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BIOCHEMICAL EVALUATION OF ANTIDIABETIC AND ANTIOXIDANT POTENTIALS OF *ANNONA SQUAMOSA* LEAVES EXTRACTS STUDIED IN STZ INDUCED DIABETIC RATS

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ABSTRACT

Diabetes mellitus is characterized by persistent fasting and postprandial blood glucose levels due to inability of the body cells to utilize glucose properly. Though drugs are plenty for the treatment of diabetes, none is found to be ideal due to undesirable side effects and diminution after prolonged use. Hence, search for novel drugs, especially from plant origin continues. Based on folkloric use, the present study was designed to evaluate the antidiabetic and antioxidant potential of *Annona squamosa* Linn. (Annonaceae) in STZ-induced experimental diabetes in rats. Daily oral administration of *Annona squamosa* leaves extract (100 mg/kg b.w./day) to diabetic rats for 30 days significantly reduced the levels of blood glucose, glycosylated hemoglobin, urea and creatinine. The observed decrease in the levels of plasma protein, plasma insulin, C-peptide and hemoglobin in the diabetic rats were elevated to near normal by the extract treatment. The altered antioxidant status of diabetic rats were reverted back to near normalcy by the administration of *Annona squamosa* leaves extract. The efficacy of the *Annona squamosa* extract was comparable with gliclazide, a known hypoglycemic drug.

INTRODUCTION: Diabetes mellitus (DM), a major metabolic disorder in the endocrine system, is characterized by elevated blood glucose levels, alterations in carbohydrate, lipid and protein metabolism. It is becoming the third "killer" of mankind after cancer and cardiovascular diseases, because of its high prevalence, morbidity and mortality [1]. The pathogenesis, progress and the possibility of its management by oral administration of hypoglycemic agents have stimulated great interest in recent decades. Numerous therapies designed for the treatment of DM have proven to be fairly effective, but none is ideal due to undesirable side effects and diminution after prolonged use. For a long time, diabetes has been treated orally with several medicinal plants or their extracts based on folk medicine.

Annona squamosa Linn. (Annonaceae) commonly known as custard apple, is well known for its edible fruits. The plant is traditionally used for the treatment of epilepsy, dysentery, cardiac problems, fainting, worm infestation, constipation, hemorrhage, fever, ulcers and also as an abortifacient [2]. The aqueous leaves extract of *A. squamosa* has been reported to ameliorate hyperthyroidism [3], which is often considered as a causative factor for DM. A detailed review of literature afforded no systemic studies carried out on the medicinal properties of *A. squamosa* levels. Hence, in the present study an attempt has been made to evaluate the antidiabetic and antioxidant potential of *A. squamosa* leaves extract in STZ-induced experimental diabetes in rats.

MATERIALS AND METHODS:

Plant material: Fresh, mature *Annona squamosa* leaves were collected during July from a tree in Kolli Hills, Tamil Nadu, India. The plant was identified at the Herbarium of Botany, CAS in Botany, University of Madras. An exemplar specimen was deposited in the department herbarium.

Preparation of plant extract: The *Annona squamosa* leaves were first washed well with distilled water and dried at room temperature. The dried leaves were powdered in an electrical grinder and stored at 5°C until further use. One hundred grams of powder was extracted with petroleum ether (60-80°C) to remove lipids. It was then filtered, and the filtrate was discarded. The residue was extracted with 95% ethanol by soxhelation. The ethanol was evaporated in a rotary evaporator at 40-50°C under reduced pressure. The yield was 1.9 g/100 g. The extract was subjected to preliminary phytochemical screening for various plant constituents [4].

Experimental animals: Animal experiments were reviewed and approved by the Institutional Animal Ethics Committee of the University of Madras (approval no. 01/022/08). Male Wistar albino rats weighing 160-180 g procured from Tamil Nadu Veterinary and Animal Sciences University, Chennai, India were used. The rats were acclimatized and maintained over husk bedding in polypropylene cages in the central animal house facility of the institution. Throughout the experimental period, the rats were fed with balanced commercial pellet diet (Hindustan Lever Ltd., Bangalore, India) with composition of 5% fat, 21% protein, 55% nitrogen-free extract, and 4% fiber (w/w) with adequate mineral and vitamin levels for the animals. Diet and water were provided *ad libitum*.

Acute toxicity study: Acute toxicity studies on *A. squamosa* leaves extract were performed in control rats. Graded doses of the ethanolic extract of *A. squamosa* leaves extract (100, 250, 500, and 1,000 mg/kg body weight) were administered orally and the animals were observed for 2 weeks following administration [5]. Change in body weight gain, food

consumption, hematological, macroscopic, and clinical biochemical findings including the activities of pathophysiological enzymes were noted.

Experimental induction of diabetes: Rats were fasted overnight and experimental diabetes was induced by intraperitoneal injection of streptozotocin (STZ) with a single dose of 50 mg/kg body weight. STZ was dissolved in a freshly prepared 0.1 M cold citrate buffer pH- 4.5 [6]. Control rats were similarly injected with citrate buffer. Because STZ is capable of inducing fatal hypoglycemia as a result of massive pancreatic insulin release, STZ-treated rats were provided with 10% glucose solution after 6 h for the next 24 h to prevent hypoglycemia. Neither death nor any other adverse effect was observed. After 3 days for development and aggravation of diabetes, rats with moderate diabetes (i.e., blood glucose concentration 250 mg/dl) that exhibited glycosuria and hyperglycemia were selected for the experiment.

