pH 7.0), 25 mM EDTA, 0.5mM Glutathione (reduced), 2mM Sodium azide, 1.5 IU Glutathione reductase, 0.1 mM NADPH and the cytosolic fraction containing about 50 µg of protein. The reaction was started by the addition of t-butyl hydroperoxide and the decrease in absorbance was monitored at 25^{o}C at 340 nm. One unit of enzyme activity is defined as 1 µmol of NADPH oxidized/min/mg protein.

Glutathione reductase:

The Glutathione reductase (GR) activity was measured in the soluble tissue extracts by the modified method of Erden et al. [21] The reaction mixture of 1 ml contained in final concentration, 4.1 mM Tris-HCl (pH 7.5), 15 mM $MgCl_2$, 5.7 mM EDTA, 60 mM KCl, 2.6 mM Glutathione (oxidized) and 0.1 mM NADPH. The reaction was started by the addition of tissue extract containing approximately 100 µg of protein. One unit of enzyme activity is defined as 1 µmol of NADPH oxidized/min/mg protein. The decrease in absorbance was monitored at 25^{o}C at 340 nm.

Glucose-6-phosphate dehydrogenase:

G6PD activity was assayed by following the method described by Cohen and Rosemeyer. [22] The assay mixture of 1ml contained in final concentration, 0.05M trisHCl buffer (pH7.6), 0.8mM glucose-6-phosphate, 8mM $MgCl_2$ and 0.1mM NADP The reaction was started by adding appropriate amount of tissue extract containing 100 µg protein. The change in absorbance was monitored at 340 nm for 3 min at 25^{o}C against blank. One unit of enzyme activity is defined as 1nmol NADP reduced/min/mg protein.

Lipid peroxidation:

The level of lipid peroxidation was assessed in blood and the brain tissue by measuring the formed malondialdehyde (MDA), an end product of fatty acid peroxidation, by using thiobarbituric acid reactive substance (TBARS) method. [1] The 10% tissue homogenate was centrifuged at 1000xg for 10 min and deproteinized with half volume of 20% trichloroacetic acid (TCA). Blood plasma was used as a whole. Precipitated proteins were removed by centrifugation. The supernatant in 10 mM

Potassium phosphate buffer (pH 7.4) was incubated at 80^{o}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.

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. [23]

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:

General parameters and Hyperglycemia:

Animals with glucose level >360mg/dl were selected for experimental analysis. These animals had glycosuria, polydipsia, polyphagia and reduced rate of growth. The changes in general parameters like body weight, tissue weight and tissue protein as observed in all experimental groups and controls are summarized 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 as compared to the diabetic rats. The brain weight, when calculated per 100g body weight did not show any statistically significant difference.

The protein content in cytosolic fraction of the brain of control and experimental groups did not show any significant difference. All the enzyme activities are expressed as per milligram protein and therefore, represent true changes under these conditions.

TABLE 1: GENERAL PARAMETERS OF THE CONTROL (NC), DIABETIC (DC), AND DIABETIC RATS TREATED WITH INSULIN (D+I), *A. INDICA* LEAF EXTRACT (D+AILE) AND A. INDICA BARK EXTRACT (D+AIBE).

General Parameters	NC	DC	D+I	D+AILE	D+AIBE
Body wt. (g)	188.3 ± 11.0	91.6 ± 9.3[b]	165 ± 9.1	155 ± 15	136± 18.4
Brain wt. (g)	1.73 ± 0.09	1.45 ± 0.09	1.6 ± 0.18	1.61 ± 0.12	1.64 ± 0.04
Brain wt./100g body wt.	0.92 ± 0.02	1.31 ± 0.04	0.96 ± 0.01	1.03 ± 0.03	1.21 ± 0.03
Protein (mg/g brain)	130.1 ± 4.2	120.7 ± 2.9	126.0 ± 4.9	133.4 ± 6.2	129.8 ± 4.7

Each value is the mean ± SEM of five separate experiments. [b]$p<0.05$ *vs*. control.

Alloxan induced diabetic rats showed marked hyperglycemia with almost 3-fold higher blood glucose concentration when compared to the control values (**Table 2**). AILE and AIBE were found significantly ($P<0.05$) effective in lowering the blood glucose levels in diabetic rats. Long term administration of AILE and AIBE for 60 days was found effective in further improving the elevated plasma glucose and reduced body weight (**Fig.1**); however insulin treatment failed to restore the altered body weight and produced hypoglycemia. 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 elevation of HbA1c was observed lesser in all the diabetic treated groups, treatment was more effective in AILE treated group.

