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EFFECTS OF EGYPTIAN *ANNONA SQUAMOSA* LEAVES EXTRACTS AGAINST ALLOXAN INDUCED HYPERGLYCEMIA IN RATS

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ABSTRACT

The objective of the present study was to investigate the hypoglycemic effect of ethanolic and aqueous extracts of Egyptian *Annona squamosa* leaves and mix of them against alloxan induced hyperglycemia in rats. The study was performed on 60 male albino rats divided into 6 groups. Healthy rats served as control group, diabetic group, Vildagliptin drug treated diabetic group, aqueous extracts of *Annona squamosa* leaves treated diabetic group, ethanolic extracts of *annona squamosa* leaves treated diabetic group and mix extracts of *Annona squamosa* leaves treated diabetic group. Experimental diabetes was induced by single intraperitoneal injection of 150 mg/kg body weight alloxan. After one month blood samples were collected for assessment of blood glucose, insulin, lipid profile (triglycerides, total cholesterol, high density lipoprotein, and low density lipoprotein, albumin, total protein, urea, creatinine and the activities of diagnostic marker liver enzymes aspartate aminotransferase, alanine aminotransferase, the antioxidant properties of the extract were determined by tissue nitric oxide, reduced glutathione and malondialdehyde. Sections of liver and pancreas organs were qualitatively examinated under light microscope. The result showed that mean concentrations of FBS, serum cholesterol, triglycerides, low density lipoprotein, liver enzymes, serum urea and creatinine were significantly decreased and serum insulin, high density lipoprotein, albumin, total protein were significantly increased in all treated diabetic groups compared with the diabetic group. The results showed that the mix of ethanol and aqueous extracts has higher significant effect in the most biochemical markers than the individual effect of aqueous and ethanolic extracts respectively.

KEYWORDS: *Annona squamosa* leaves extracts, blood glucose, diabetes mellitus, insulin, oxidative stress.

INTRODUCTION

Diabetes mellitus (DM) is a complex metabolic disorder that characterized by disturbance in glucose metabolism, insulin action and/or production with prolonged exposure of the cells and tissues to hyperglycemia.[1] DM markedly increases the risk of microvascular and macrovascular complications.[2] The most complication of hyperglycemia is a long-term damage, dysfunction, and failure of various organs, specially the eyes, kidneys, nerves, heart and blood vessels.[3]

Oxidative stress as well as defects in antioxidant defense systems is recognized as causative factors for the development of major diabetic complications.[4] Hyperglycemia in diabetic states can induce increased formation of reactive oxygen species (ROS) and oxidative stress that cause damage to biological macromolecules such as lipids, proteins and DNA.[5] Common antioxidants include glutathione, and the enzymes superoxide dismutase, catalase, glutathione peroxidase, and glutathione reductase.[6] Decreased levels of glutathione and elevated concentrations of thiobarbituric acid reactants are consistently observed in diabetes. In addition, changes in nitric oxide and glycated proteins are also seen in diabetes.[7]

Impairment of the biological action of insulin at the cellular level is believed to be a cardinal and possibly primary underlying metabolic defect in the development of the characteristic dyslipidemia observed in diabetes.[8] The key components of diabetic dyslipidemia are elevated plasma low density lipoproteins (LDL), very low-density lipoproteins (VLDL), triglycerides (TGs), circulating free fatty acids (FFAs) and lowered high density lipoprotein cholesterol (HDL-C).[9] Small dense LDL is a strong risk factor for cardiovascular disease, it is highly atherogenic and serves as a marker for atherosclerosis where the oxidation of LDL leads to its increased penetration of arterial walls.[10]

Management of DM without any side effects is still a challenge to the medical system. There is an increasing demand by patients to use natural products with antidiabetic activity, because insulin and oral hypoglycaemic drugs have undesirable side effects.[11]

Over the past centenary, the phytochemicals and active constituents in plants have played a pivotal role in pharmaceutical discovery.[12] One of such plants with bioactive materials is

Annona squamsoa (A.squamosa). A. squamosa L. is commonly known as sugar apple, custard apple, sweet sop and sweet apres, is a member of Annonaceae family.[13] In recent years, many biological activities of custard apple have been found in a large number of studies, such as the vitro antioxidant activities of methanol and aqueous extract of A. squamosa fruit pulp[14]; the antidiabetic activity of the aqueous extract of *Annona squamosa*[15]; the antitumour activity and cytotoxic effects of A. squamosa seed extracts.[16]

Annona squamosa leaves has been reported to contain alkaloids, saponins, flavonoids, terpenoids, glycosides and phenolic compounds that exhibited the medicinal activities like antimicrobial, antioxidant, anticancerous and antidiabetic activities.[17]

The objective of present study is to investigate hypoglycemic, hypolipidemic effects and antioxidant activity of ethanolic and aqueous extracts of A.squamosa leaves against alloxan induced hyperglycemia in rats.

MATERIALS AND METHODS

Materials

- **Animals:** 60 male albino rats, weighted 150-200 gm, were obtained from the animal house of the National Research Centre, Giza, Egypt.
- **Chemicals**: Alloxan was purchased from Sigma Aldrich medical company St.Louis USA.
- **Leaves of A. squamosa:** Fresh leaves of *Annona squamosa* were collected in the month of November in 2016 from Al-Nobaria, EL-Behera, Egypt. The leaves were identified by the Taxonomist/curator of Botany Department, Faculty of Science, Port-Said University.