Dosage fixation study: A suitable optimum dosage schedule was identified by administering the *A. squamosa* leaves extract at different dosages (50, 100, 150 and 200 mg/kg b.w./day) for 30 days) to STZ induced diabetic rats. At the end of experimental period, all the rats were performed glucose tolerance test as described earlier. After conducting glucose tolerance test, the rats were sacrificed and blood was used to assess the biochemical changes. The dosage of *A. squamosa* leaves extract, which offered maximum hypoglycemic activity (as elicited by glucose tolerance, levels of blood glucose, glycosylated hemoglobin, blood urea and urine sugar) against streptozotocin-induced diabetic rats, was fixed up for all the subsequent experiments. The optimum dosage for *A. squamosa* leaves extract was fixed as 100 mg/kg body weight/day for 30 days.

Experimental design: The rats were divided into four groups comprising of six animals in each group as follows:

- Group I : Control rats receiving 0.1 M cold citrate buffer (pH 4.5).
- Group II : Diabetic control rats.
- Group III : Diabetic rats treated with *Annona squamosa* leaves extract (100mg/kg b.w. /day) in aqueous solution orally for 30 days.
- Group IV : Diabetic rats treated with gliclazide (5mg/kg b.w. /day) in aqueous solution orally for 30 days.

During the experimental period, body weight and blood glucose level of all the rats were determined at regular intervals of time. At the end of the experimental period, the rats were anaesthetized and sacrificed by cervical dislocation. Blood was collected with anticoagulant and used for the preparation of plasma. Blood collected without anticoagulant was used for serum separation.

Biochemical parameters: Whole blood was used for glucose [7] and urea [8] estimation. Plasma was separated and used for insulin and C-peptide assay using radioimmunoassay (RIA) kit for rats (Linco Research, Inc., USA). Levels of hemoglobin and glycosylated hemoglobin were estimated according to methods of Drabkin and Austin [9] and Nayak and Pattabiraman [10] respectively. Plasma was used for proteins assay [11] and serum for determination of creatinine [12]. Activities of pathophysiological enzymes such as serum aspartate transaminase (AST), serum alanine transaminase (ALT), and serum alkaline phosphatase (ALP) were assayed by the method of King [13, 14]. Levels of vitamin C, vitamin E, and glutathione (GSH) were determined by the methods of Omaye *et al*., [15] Desai [16] and Sedlak and Lindsay [17] respectively.

Liver tissue was excised, washed in ice-cold saline, and then homogenized in Tris–HCl buffer (pH 7.4) using a Teflon homogenizer. The liver homogenate was then centrifuged at 5,000 9 g to remove cellular debris and supernatant was used for determination of levels of lipid peroxides, hydroperoxides and enzymatic antioxidants. Lipid peroxidation was determined using thiobarbituric acid reactive substances by the method of Ohkawa *et al*.,[18] and hydroperoxides were estimated by the method of Jiang *et al*.,[19]. Enzymatic antioxidants such as superoxide dismutase [20], catalase [21] and glutathione peroxidase [22] were assayed.

Histological studies: The liver, kidney and pancreatic tissues were dissected out and washed in ice-cold saline immediately. A portion of the liver, kidney and pancreatic tissues was fixed in 10% buffered neutral formalin solution for histological studies. After fixation, tissues were embedded in paraffin; solid sections were cut at 5µm. Liver and kidney sections were stained with haematoxylin and eosin and pancreas was stained with aldehyde fuschin. The sections were examined under light microscope and photomicrographs were taken [23].

Statistical analysis: All the grouped data were statistically evaluated with SPSS 16.00 software. Hypothesis testing methods included one-way analysis of variance followed by least significant difference (LSD) test. $p<0.05$ was considered to indicate statistical significance. All results are expressed as mean ± standard deviation (SD) for six rats in each group.

RESULTS:

Phytochemical analysis: Preliminary phytochemical screening proves the presence of biologically active principles like flavonoids, Alkaloids, Triterpenoids and Tannins.

Acute toxicity and dosage fixation: The results of acute toxicity studies of the ethanolic extract of *A. squamosa* leaves extract (100, 250, 500, and 1,000mg/kg body weight) on the normal rats indicated that the leaves extract was non-toxic up to the maximum dosage of 1000 mg/kg body weight. The optimum dosage for *A. squamosa* leaves extract was fixed as 100 mg/kg body weight/day for 30 days as the rats showed well improved glucose tolerance and controlled blood glucose, glycosylated hemoglobin and urine sugar (data not shown).

Effect of *A. squamosa* leaves extract on basic biochemical parameters: The biochemical parameters (**Table 1**) such as blood glucose, protein, urea and creatinine in control and experimental groups of rats shows significant increase in the levels of glucose, urea, creatinine and a significant decrease in the levels of plasma total protein in STZ-induced diabetic rats, when compared with control rats. Administration of *A. squamosa* leaves extract to diabetic rats for 30 days resulted in the restoration of blood glucose, total plasma protein, urea, and creatinine levels towards near normalcy as in gliclazide treated diabetic rats.

TABLE 1: BIOCHEMICAL MARKERS IN CONTROL AND EXPERIMENTAL GROUPS OF RATS

Groups	Control	Diabetic Control	Diabetic + *A. squamosa*	Diabetic + Gliclazide
Blood glucose (mg/dl)	82.63 ± 4.27	292.72 ± 12.33 [a*]	91.54 ± 2.74 [b*]	83.64 ± 4.02 [b*]
Total protein (g/dl)	7.14 ± 0.23	4.35 ± 0.35 [a*]	6.87 ± 0.35 [b*]	6.90 ± 0.34 [b*]
Serum creatinine (mg/dl)	0.71 ± 0.03	1.24 ± 0.22 [a*]	0.66 ± 0.03 [b*]	0.69 ± 0.02 [b*]
Blood urea (mg/dl)	17.44 ± 1.53	36.77 ± 2.33 [a*]	18.28 ± 1.41 [b*]	18.37 ± 0.71 [b*]

Results are expressed as mean ± S.D. (n=6). One way ANOVA followed by *post hoc* test LSD. *p<0.05; the results were compared with [a] control; [b] Diabetic control

Effect of *A. squamosa* leaves extract on Insulin and C-peptide levels: The levels of insulin and C-peptide in control and experimental groups of rats are shown in **Fig. 1** and **2** respectively. In diabetic rats, significant decrease was noted in the levels of insulin and C-peptide when compared with the control rats. Administration of *A. squamosa* leaves extract to diabetic rats increased the levels of insulin and C-peptide as compared with diabetic rats.