TABLE 2: CHANGES IN GLYCEMIC INDEX IN CONTROL (NC), DIABETIC (DC), AND DIABETIC RATS TREATED WITH INSULIN (D+I), *A. INDICA* LEAF EXTRACT (D+AILE) AND A. INDICA BARK EXTRACT (D+AIBE).

Parameters	NC	DC	D+I	D+AILE	D+AIBE
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
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.

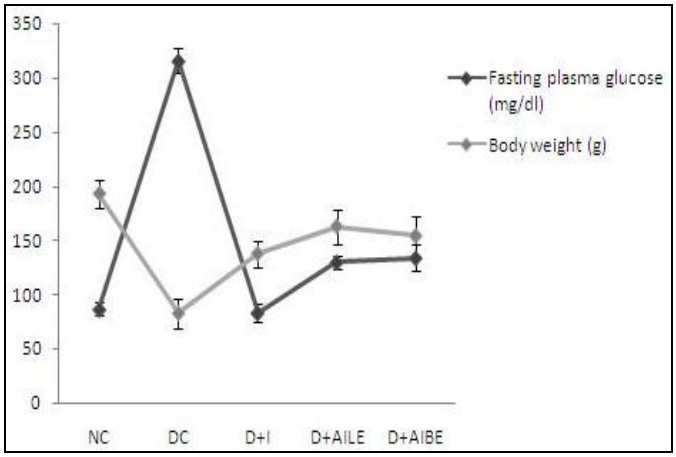

FIG.1: EFFECT OF INSULIN (D+I), *A. INDICA* LEAF EXTRACT (D+AILE) AND A. INDICA BARK EXTRACT (D+AIBE) ON CHANGE IN BODY WEIGHT AND FASTING PLASMA GLUCOSE AFTER 60 DAYS OF TREATMENT.
**p*<0.05, ***p*<0.01

Effect on antioxidant enzymes:

The diabetic state is associated with a generalized increase in tissue oxidative stress, which might be reflected in the changes in the tissue antioxidant system. . Therefore, the activities of some major antioxidant enzymes were measured in control and experimental rats. SOD and CAT activity significantly (p<0.05) decreased in diabetic rats (**Fig. 2**). However, GPx and GR treatment showed an increase in the activity after 21 days of diabetes (**Fig.3**). Treatment of the diabetic animals with Insulin, AILE and AIBE reversed the changes of these enzymes to control levels. Diabetic rats treated with AIBE showed maximum restoration in SOD, CAT, GPx and GR activities.

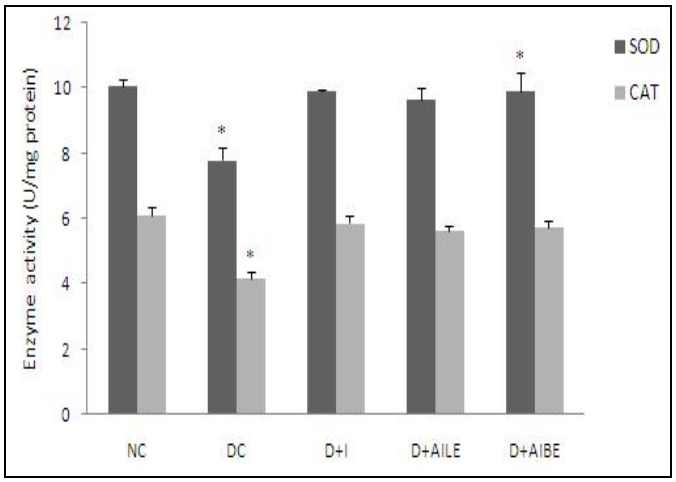

FIG.2: CHANGES IN ENZYME ACTIVITIES (SOD, CAT) IN CONTROL, DIABETIC AND DIABETIC TREATED RATS IN CYTOSOLIC FRACTION OF BRAIN. RESULTS ARE EXPRESSED AS MEAN ± SEM AND COMPARED WITH CONTROL VALUES.
*p<0.05.