Methods

- **Induction of diabetes**

Alloxan monohydrate was dissolved in normal saline and freshly prepared solution was injected intraperitoneally at a dose of (150 mg/kg.) after 18 hr fasting of animals.[18] After 1 hr of alloxan administration, the animals were fed on standard pellets and water *ad libitum*. After 48 hours of the injection by alloxan the blood glucose was estimated. The animals were considered to be diabetic if fasting glucose level was 200mg/dl or over.[19]

- **Preparation of aqueous extract of leaves of A.squamosa**

Fresh leaves of A.squamosa were collected. leaves were cleaned with water then dried at 60C$^{\circ}$ for three consecutive days in the oven. Dried leaves (500 g) were extracted with warm water for 2 hrs. The resulting dark brown extract was cooled and filtered using gauze then

two times by Whatman no.1 filter paper. The filtrate was centrifuged at 10,000 rpm for 10 min in sorvall centrifuge at room temperature and the sediment was discarded. The supernatant of extract was concentrated on rotavapour under reduced pressure. The concentrated crude extract was lyophilized to get a powder (50 g) and was used for the study. The obtained crude extract was weighed and stored at deep freezer until dissolved with (0.9% NaCl) before animal treatment.[15]

- **Preparation of ethanolic extract of leaves of A. squamosa**

The leaves were washed with water and dried. About 500 g of crushed leaves were extracted twice with 70% ethanol for 6 h. The resulting extract was filtered. The filtrate was evaporated in vaccum evaporator to give a residue (yield: 8.2% w/w).[20]

Experimental design

All rats were housed individually in stainless steel cages for one month; they were fed standard rodent chow and kept in standard conditions of temperature and light. The animals were divided into 6 groups with 10 animals in each group:

Group I: Normal control rats received only normal saline intraperitoneally.

Group II: Diabetic rats received only alloxan (150 mg/kg i.p.).

Group III: Diabetic rats received vildagliptin drug (10 mg/kg bw).[21]

Group IV: Diabetic rats received aqueous extract of A. squamosa (300 mg/kg/day).[11]

Group V: Diabetic rats received ethanolic extract of A. squamosa (300 mg/kg/day).[11]

Group VI: Diabetic rats received mix of ethanolic and aqueous extract of A.squamosa (300 mg/kg/day).

Blood sample and tissue collection

At the end of the experiment, one month, the animals were fasted for 14-16 hours. Blood samples were withdrawn from the orbital vein using ether as general anesthetic. Blood samples were divided into two portions, the first portion was collected in tubes contain sodium floride for blood glucose estimation. A second portion collected in plan tube, and serum was separated at 4000 rpm for 15min for estimation levels of total cholesterol, triglycerides, HDL-cholesterol, urea, creatinin, AST, ALT insulin, albumin and total protein.

Liver and pancreas were removed quickly on ice and homogenized, prepared for estimation of MDA in pancreas tissue and NO and GSH in liver tissue.

Preparation of liver and pancreas homogenate for oxidant / antioxidant parameters

Liver and pancreas were removed quickly and placed in iced normal saline, perfused with the same solution to remove blood cells, blotted on filter paper and frozen at -80°C. The frozen tissues were cut into small pieces and homogenized in 5 ml cold buffer (0.5g of Na_2HPO_4 and 0.7 g of NaH_2PO_4 per 500 ml deionized water (pH 7.4) per gram tissue, then centrifuged at 4000 rpm for 15 minutes at 4°C and the supernatant was removed for chemical parameters estimation.[22]

Histopathological studies

Liver and pancreas organs of different animal groups were collected for microscopic examinations and stored in 10% formalin. After usual processing 5 µm thick sections were cut and stained with hematoxylin and eosin.[23] Sections were qualitatively observed under light microscope.

Biochemical Analysis: The following parameters were investigated:

Fasting blood glucose was performed according to the method of Passing and Bablok[24] using commercially kit supplied by Biodiagnostic, Egypt.

Serum insulin was estimated by enzyme-linked immunosorbent assay according to Judzewitsch et al.[25] by the kit provided by DRG, USA.

Serum cholesterol and triglycerides were estimated according to established methods of Allain et al.[26] and Glick et al.[27] respectively. **HDL- cholesterol** was measured by the enzymatic method according to Lopez–Virella[28], while LDL cholesterol was calculated according to the equation of Friedwald.[29]

Pancreatic malondialdehyde (MDA) was determined using colorimetric kit (from Biodiagnostics, Egypt) according to the method of Ohkawa et al.[30]

Liver nitric oxide (NO) was estimated by colorimetric methods according to Lisa et al.[31]

Liver glutathione reduced (GSH) was determined using colorimetric kit (from Biodiagnostics, Egypt) according to the method of Beutler et al.[32]

Serum urea was measured by colorimetric monoreagent according to the method of Fawcett et al.[33] Kit was purchased from Biodiagnostics, Egypt.

Serum creatinine was measured by kinetic kit (Biodiagnostic, Egypt) according to Belfield and Goldberg[34] method.

Serum alanine amino transferase (ALT) and aspartate amino transferase (AST) were determined using commercial kit purchased from (BioMed Diagnostics, Egypt) based on the method described by Reitman and Frankel.[35]

Serum albumin was measured by colorimetric kit (Biodiagnostic, Egypt) according to the method of Doumas et al.[36]

Serum total protein was measured by colorimetric kit (Biodiagnostic, Egypt) according to the method of Gornal et al.[37]

STATISTI CAL ANALYSIS

Results were expressed as mean ± standard error. Data were analyzed by independent sample test (SPSS) version 15 followed by (LSD) test to compare significance between groups. Difference was considered significant when P value <0.05.