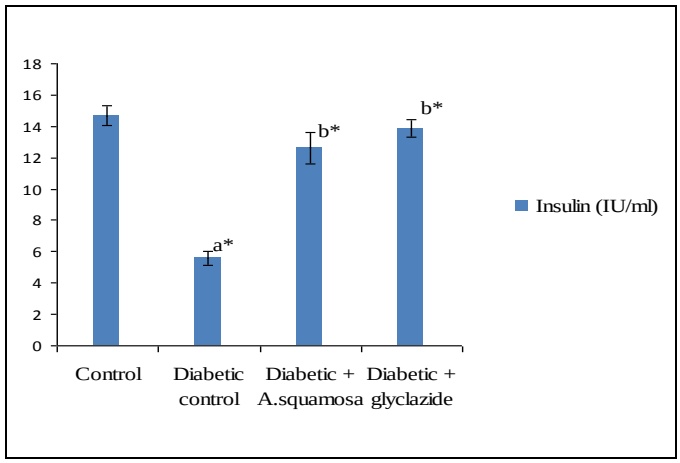

FIG. 1: THE LEVELS OF PLASMA INSULIN IN CONTROL AND EXPERIMENTAL GROUPS OF RATS

Results are expressed as mean ± S.D. (n=6). One way ANOVA followed by *post hoc* test LSD. *p<0.05; the results were compared with [a] control; [b] Diabetic control.

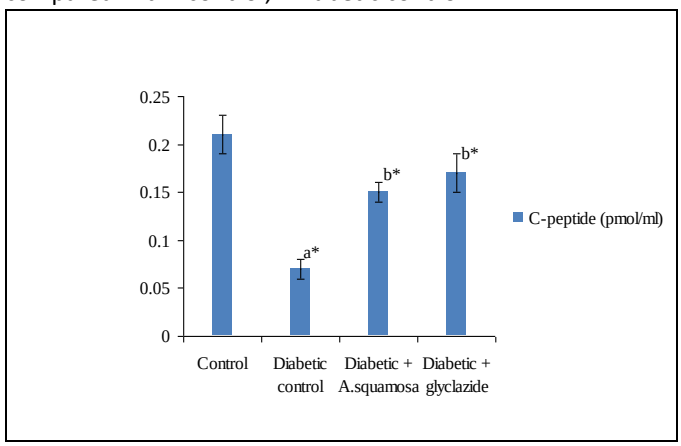

FIG. 2: THE LEVELS OF PLASMA C-PEPTIDE IN CONTROL AND EXPERIMENTAL GROUPS OF RATS

Results are expressed as mean ± S.D. (n=6). One way ANOVA followed by *post hoc* test LSD. *p <0.05; the results were compared with [a] control; [b] Diabetic control

Effect of *A. squamosa* leaves extract on hemoglobin and glycosylated hemoglobin levels: Fig. 3 shows the levels of hemoglobin and glycosylated hemoglobin in control and experimental groups of rats. A significant decrease in the level of hemoglobin and a significant increase in the level of glycosylated hemoglobin were observed in diabetic rats when compared to control rats. Administration of *A. squamosa* leaves extract to diabetic rats resulted in the restoration of hemoglobin and glycated hemoglobin levels to near normal.

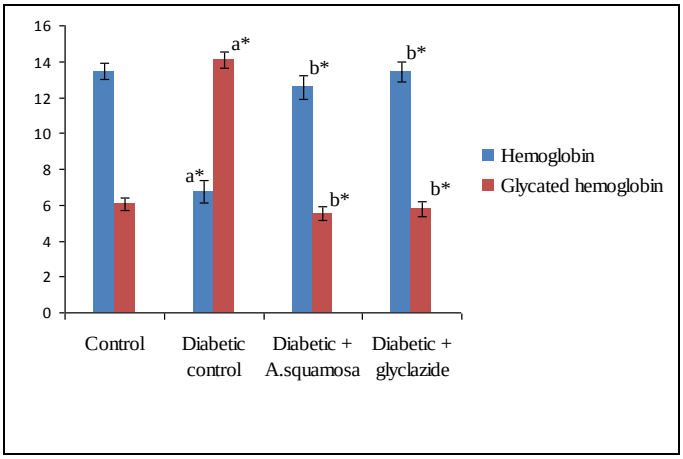

FIG. 3: THE LEVELS OF HAEMOGLOBIN AND GLYCOSYLATED HEMOGLOBIN IN CONTROL AND EXPERIMENTAL GROUPS OF RATS

Units: g/dl for Hemoglobin; % Hemoglobin for Glycosylated hemoglobin. Results are expressed as mean ± S.D. (n=6). One way ANOVA followed by *post hoc* test LSD. *p <0.05; the results were compared with [a] control; [b] Diabetic control

Effect of *A. squamosa* leaves extract on the activities of pathophysiological enzymes: Table 2 depicts the activities of AST, ALT and ALP in serum of control and experimental groups of rats. There was a significant increase in the activities of AST, ALT and ALP in serum of diabetic rats when compared with control rats. Administration of *A. squamosa* leaves extract brought down these enzyme activities to near normal.