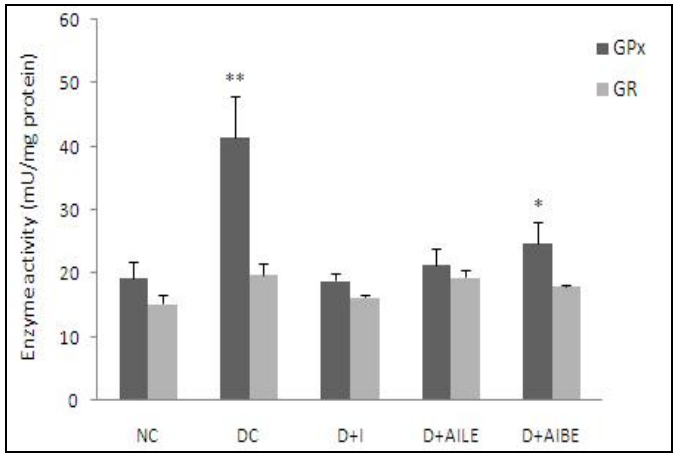

FIG.3: CHANGES IN ENZYME ACTIVITIES (GPx, GR) IN CONTROL, DIABETIC AND DIABETIC TREATED RATS IN CYTOSOLIC FRACTION OF BRAIN. RESULTS ARE EXPRESSED AS MEAN ± SEM AND COMPARED WITH CONTROL VALUES. *p<0.05, **p<0.05

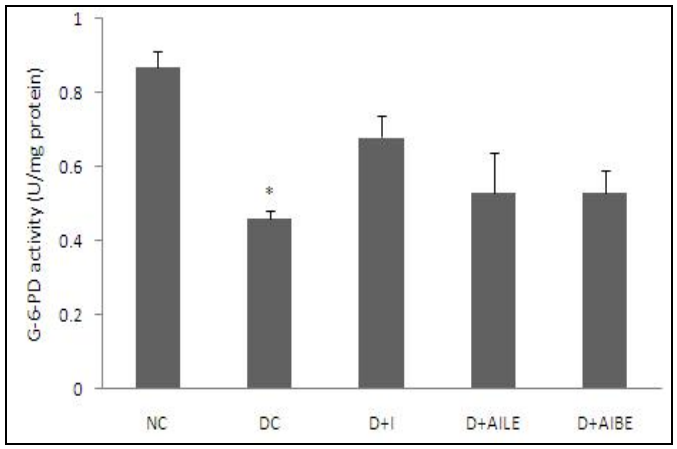

FIG.4: CHANGES IN GLUCOSE-6-PHOSPHATE DEHYDROGENASE ACTIVITY IN CONTROL, DIABETIC AND DIABETIC TREATED RATS IN CYTOSOLIC FRACTION OF BRAIN. RESULTS ARE EXPRESSED AS MEAN ± SEM AND COMPARED WITH CONTROL VALUES.
*p<0.05.

Significant decrease (p<0.05) was observed in the activity of G-6-PD in untreated diabetic rats **(Fig. 4**). Treatment with Insulin, AILE and AIBE restore the activity of enzyme to control levels.

Effect on lipid peroxidation level:

The level of lipid peroxidation was determined in control, diabetic and diabetic treated rats with Insulin, AILE and AIBE. **Table 3** shows the change in lipid peroxidation level in blood plasma. The result showed significant increase in the level of MDA in untreated diabetic rats (p<0.05) when compared to controls. Treatment for 21 days showed marked reduction in MDA level in all the treated groups.

TABLE 3: CHANGES IN MDA LEVELS IN CONTROL, DIABETIC AND DIABETIC TREATED RATS IN BLOOD PLASMA AND CYTOSOLIC FRACTION OF BRAIN

Parameters	Malondialdehyde(MDA) level				
	NC	DC	D+I	D+AILE	D+AIBE
Blood plasma (nmol MDA/ml)	2.89 ± 0.32	4.73 ± 0.64[b]	2.26 ± 0.06	2.46 ± 0.21	2.12 ± 0.19
Brain cytosolic fraction (nmol MDA/mg protein)	1.47 ± 0.13	2.44 ± 0.23[b]	1.59 ± 0.18	1.72 ± 0.09	1.68 ± 0.17

Each value is the mean ± SEM of five separate experiments. [b]$p<0.05$ *vs* control.

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There was an increase in lipid peroxidation in the cytosolic fraction of the brain of diabetic rats, and results showed significant increase ($p<0.05$) in MDA levels (**Table 3**). Treatment with AILE and AIBE reversed the above altered parameter to normal values. AIBE was found to be more effective in controlling diabetes induced changes in lipid peroxidation.