RESULTS AND DISCUSSION

Medicinal plants are a good source of natural antioxidants believed to exert their effect by reducing the formation of the final active metabolite of the drug-induced systems or by scavenging the reactive molecular species to prevent their reaching a target site.[38] The aim of this work was studying the efficacy of the aqueous and ethanolic extracts of *Annona* leaves extract as a natural antidiabetic treatment rather than synthetic chemical drugs in the experimental treatment of alloxan induced diabetes in rats, and how we can avoid major side effects of currently used chemical drugs. In the present study alloxan induce diabetes by pancreatic cell damage mediated through generation of oxygen free radicals. The primary target of these radicals is the DNA fragmentation of pancreatic cells.[39]

As regard administration of aqueous and ethanolic extract of *A. squamosa* leaves to diabetic rats showed a significant decrease in the levels of blood glucose and an increase in the levels of serum insulin. These results are in agreement with Gupta et al.[20] and Shirwaikar et al.[40] who reported the anti-diabetic effect of aqueous and ethanolic extract of leaves of A. squamosa in experimental diabetic animals table 1.

Table (1): Levels of fasting blood sugar and serum insulin in different treated groups.

Parameter / Groups	F.B.S mg/dl	Serum Insulin µIU/ml
Control	76.2 ±2.2	15.1 ±0.7
Diabetic	287.8 ±6.1[a]	6.2 ±0.2 [a]
Diabetic treated with vildagliptin drug	144.0 ±3.2[b]	8.1 ±0.1 [b]
Diabetic treated with aqueous extract of leaves	117.4 ±2.8[bce]	9.9 ±0.2 [bce]
Diabetic treated with ethanolic extract of leaves	119.2 ±2.8[bce]	9.5 ±0.2 [bce]
Diabetic treated with mix of the aqueous and ethanolic extracts	93.4 ±2.6[b]	11.9±0.4[b]

a = significant versus control group, b = significant versus diabetic group, c = significant versus diabetic treated with vildagliptin drug, d = significant versus diabetic treated with aqueous extract of leaves. e = significant versus mix.

The possible mechanism by which *A. squamosa* achieved its hypoglycemic action in diabetic rats may be by potentiating the insulin action by either increasing the pancreatic secretion of insulin from the existing beta cells or by its release from the bound form.[40] Moreover, Ren et al.[41] reported that *A. squamosa* might exert anti-diabetic effect by inhibiting of alpha-glycosidase enzyme.

After treatment with *A. squamosa* leaves extracts at a dose of 300 mg/ kg, islets of Langerhans showed an improvement and restoration of normal cellular population size. The histopathological finding of diabetic liver and pancreas treated with both extracts of *A.squamosa* leaves showed marked improvement and minimal pathological changes. These results are in agreement with Raj et al.[42] who reported the hepatoprotective potential of the alcoholic extract of A. squamosa leaves in DEN-induced liver injury in mice thus the histopathological pattern of treated groups showed a minimal inflammation with moderate portal triaditis and their lobular architecture were normalized.

The current results of the pancreatic tissues of diabetic group illustrated in figure (1) showed moderate degeneration of pancreatic acini (A), with markedly shrunken islets size with moderate edema and congestion (C and E). There was moderate inflammatory cells infiltration formed of lymphocytes surrounding pancreatic islets (dashed arrow) with vacuolar degenerated islets cells (arrow), vessels showed slight wall thickening (arrowhead). As regard diabetic treated with mix of the two extracts, the pancreatic tissues showed marked improvement, islets size were restored, cells (arrow) showed pale eosinophilic cytoplasm

with rounded nuclei. No degenerative changes were seen in normal pancreatic acini (A). While, residual focal edema (E) and small capillary sized islets vessels (arrowhead) were showed as compared with other treated groups.

As shown in figure (2), the liver tissue showed partially disturbed architecture, hepatocytes are arranged in thick and thin cell plates with distended cytoplasm showing moderate hydropic degeneration and steatosis (Arrow), enlarged nuclei (Arrowhead) with few sinusoids (S). There are foci showing an inflammatory cells infiltrate formed of lymphocytes and plasma cells (Dashed arrow). The central veins showed marked congestion (C). On the other hand, in diabetic treated mix of extracts the liver tissue is formed of hepatocytes (arrow) that have abundant cytoplasm with marked improvement, minimal hydropic degeneration and arranged in thin cell plates with normal sinusoids (S), some cells showed nucleoli (arrowhead). There is minimal residual inflammation (dashed arrow) surrounding central vein (C) as compared with other treated group.