TABLE 2: THE ACTIVITIES OF PATHOPHYSIOLOGICAL ENZYMES IN CONTROL AND EXPERIMENTAL GROUPS OF RATS

Groups	Control	Diabetic Control	Diabetic + A. squamosa	Diabetic + Gliclazide
Serum AST	76.61 ± 2.45	133.50 ± 2.54 [a*]	81.13 ± 1.80 [b*]	78.42 ± 3.38 [b*]
Serum ALT	16.22 ± 0.73	35.21 ± 1.74 [a*]	15.23 ± 0.55 [b*]	14.42 ± 0.91 [b*]
Serum ALP	68.93 ± 1.11	107.79 ± 3.02 [a*]	69.29 ± 1.93 [b*]	71.79 ± 2.50 [b*]

The enzyme activities expressed as: AST and ALT - µmoles of pyruvate/min/mg of protein. ALP - µmoles of phenol liberated/min/mg of protein. Results are expressed as mean ± S.D. (n=6). One way ANOVA followed by *post hoc* test LSD. *p <0.05; the results were compared with [a] control; [b] Diabetic control

Effect of *A. squamosa* leaves extract on the plasma non-enzymatic antioxidants levels: The levels of non-enzymatic antioxidants such as vitamin C, vitamin E and reduced glutathione in plasma of control and experimental groups of rats are as shown in **Table 3**. A significant decrease in the levels of vitamin C, vitamin E and reduced glutathione were found in diabetic rats when compared with control rats. These alterations were restored back to near normalcy in *A. squamosa* leaves extract treated diabetic rats which is similar to gliclazide treated diabetic rats.

TABLE 3: THE LEVELS OF VITAMIN C, VITAMIN E AND REDUCED GLUTATHIONE IN PLASMA OF CONTROL AND EXPERIMENTAL GROUPS OF RATS

Groups	Control	Diabetic Control	Diabetic + *A. squamosa*	Diabetic + Gliclazide
Vitamin C (mg/dl)	1.73 ± 0.13	0.60 ± 0.40 [a*]	1.46 ± 0.10 [b*]	1.63 ± 0.50 [b*]
Vitamin E (mg/dl)	1.20 ± 0.02	0.52 ± 0.06 [a*]	0.92 ± 0.05 [b*]	0.98 ± 0.03 [b*]
Reduced Glutathione (mg/dl)	28.73 ± 1.41	16.29 ± 1.46 [a*]	24.94 ± 0.91 [b*]	26.95 ± 0.82 [b*]

Results are expressed as mean ± S.D. (n=6). One way ANOVA followed by *post hoc* test LSD. *p <0.05; the results were compared with [a] control; [b] Diabetic control

Effect of *A. squamosa* leaves extract on TBARS and hydroperoxides levels in liver tissues: The levels of TBARS and hydroperoxides in liver tissues of control and experimental groups of rats are presented in **Fig. 4** and **5** respectively. Diabetic rats showed marked increase in TBARS and hydroperoxides when compared with control rats. Administration of both *A. squamosa* leaves extract as well as gliclazide to diabetic rats tends to bring the concentration of TBARS and hydroperoxides significantly to near normal levels.

Effect of *A. squamosa* leaves extract on the liver enzymatic antioxidants: The activities of SOD, CAT and GPx in liver tissues of control and experimental groups of rats are represented in **Table 4**. A significant decrease in the activities of these antioxidant enzymes was found in liver tissues of diabetic rats when compared with control rats. The altered activities of antioxidant enzymes were brought back to near normalcy by *A. squamosa* leaves extract as well as gliclazide treated diabetic rats.

TABLE 4: ACTIVITIES OF ANTIOXIDANT ENZYMES IN LIVER TISSUES OF CONTROL AND EXPERIMENTAL GROUPS OF RATS

Groups	Control	Diabetic Control	Diabetic + *A. squamosa*	Diabetic + Gliclazide
SOD	13.48±0.43	6.97 ± 0.18[a*]	11.86 ± 0.46[b*]	13.01 ± 0.66[b*]
CAT	76.52±2.85	43.37 ± 2.4[a*]	71.16 ± 0.9[b*]	75.50 ± 3.15[b*]
GPx	11.64±0.83	5.53 ± 0.67[a*]	10.21 ± 0.43[b*]	10.92 ± 0.48[b*]

Activities were expressed as: 50% of inhibition of epinephrine auto oxidation/mg protein/ min for superoxide dismutase (SOD); µmoles of hydrogen peroxide decomposed per min per mg of protein for Catalase (CAT); µmoles of glutathione oxidized per min per mg of protein for Glutathione peroxidase (GPx). Results are expressed as mean ± S.D. (n=6). One way ANOVA followed by *post hoc* test LSD. *p <0.05; the results were compared with [a] control; [b] Diabetic control

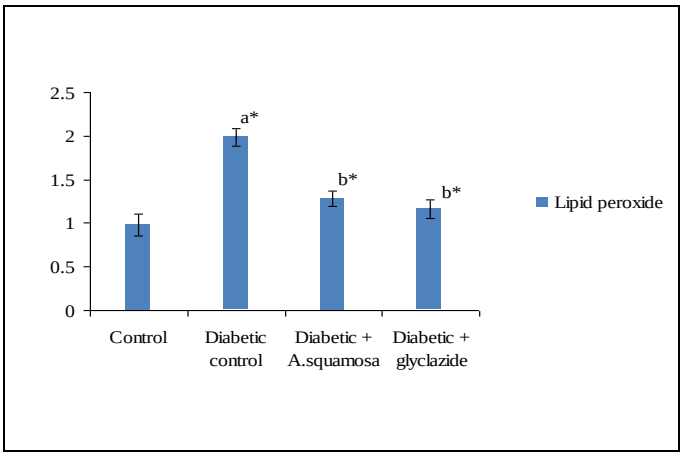

FIG. 4: THE LEVELS OF LIPID PEROXIDE IN LIVER TISSUES OF CONTROL AND EXPERIMENTAL GROUPS OF RATS

Units: nmoles of TBARS/100 g of wet tissue. Results are expressed as mean ± S.D. (n=6). One way ANOVA followed by *post hoc* test LSD. *p<0.05; the results were compared with [a] control; [b] Diabetic control