DISCUSSION: The present study explored the effect of experimental diabetes on different parameters related to antioxidant defense system in brain tissue, and whether the treatment with AILE and AIBE can restore the alterations occurred due to hyperglycemia induced oxidative stress. Oxidative stress is the imbalance between production and removal of reactive oxygen species (ROS). Increased oxidative stress, which contributes substantially to the pathogenesis of diabetic complications, is the consequences of either enhanced ROS production or attenuated ROS scavenging capacity. Several reports have shown the alterations in the antioxidant enzymes during diabetic condition.[1, 24] Mammalian brain is most vulnerable to oxidative stress because of its high rate of oxidative metabolism, intensive production of reactive oxygen metabolites, relative low antioxidant capacity and low repair mechanism. [25, 26] Oxidative stress is believed to be implicated in a variety of human degenerative disorders of the CNS, including Alzheimer's disease, Parkinson's disease and Amylotropic lateral sclerosis and in pathological conditions such as ischemia.[27, 28]

The antioxidant defense system like SOD and CAT showed lower activities in brain during diabetes and the results agree well with the earlier published data of El-Missiry et al. [29] The decreased activity of SOD and CAT may be a response to increased production of H_2O_2 and O_2^- by the auto oxidation of excess glucose and non-enzymatic glycation of proteins. [30] Hodgson and Fridovich have reported the partial inactivation of these enzyme activities by hydroxyl radicals and hydrogen peroxide. [31] The decreased activity of SOD and CAT could also be due to their decreased protein expression levels in the diabetic condition as reported recently in liver. [32] However, the activity of GPx and GR are enhanced in the diabetic rat brains. The result is consistent with the studies of Ulusu et al which suggest that the increased GPx and GR activity represents compensatory mechanism to degrade H_2O_2, which are produced in excess during the metabolism of catecholamines. [33] Treatment of the diabetic animals with AILE and AIBE restored the altered activity of SOD, CAT, GPx and GR, and the results were comparable to the insulin treatment.

The different brain areas are endowed with enzymatic and non-enzymatic defense system to participate in the cellular defense against reactive oxygen species. Among the antioxidant enzyme system SOD, CAT and GPx primarily constitute the antioxidant system. G-6-PD by virtue of their ability to produce NADPH along with GR is conventionally considered as supporters of the antioxidant systems. [34] G-6-PD is the principle source of NADPH which is of central importance to cellular redox regulation and any changes in G-6-PD will alter NADPH levels and thus impact the entire antioxidant system. Also, G-6-PD is the rate limiting enzyme of pentose phosphate pathway which helps in maintaining the normal blood glucose level. [35]

Any imbalance in glucose homeostasis will result in altered activity of the enzyme. In the present study, AILE and AIBE treatment restored the altered activity of G-6-PD in brain tissues of diabetic models. Until recently, the role of this housekeeping enzyme in the cell response to the oxidative stress was limited to human erythrocytes that lack any other NADPH producing route. However, recent observations have shown that the G6PD also plays a protective role against reactive

oxygen species in eukaryotic cells that possess alternative routes for the production of NADPH and that G6PD expression is up-regulated by oxidants through a mechanism acting mainly on the rate of transcription of this gene. [36, 37]

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 membrane in diabetes. One of the consequences of lipid peroxidation degenerative processes can result in enzyme activity changes. In the present study, the formation of MDA, a measure of TBARS and product of lipid peroxidation reaction, was significantly increased in diabetic brain tissue. The results were in agreement with earlier published data. This increased lipid peroxides formation during diabetes could disturb the anatomical integrity of the membrane, leading to the inhibition of several membrane bound enzymes.[38]

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. The present study showed the hypoglycemic and antioxidant properties of *A. indica.* Both AILE and AIBE were found significantly effective in improving glucose homeostasis. A reduction in the production of free radicals and lipid peroxides formation by restoring the antioxidant enzymes was observed which can beneficially prevent the diabetes associated tissue damage. The therapeutic effect observed in this study might be attributed to the insulin mimetic effect of AILE and AIBE. It is also suggested that the hypoglycemic action of *A. indica* may partly be due to extra-pancreatic sites of action i.e., by increased peripheral glucose utilization or by direct metabolic effect on tissues.

CONCLUSION: This study concludes the antioxidant potential of *A. indica* 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 brain. Treatment with *A. indica* was found effective in improving the antioxidant status in brain. *A. indica* may exhibit its therapeutic effects through modulation of insulin secretion. 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.

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