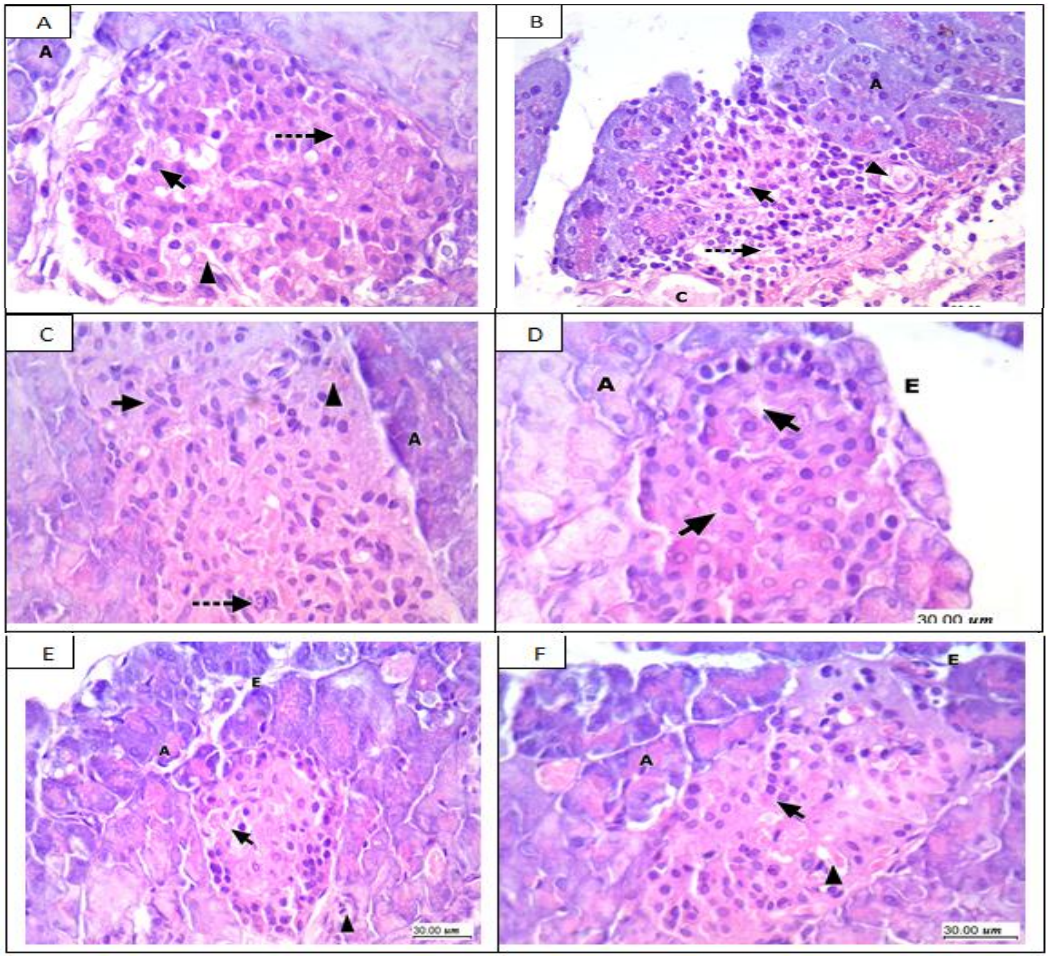

Fig. 1 – Effect of different extracts on histopathological features of pancreas: (A) normal control group; (B) diabetic control group; (C) diabetic treated Vildagliptin drug; (D) diabetic treated aqueous extract of leaves; (E) diabetic treated alcohol extract of leaves (F) diabetic treated mix of extracts.

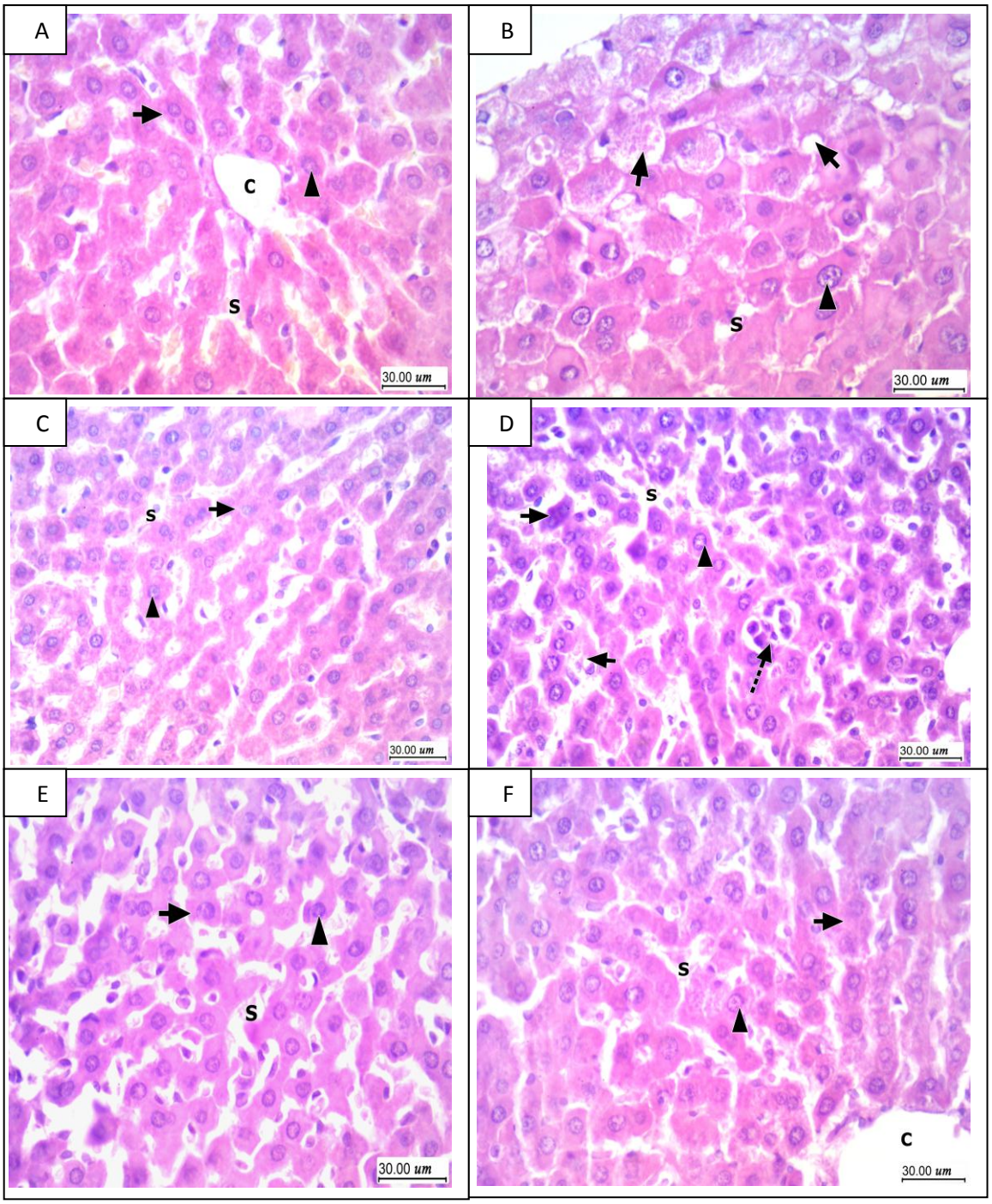

Fig. 2 – Effect of different extracts on histopathological features of liver: (A) normal control group; (B) diabetic control group; (C) diabetic treated Vildagliptin drug; (D) diabetic treated aqueous extract of leaves; (E) diabetic treated alcohol extract of leaves (F) diabetic treated mix of extracts.