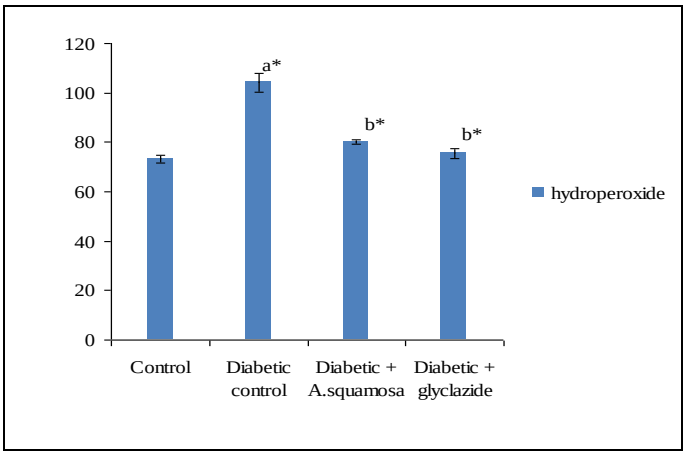

FIG. 5: THE LEVELS OF HYDROPEROXIDE IN LIVER TISSUES OF CONTROL AND EXPERIMENTAL GROUPS OF RATS

Units: nmoles/100 g of wet tissue. Results are expressed as mean ± S.D. (n=6). One way ANOVA followed by *post hoc* test LSD. *p <0.05; the results were compared with [a] control; [b] Diabetic control

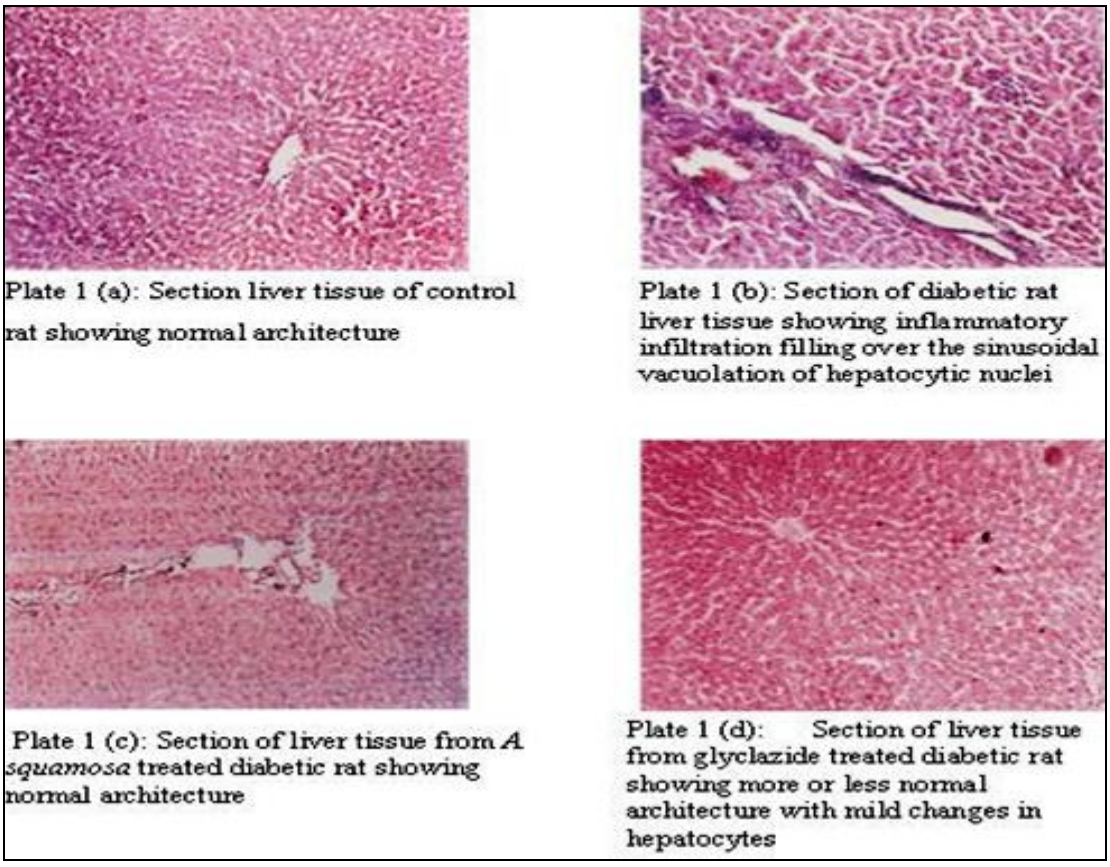

PLATE 1 (A-D): HISTOPATHOLOGICAL OBSERVATIONS IN THE LIVER TISSUE OF CONTROL AND EXPERIMENTAL GROUPS OF RATS (HE, 100X)

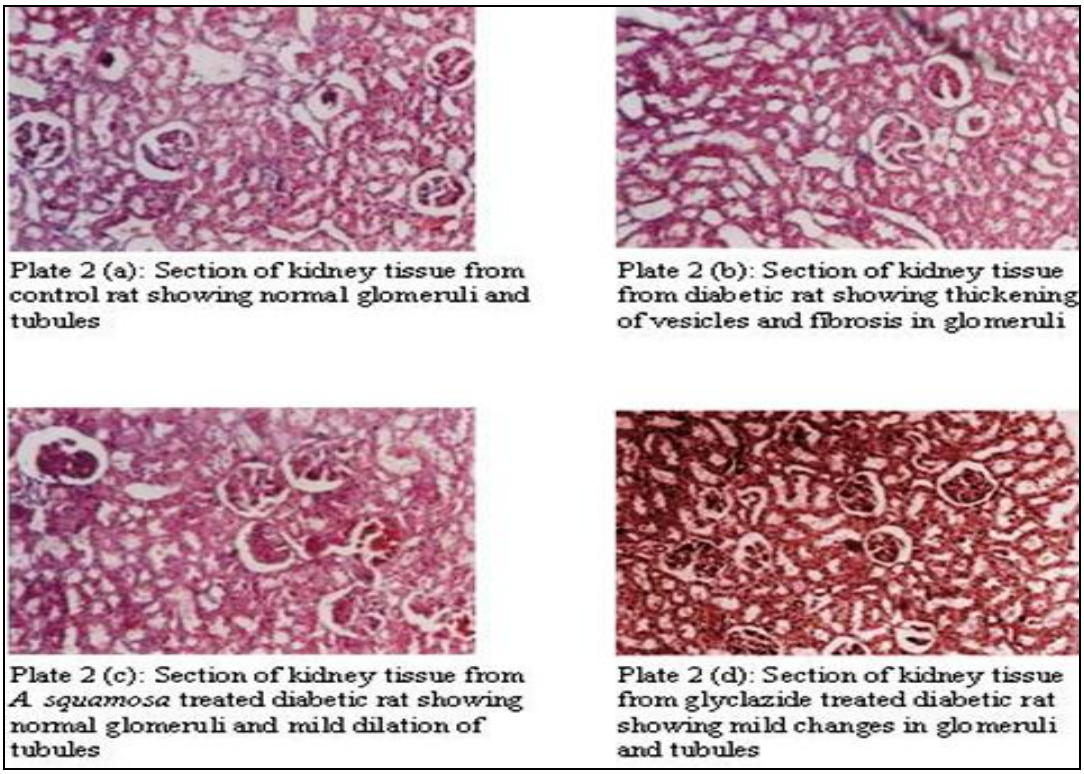

Plate 2 (a): Section of kidney tissue from control rat showing normal glomeruli and tubules

Plate 2 (b): Section of kidney tissue from diabetic rat showing thickening of vesicles and fibrosis in glomeruli

Plate 2 (c): Section of kidney tissue from *A. squamosa* treated diabetic rat showing normal glomeruli and mild dilation of tubules

Plate 2 (d): Section of kidney tissue from glyclazide treated diabetic rat showing mild changes in glomeruli and tubules

PLATE 2 (A-D): HISTOPATHOLOGICAL OBSERVATIONS IN THE KIDNEY TISSUE OF CONTROL AND EXPERIMENTAL GROUPS OF RATS (HE, 100X)

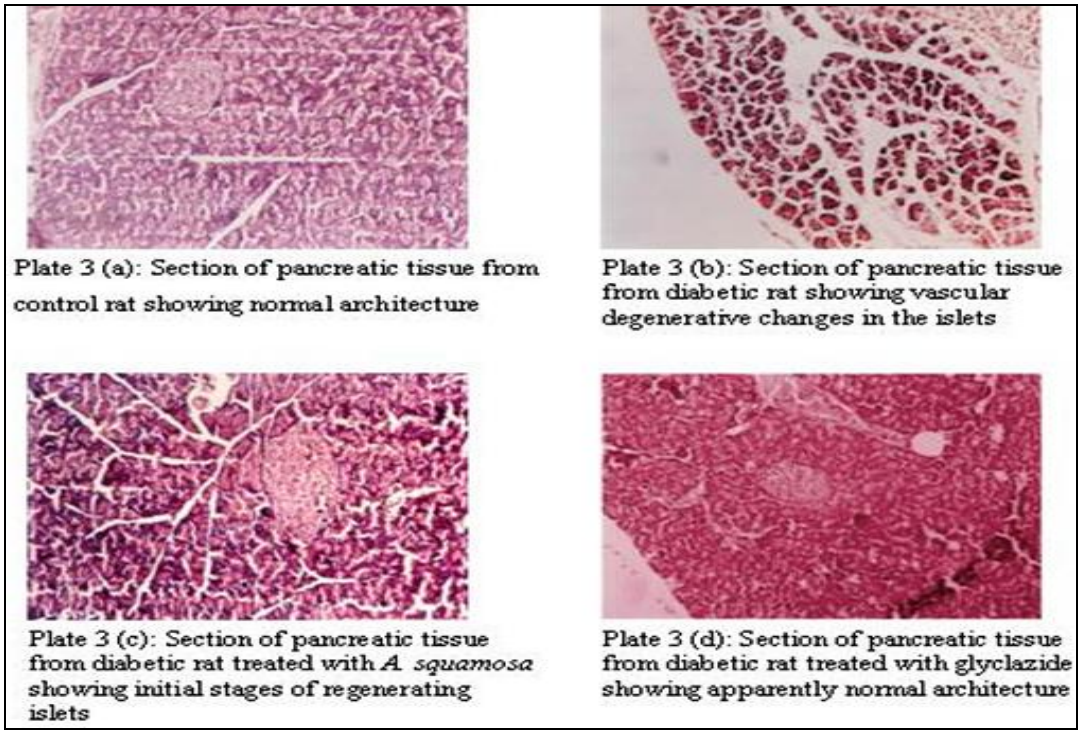

Plate 3 (a): Section of pancreatic tissue from control rat showing normal architecture