The liver and the kidney are the major organs that affected by diabetes. If the liver enzyme activity and kidney functions increased, it may indicated to liver and kidney damage.[43] Liver enzymes are good indicators for liver functioning. Diabetic conditions also caused secretion and release of liver enzymes to blood circulation due to destruction of cells due to changes in

membrane structure.[44] Oral administration of aqueous and alcoholic extracts by 300 mg/kg doses significantly reduces the levels of AST and ALT enzymes in blood. In accordance with our results, Saleem et al.[45] were reported the protective effect of alcoholic extract of *A. squamosa* leaves on liver and improvement of its functional efficiency in rifampicin induced hepatotoxic rats through significant decrease in total bilirubin, alkaline phosphatase (ALP), AST and ALT.

In the present work, alloxan-induced diabetic rats exhibited significantly higher serum urea and creatinine levels compared to the control group. These results were in agreements with Kaleem et al.[11] who reported that DM increased production of reactive oxygen species that causes renal damage and abnormal glucose regulation causes haemodynamic changes within the kidney tissue that could be due to increased oxidative stress.

As well as, reduction in serum total protein and albumin levels was observed in alloxan-induced diabetic group compared to control group and this was consistent with the results obtained by Kaleem et al.[11] The decrease in protein and albumin may be due to microproteinuria and albuminuria, which are important clinical markers of diabetic nephropathy [46] and may be due to increased protein catabolism.[47] The results of the present study demonstrated that the administration of aqueous and alcoholic extracts of *A. squamosa* to diabetic rats was significantly elevate the levels of total protein and albumin and significantly decrease urea and creatinine levels compared with diabetic group.

The hepatoprotective effect and enhancing renal function of the extracts may be due to that *A. squamosa* leaves contain flavonoids which posses antioxidative effects might have scavenged the free radical, so offering hepato and renal protection[11,38] table 2.

Table (2): Levels of kidney function parameters in different treated groups.

Parameters / Groups	Creatinine mg/ dl	Urea mg/dl	Total protein g/dl
Control	0.7 ±0.06	15.8 ±0.5	10.0 ±0.2
Diabetic	1.9 ±0.1^{a}	34.4 ±1.2^{a}	4.3 ±0.2^{a}
Diabetic treated with vildagliptin drug	1.2 ±0.07^{b}	27.4 ±0.8^{b}	6.6 ±0.3^{b}
Diabetic treated with aqueous extract of leaves	0.98 ±0.05^{bce}	24.8 ±0.6^{bce}	6.9 ±0.4^{be}
Diabetic treated with alcoholic extract of leaves	1.1 ±0.04^{be}	25.4 ±0.7^{be}	7.2 ±0.4^{be}
Diabetic treated with mix of aqueous and alcoholic extracts	0.8 ±0.07^{b}	20.2 ±0.4^{b}	8.4 ±0.24^{b}

a = significant versus control group, b = significant versus diabetic group, c = significant versus diabetic treated with vildagliptin drug, d = significant versus diabetic treated with aqueous extract of leaves. e = significant versus mix.

Table (3): Levels of liver functions parameters in different treated groups.

Parameters / Groups	Albumin g/dl	GOT U/L	GPT U/L
Control	4.4 ±0.1	35.0 ±1.6	21.0 ±1.2
Diabetic	2.3 ±0.2 [a]	94.8 ±0.3 [a]	74.6 ±2.6 [a]
Diabetic treated with vildagliptin drug	3.3 ±0.05 [b]	62.2 ±1.6 [b]	39.0 ±1.2 [b]
Diabetic treated with aqueous extract of leaves	3.9 ± 0.17[bc]	46.8 ±1.6[bc]	37.6 ±1.8[be]
Diabetic treated with alcoholic extract of leaves	3.5 ±0.06 [b]	44.8 ±0.6 [bc]	40.0 ±1.7 [be]
Diabetic treated with mix of aqueous and alcoholic extracts	3.9 ±0.2 [b]	43.4 ±0.4 [b]	28.4 ±1.0 [b]

a = significant versus control group, b = significant versus diabetic group , c = significant versus diabetic treated with vildagliptin drug, d = significant versus diabetic treated with aqueous extract of leaves. e = significant versus mix.

It has been demonstrated that insulin deficiency in diabetes mellitus leads to a variety of derangements in metabolic and regulatory processes.[48] In the present study, level of cholesterol, TGs, LDL-cholesterol was significantly increased and HDL-cholesterol was significantly decreased in diabetic group compared to control group. These results were in agreement with finding of Otamere et al.[9] who reported that the most common lipid disorders associated with diabetes are increased levels of cholesterol, triglyceride-rich lipoproteins, low level of HDL and the presence of small dense LDL particles. Defect in insulin secretion associated with a diminished level of LDL receptor causes the increase in LDL particles.[10]

Deficiency of insulin is associated with increase in cholesterol levels due to the enhanced mobilization of lipids from the adipose tissue to the plasma.[48] On the other hand, hypertriglyceridemia results from accumulation of VLDL particles, either by overproduction, decreased catabolism or both due to lack of insulin, which normally activates the enzyme lipoprotein lipase which catalyses the hydrolysis of the TGs component of circulating chylomicrons and VLDL particles.[50]

The decreased HDL level in diabetic rats may be due to the decreased production of HDL by intestine and liver or due to the nonenzymatic glycation of HDL or its apolipoprotein A-I which results in a decrease in Lecithin cholesterol acyltransferase (LCAT) activity which catalyzes the maturation of HDL particles by esterifying free cholesterol molecules [51]. These findings indicate that DM is accompanied with increased risk of atherosclerosis and coronary artery diseases. Lowering of serum lipid levels through dietary or drug therapy seems to be associated with a decrease in the risk of vascular disease.[52]