Plate 3 (b): Section of pancreatic tissue from diabetic rat showing vascular degenerative changes in the islets

Plate 3 (c): Section of pancreatic tissue from diabetic rat treated with *A. squamosa* showing initial stages of regenerating islets

Plate 3 (d): Section of pancreatic tissue from diabetic rat treated with glyclazide showing apparently normal architecture

PLATE 3 (A-D): HISTOPATHOLOGICAL OBSERVATIONS IN THE PANCREATIC TISSUE OF CONTROL AND EXPERIMENTAL GROUPS OF RATS (ALDEHYDE FUSCHIN, 320X)

DISCUSSION: The herbal extracts contain different phytochemicals with wide range of biological activity. For example, phytochemicals such as saponins, terpenoids, flavonoids and tannins found to inhibit cancer cell proliferation, regulate inflammatory, immune response and protect against lipid peroxidation. Most of the phytochemicals have the ability to inhibit lipid peroxidation and also possess hypoglycemic and hypolipidemic properties [24].

In the present study, we have found that most of the biologically active phytochemicals were present in the ethanolic leaves extract of *A. squamosa*. The antidiabetic and antioxidant properties of *A. squamosa* leaves extract may be due to the presence of such phytochemicals. Streptozotocin- induction cause specific damage to islet β-cells and thus exerts a pronounced increase in the concentrations of blood glucose. It is well established that gliclazide produce hypoglycemia and is often used as a standard drug in STZ-induced moderate diabetic models to compare the antidiabetic properties of a variety of compounds [25]. Administration of *A. squamosa* leaves extract to STZ-induced diabetic rats resulted in a significant reduction in blood glucose level.

Insulin deficiency is manifested in a number of biochemical and physiological alterations. Insulin estimations and more specifically assessment of C-peptide are generally accepted as an index of β-cell function. In the present study, we have observed a significant decrease in the levels of insulin and C-peptide in streptozotocin-induced diabetic rats. C-peptide deficiency is a contributing pathogenic factor in type 1 diabetic complications. C-peptide promotes insulin action at low hormone concentration and inhibits it at high hormone levels suggesting a modulatory effect by C-peptide on insulin signaling. C-peptide has insulinomimetic effects on its own by activating insulin receptor and increases glycogen synthesis and amino acid uptake. Oral administration of *A. squamosa* leaves extract increased the levels of insulin and C-peptide and decreased the levels of blood glucose. This decreased level of blood glucose might be due to the increased formation of glycogen and amino acid uptake by C-peptide activity [26]. The increased level of insulin as well as C-peptide in *A. squamosa* leaves extract treated diabetic rats might be due to the activation of remnant β-cells of the pancreas.

During diabetes the excess glucose present in blood reacts non-enzymatically with hemoglobin to form glycosylated hemoglobin (HbA_1C). As a result, the total hemoglobin level is decreased in diabetic rats [27]. The rate of glycosylation is proportional to the concentration of blood glucose [28]. Hence, estimation of glycosylated hemoglobin is a well-accepted biochemical parameter useful for the diagnosis and management of the disease. The increased glycated hemoglobin is associated with loss of β-cell function and has been implicated in the complications of diabetes mellitus [29]. Glycated hemoglobin (HbA_1C) was found to increase in patients with diabetes mellitus and the amount of increase being directly proportional to the fasting blood glucose level [30]. Oral administrations of *A. squamosa* leaves extract as well as gliclazide tend to decrease the level of glycosylated hemoglobin by improving the blood glucose homeostasis.

During diabetes, there is increased protein catabolism with flow of amino acids into liver, which feeds gluconeogenesis [31]. STZ-induced diabetic rats manifest a negative nitrogen balance related to enhanced proteolysis particularly in the muscles coupled with lowered protein synthesis. The accelerated proteolysis of uncontrolled diabetes occurs as a result of deranged glucagon-mediated regulation of cyclic AMP formation in insulin deficiency [32]. This readily accounts for the observed decrease in the total protein content in diabetes mellitus. Administration of *A. squamosa* leaves extract to diabetic rats significantly

improves the total plasma protein level to near normal.

Elevated protein catabolism with inflow of amino acids to the liver in diabetes facilitates urea synthesis thereby resulting in hyperuremia [31]. Thus the hyperuremia in blood reflects either increased synthesis of urea or its decreased excretion. Administration of *A. squamosa* leaves extract to diabetic rats decreased the level of blood urea to near normal and was comparable with gliclazide treated rats.

Creatinine is a break-down product of creatine phosphate in muscle, and is usually produced at a fairly constant rate by the body. Creatinine is chiefly filtered out of the blood by the kidneys. If the filtering ability of the kidney is deficient, blood creatinine levels rise. Therefore, creatinine levels in blood and urine may be used to assess the renal function. Oxidative stress in diabetes causes renal dysfunction [33]. The observed increase in creatinine level in diabetic rats is mainly due to renal dysfunction and is altered to near normal by oral administration of *A. squamosa* leaves extract for 30 days.

The clinical and diagnostic values associated with changes in serum enzyme activities such as AST, ALT and ALP have long been recognized. The increased activity of these enzymes during diabetic condition is probably due to the alterations in the normal function of the cell.[34] Increase in serum ALT activity is almost always due to hepatocellular damage followed by cardiac damage and is usually accompanied by an increase in AST activity [35]. The normalization of the activities of AST and ALT by the administration of *A. squamosa* leaves extract as well as gliclazide to STZ induced diabetic rats indicates amelioration of cellular dysfunction and tissue damage caused by hyperglycemia. Alkaline phosphatase is a non-specific hepatic marker enzyme. ALP and ACP activities were markedly increased in insulin deficient animals leading to tissue necrosis.[36] The altered activities were reverted back to near normal by *A. squamosa* leaves extract as well as glyclazide treatment. The reversal of AST, ALT and ALP activities in *A. squamosa* leaves extract treated diabetic rats towards near normalcy indicates the tissue protective and non-toxic nature of the *A. squamosa* leaves extract.