As regard to aqueous and ethanolic extracts of *A.squamosa* leaves treatment to diabetic group, the mean level of cholesterol, triglyceride, LDL-cholesterol was significantly decreased and HDL-cholesterol was significantly increased compared to diabetic group. These results were in agreement with Tomar and Sisodia[53] who reported that *A. squamosa* L. extract at a dose of 350 mg/kg b.w. caused significant decrease in serum lipids cholesterol and triglycerides and significant increase in serum HDL levels after 28 days of treatment when compared to diabetic control group. Also, Gupta et al.[20] reported that after 15-day treatment of ethanolic extract of *A.squamosa* leaves brought down the elevated levels of TC, LDL and VLDL cholesterol and TG in diabetic animals to nearly normal level and increased the HDL cholesterol which considered a desirable effect. Shirwaikar et al.[40] attributed the significant control of the levels of serum lipids in the aqueous extract of *A. squamosa* leaves treated diabetic to improvements in insulin levels upon *A. squamosa* therapy table (4).

Disturbances in the normal redox state of cells can cause toxic effects through the production of peroxides and free radicals that damage all components of the cell, including proteins, lipids, and deoxyribonucleic acid (DNA). Chemically, oxidative stress is associated with increased production of oxidizing species or a significant decrease in the effectiveness of antioxidant defenses, such as glutathione. Oxidative stress has been considered to be a pathogenic factor of diabetic complications.[54]

Table (4): Levels of lipid profile parameters in different treated groups.

Parameters / Groups	Cholesterol mg/dl	TGs mg/dl	HDL-cholesterol mg/dl	LDL-cholesterol mg/dl
Control	57.8 ±2.9	67.0 2.±3	49.4 ±2.3	54.0± 1.3
Diabetic	131.0 ±2.7[a]	145.0 ±3.2 [a]	29.0 ±0.9 [a]	111 ±1.2 [a]
Diabetic treated with vildagliptin drug.	71.8 ±1.7[b]	109.2 ±2.0 [b]	30.2 ±0.5 [b]	76± 1.1[b]
Diabetic treated with aqueous extract of leaves.	64.4 ±1. 4[bce]	77.4 ±3.4[bc]	33.8 ±1.2[bc]	73± 1.3[be]
Diabetic treated with alcoholic extract of leaves.	65.4 ±3.1[b]	92.2 ±1.6[bcde]	32.6 ±1.2[be]	77 ±1. 8[bde]
Diabetic treated with mix of aqueous and alcoholic extracts	72.0± 2.7 [b]	73.3 ±1.6 [b]	40.6 ±2.0 [b]	64 ±1.2 [b]

a = significant versus control group, b = significant versus diabetic group, c = significant versus diabetic treated with vildagliptin drug, d = significant versus diabetic treated with aqueous extract of leaves. e = significant versus mix.

Lipid peroxidation is a free radical-induced process leading to oxidative deterioration of polyunsaturated fatty acids. lipid peroxidation is evaluated by measuring the level of MDA which is formed when polyunsaturated fatty acyl chains are attacked by hydroxyl radicals.[55] We have observed a significant increase in MDA level in pancreatic tissues of diabetic rats compared to control group. This result was in agreement with a study of Bagri et al.[56] who reports that MDA was significantly increased in pancreas of STZ induced diabetic animals (table 5).

Nitric oxide is a potent vasodilator and cytostatic agent and may therefore be involved in diabetic angiopathies. NO interact with superoxide to produces another potent oxidant, the peroxynitrite anion, which rapidly decomposes to give significant quantities of hydroxyl radical, nitrate and nitrite.[57] The present data indicated that alloxan-induced diabetes disrupted the level of NO. Bhatia et al. [57] observed a significant increase of NO end products in serum of diabetic patients accompanied with nephropathy.

GSH is a well known and an important inhibitor of free radical mediated lipid peroxidation.[58] Data of the current work showed a marked depletion in the GSH content of liver in alloxan-induced diabetic rats compared to control rats. Several studies have also reported the decreased levels of plasma GSH in diabetic rats.[56] This decreased level of GSH in diabetes may result from increased utilization in trapping the oxyradicals.[56]

As regard, aqueous and ethanolic extracts of *A.squamosa* leaves treatment showed a significant increase in liver GSH content, decrease in pancreatic MDA and liver NO levels of diabetic rats. In agreement with these results, Nandhakumar and Indumathi[14] reported that the aqueous and alcoholic extracts of *A.squamosa* leaves counteract the effect of nitric oxide, exhibited NO scavenging activity and arrest the chain of reactions, initiated by excess generation of NO in vitro. Furthermore; Gupta et al.[15] reported that the water extract significantly decreased the MDA and increased GSH levels in diabetic rats in comparison to diabetic control rats. Kalidindi et al.[59] reported that antioxidant and free radical scavenging activities of *A. squamosa* leaves extracts contributed to the presence of the flavonoids and phenols.

Table (5): Levels of oxidant and antioxidant parameters in different studies groups.

Parameters / Groups	MDA nmol/gm	NO µmol / gm	GSH mg/gm
Control	49.8 ±2.3	7.2 ±0.4	48.0 ±1.5
Diabetic	117.2 ±3.07[a]	17.3 ±0.5 [a]	25.6 ±1.3 [a]
Diabetic treated with vildagliptin drug	80.6 ±1.7[b]	10.4 ±0.5 [b]	33.4 ±1.2 [b]
Diabetic treated with aqueous extract of leaves	63.6 ±1.9[bce]	11.0 ±0.3[be]	36.2 ±1.3[b]
Diabetic treated with alcoholic extract of leaves	63.2 ±1.8[bce]	12.9 ±0.4[bcde]	33.4 ±1.2[be]
Diabetic treated with mix of aqueous and alcoholic extracts	44.2 ±1.6[b]	9.5 ±0.4 [b]	38.8 ±1.0 [b]

a = significant versus control group, b = significant versus diabetic group , c = significant versus diabetic treated with vildagliptin drug, d = significant versus diabetic treated with aqueous extract of leaves. e = significant versus mix.

The present results showed that no significant difference was found between diabetic group treated with aqueous extract of leaves and diabetic group treated with alcoholic extract of leaves in the most markers. Furthermore, the results obtained clearly suggested that the mix of ethanol and aqueous extracts has an effective anti-diabetic and antioxidant activities than the aqueous extract or ethanol extract individually that could be due to a higher presence of phenolic and flavonoidal constituents in the mixes of extracts.

CONCLUSIONS

No significant difference was found between diabetic groups treated aqueous extract of leaves and diabetic group treated alcohol extract of leaves in the most markers. Mix extracts ethanolic and aqueous of A. squamosa leaves showed more potent hypoglyceamic,

hypolipidemic effect and antioxidant activities in most marker compared with the aqueous extract or ethanolic extracts individually.

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REFERENCES

1. American Diabetes Association. Diagnosis and Classification of Diabetes Mellitus. Diabetes Care, 2014; 37, Supplement 1.
2. Marshall SM and Flyvbjerg A. Prevention and early detection of vascular complications of diabetes. British Medical Journal, 2006; 333: 475-480.
3. Pasupathi P, Chandrasekar V and Kumar SU. Evaluation of oxidative stress, enzymatic and non-enzymatic antioxidants and metabolic thyroid hormone status in patients with diabetes mellitus. Diabetes and Metabolic Syndrome: Clinical Research and Reviews, 2009; 3: 160–165.
4. Tangvarasittichai S. Oxidative stress, insulin resistance, dyslipidemia and type 2 diabetes mellitus. World J Diabetes, 2015; 6(3): 456-480.
5. Brownlee M. The pathobiology of diabetic complications: a unifying mechanism. Diabetes, 2005; 54: 1615-1625.
6. Maritim AC, Sanders RA, and Watkins JB. Diabetes, oxidative stress and antioxidants: a review. Journal of Biochemical and Molecular Toxicology, 2003; 17(1): 24-38.
7. Erejuwa O. Oxidative Stress in Diabetes Mellitus: Is There a Role for Hypoglycemic Drugs and/or Antioxidants?. Oxidative Stress and Diseases, 2012.
8. Solano M P, Goldberg R B. Management of dyslipidemia in diabetes. Cardiology in Review, 2006; 14: 125– 135.
9. Ansar S, Koska J, Reaven PD. Postprandial hyperlipidemia, endothelial dysfunction and cardiovascular risk: Focus on incretins. Cardiovascular Diabetology, 2011; 10: 61.
10. Arcari DP, Porto VB, Rodrigues ERV, Martins F, Lima RJ D, Sawaya A C HF, Ribeiro ML, Carvalho PDO . Effect of mate tea (Ilex paraguariensis) supplementation on oxidative stress biomarkers and LDL oxidisability in normo- and hyperlipidaemic humans. Journal of Functional Foods, 2011; 3: 190–197.

11. Kaleem M, Medha P, Ahmed Q U, Asif M and Bano B. Beneficial effects of Annona squamosa extract in streptozotocin-induced diabetic rats. Singapore Med J Original Article, 2008; 49(10): 800.
12. Moghadamtousi SZ, Goh BH, Chan CK, Shabab T and Kadir HA. Biological activities and phytochemicals of swietenia macrophylla king. Molecules, 2013; 18: 10465–10483.
13. Srivastava S, Lal VK, Pant KK. Medicinal potential of Annona squamosa: At a glance. J. Pharm. Res, 2011; 4: 4596–4598.
14. Nandhakumar E and Indumathi P .In vitro Antioxidant Activities of Methanol and Aqueous Extract of Annona squamosa (L.) Fruit Pulp. J Acupunct Meridian Stud, 2013; 6(3): 142-148.
15. Gupta RA, Kesari AN, Diwakar S, Tyagi A, Tandon V, Chandra R and Watal G.In vivo evaluation of anti-oxidant and anti-lipidimic potential of Annona squamosa aqueous extract in Type 2 diabetic models. J Ethnopharmacol, 2008; 118: 21-25.
16. Qing, A. Clinical observation for treatment of bujing jiedu for 86 cases of advanced non-small cell lung cancer. Zhongguo Zhong Yi Yao Za Zhi, 2012; 18: 26–27.
17. Pandey N and Barve D. Phytochemical and Pharmacological Review on Annona squamosa Linn. International Journal of Research in Pharmaceutical and Biomedical Sciences, 2011; 2: 4.
18. Alam MM, Meerza D and Naseem I. Protective effect of quercetin on hyperglycemia, oxidative stress and DNA damage in alloxan induced type 2 diabetic mice. Life Sci, 2014; 109(1): 8–14.
19. Kohli R, Meininger JC, Haynes ET, Yan W, Self TJ and Wu G. Dietary L-arginine supplementation enhances endothelial nitric oxide synthesis in streptozotocin induced diabetic rats. Nutr, 2004; 134: 600-608.
20. Gupta RK, Kesari AN, Murthy PS, Chandraa R, Tandon Chandraa V and Watal G. Hypoglycemic and antidiabetic effect of ethanolic extract of leaves of Annona squamosa L. in experimental animals. Journal of Ethnopharmacology, 2005; 99: 75–81.
21. Pari L, Umamaheswari J. Antihyperglycaemic activity of Musa sapientum flowers: effect on lipid peroxidation in alloxan diabetic rats, Phytother. Res, 2000; 14(2): 136–138.
22. Manna F, Ahmed HH., Estefan SF, Sharaf HA, Eskander EF. Saccharomyces cerevisiae intervension for relieving flutamideinduced hepatotoxicity in male rats. Pharmazie, 2005; 60: 689-695.
23. Luna LG. Manual of histological technique methods of armed forces. London: Institute of Pathology, 1996; p. 1–31.