Non-enzymatic antioxidants such as vitamin C (ascorbic acid) vitamin E (α-tocopherol), reduced glutathione (GSH) may work synergistically in cellular antioxidant defense [37.] Vitamin C is one of the four dietary antioxidants, the other three being vitamin E, vitamin A and selenium. Also, vitamin C regenerates vitamin E from its oxidized form [38]. Vitamin C has been recognized as an outstanding plasma antioxidant and its depletion leads to formation of hydroperoxides even when the other antioxidants are still present [39]. The levels of plasma vitamin C were found to be lowered in diabetic rats.

Thus, the elevation in glucose concentration may depress natural antioxidant like vitamin C or due to decrease in GSH levels, since GSH is required for recycling of vitamin C. Vitamin E is a lipophilic antioxidant and inhibits lipid peroxidation, scavenging lipid peroxyl radicals to yield lipid hydroperoxides and the α-tocopheroxyl radial [40]. Vitamin E is also responsible for protecting poly unsaturated fatty acid (PUFA) against lipid peroxidation and its deficiency in diabetes may be due to their exhaustion during detoxification of free radicals produced by membrane lipid peroxidation [41]. Diabetic rats administered with *A. squamosa* leaves extract which contains antioxidant phytochemicals significantly reduces the generated free radicals thereby maintaining the normal levels of vitamin C and vitamin E.

GSH is an essential antioxidant for recycling of vitamin E and C [42]. During diabetes the decreased levels of vitamin C diminish the recycling of vitamin E [43]. GSH has a multifaceted role in antioxidant defense. It is a direct scavenger of free radicals as well as a co-substrate for glutathione peroxidase activity and as a cofactor for many enzymes and also act as a conjugates in endo and xenobiotic reactions [44]. The present study confirms with the finding that decreased level of glutathione in diabetes may represent increased utilization due to oxidative stress.

Several studies support the hypothesis that in diabetes, chronic hyperglycemia increases the polyol pathway as well as advanced glycation end products (AGEs) formation and free radical generation rates, leading to increased GSH oxidation. A relative depletion of NADPH due to aldose reductase activation and secondary to reduced production through the pentose cycle impairs GSH generation and leads to depletion of this free radical scavenger [45].

Glutathione reductase (GR) plays a major role in regenerating endogenous GSH from GSSG, thus maintaining the balance between the redox couple. Decrease in the activity of G6PD in diabetes results in reduced availability of NADPH and hence decreased levels of GSH. Reversal of hyperglycemia and scavenging of free radicals by the *A. squamosa* leaves extract may itself decrease the oxidative stress and thus may enhance glutathione content.

The endogenous antioxidant enzymes such as SOD, CAT and GPx are responsible for the detoxification of deleterious oxygen radicals. SOD has been postulated as one of the most important enzymes in the enzymatic antioxidant defense system which catalyses the dismutation of superoxide radicals to produce H_2O_2 and molecular oxygen hence diminishing the toxic effects caused by these radicals [46]. Catalase (CAT) is a hemeprotein which catalyses the reduction of hydrogen peroxides and protects the tissues from highly reactive hydroxyl radicals. The superoxide anion has been known to inactivate CAT, which is involved in the detoxification of hydrogen peroxide. GPx plays a primary role in minimizing oxidative damage. It has been proposed that GPx is responsible for the detoxification of H_2O_2 in low concentration whereas catalase comes into play when GPx pathway is reaching saturation with the substrate. GPx catalyze the reduction of hydrogen peroxide and hydroperoxides to non-toxic metabolites.

The reduction in SOD and catalase activities in diabetic condition may be due to direct glycation of enzyme protein [47]. The increase in superoxide radical in diabetes may inhibit the activity of catalase and glutathione peroxidase [48, 49]. GPx function is concert with GSH in decomposing hydrogen peroxide generated from free radical mediated reactions. Due to decrease in the concentration of GSH, the activity of GPx is also decreased in diabetes. The diminished activity of GPx in diabetes elevates hydrogen peroxide and lipid peroxides leading to the accumulation of these oxidants and thus the subsequent oxidation of lipids. It has been concluded that the restoration of antioxidant defense system by the administration of *A. squamosa* leaves extract may be attributed mainly due to the presence of antioxidant phytochemicals.

The tremendous increase in TBARS and hydroperoxides in liver tissues suggests an increase in oxygen radicals that could be due to either increased production or decreased detoxification which could be due to persistent hyperglycemia in diabetes [50]. Maintenance of persistent normoglycemia by the administration of *A. squamosa* leaves extract may attenuate lipid peroxidation in tissues and thus prevent tissue damage.

The pathological changes observed in liver **[Plate 1(a-d)]**, kidney **[Plate 2(a-d)]** and pancreas [Plate **3(a-d)]** of STZ diabetic rats may be due to the hyperglycemia and its mediated oxidative stress. *A. squamosa* leaves extract resulted in glucose homeostasis and attenuation of oxidative stress by optimization of antioxidant enzymes, which could have protected tissue damages. The histological evidence authenticated the extent of tissue injury by streptozotocin and the protection offered to hepatic, renal and pancreatic β cells by leaves extract and also revealed the non-toxic nature of *A. squamosa* leaves extract.

From the results of the present study it may be concluded that the antidiabetic and antioxidant nature of *A. squamosa* leaves might be due to the presence of biologically active phytochemicals present in the extract. Further, the present study demonstrates the scientific rationale for the use of *A. squamosa* leaves in the traditional medicine for the treatment of diabetes.

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