24. Passing H and Bablok WA. New biochemical procedure for testing the equality of measurements from two different analytical methods. Journal of Clinical Chemistry and Clinical Biochemistry, 1983; 21: 709-720.
25. Judzewitsch RG, Pfeifer MA, Best JD, Beard JC., Halter JB, and Porte Jr. Chronic Chlorpropamide therapy of noninsulin-dependent diabetes augments basal and stimulated insulin secretion by increasing islet sensitivity to glucose. J. Clin. Endocrino. Metab, 1982; 55: 321-328.
26. Allain C, Lucy C, Poon S, Cicely S, Chen G, Richmed W and paul CF.: Enzymatic determination of total cholesterol. Clinical Chemistry; 1974; 20: 470-475.
27. Glick MR, Ryder KW and Jackson SA .Graphical comparisons of interferences in Clinical Chemistry Instrumentation. Clinical Chemistry, 1986; 32: 470-474.
28. Lopez-Virella MF ,Stone P, Ellis S and Collwel J. Cholesterol determination in high density lipoprotein by three different methods. Clinical Chemistry, 1977; 23(5): 882-884.
29. Friedwald WT, Levy RI and Fredrickson DS. Estimation of the concentration of low density lipoprotein cholesterol in plasma without use of the preparative ultracentrifuge. Clin Chem, 1972; 18: 499-502.
30. Ohkawa I et al. Assay for lipid peroxidase in animal tissue by thiobarbituric acid reaction.Ann Biochem 1979; 95: 351-358.
31. Lisa A et al. Spectrophotometric method for the direct detection and quantification of nitric oxide, nitrates and nitrite in cell culture media.Analytical Biochemistry 2000; 281,223,229.
32. Beutler E., Duron O., Kelly MB. J. Lab Clin. Med, 1963; 61: 882.
33. Fawcett, J.K and Soctt, J.E. J, Clin., Path,1960; 13: 156 - 159
34. Belfield A. and Goldberg D.M. Enzyme, 1971; 12: 561.
35. Reitman A, and Frankel S. Amer J. Clin. Path, 1957; 28: 56.
36. Doumas B.T et al., Clin. Chim. Acta, 1971; 31-87.
37. Gornal AC, Bardawill CJ.and David MM. J. Biol. Chem, 1949; 177: 751.
38. Kaleem M, Sheema, Sarmed H, Bano B. Protective effects of Piper nigrum and Vinca rosea in alloxan-induced diabetic rats. Indian J Physiol Pharmacol, 2005; 49: 65-71.
39. Shankar PK, Kumar V, Rao N. Evaluation of antidiabetic activitiy of Ginkgo biloba in Streptozotocin induced diabetic rats. IJPT, 2005; 4(1): 16-19.
40. Shirwaikar A, Rajendran K, Kumar CD and Bodla R .Antidiabetic activity of aqueous leaf extract of Annona squamosa in streptozotocin–nicotinamide type 2 diabetic rats. Journal of Ethnopharmacology, 2004; 91: 171–175.

41. Ren YY, Zhu ZY, Sun HQ and Chen LJ . Structural characterization and inhibition on α -glucosidase activity of acidic polysaccharide from Annona squamos. Carbohydr, 2017; Polym.174: 1-12.
42. Raj DS, Vennila JJ, Aiyavu C and Panneerselvam K. The hepatoprotective effect of alcoholic extract of Annona squamosa leaves on experimentally induced liver injury in swiss albino mice. Int. J. Int. Biol, 2009; 5: 182–186.
43. Amraie E, Farsani MK, Sadeghi L, Khan TN, Babadi VY, Adavi Z. The effects of aqueous extract of alfalfa on blood glucose and lipids in alloxan-induced diabetic rats. Interv Med Appl Sci, 2015; 7(3): 124–8.
44. Udayakumar R, Kasthurirengan S, Mariashibu TS, Rajesh M, Anbazhagan VR, Kim SC, et al. Hypoglycemic and hypolipidaemic effects of Withania somnifera root and leaf extracts on alloxan-induced diabetic rats. Int J Mol Sci, 2009; 10: 2367–82.
45. Saleem TS, Christina AJM, Chidambaranathan N, Ravi V and Gauthaman K .Hepatoprotective activity of Annona squamosa Linn. On experimental animal model. International Journal of Applied Research in Natural Products, 2008; 1(3): 1-7.
46. Mauer SM, Steffes MW and Brown DM. The kidney in diabetes. Am J Med, 1981; 70: 63-6.
47. Almdal JP and Vilstrup H. Strict insulin therapy normalizes organ nitrogen contents and the capacity of urea nitrogen synthesis in experimental diabetes in rats. Diabetologia, 1988; 31:114-8.
48. Shepherd J.: Does statin mono therapy address the multiple lipid abnormalities in type-2 diabetes. Atherosclerosis Supplements, 2005; 6: 15–19.
49. Otamere H.O., Aloamaka C.P., Okokhere P.O. and Adisa W.A. Lipid Profile in Diabetes Mellitus; What Impact Has Age and Duration. British Journal of Pharmacology and Toxicology, 2011; 2(3): 135-137.
50. Pandhare R B, Sangameswaran B, Mohite P B, KhanageS G. Anti-hyperglycaemic and lipid lowering potential of Adenanthera pavonina Linn. in streptozotocin induced diabetic rats. Oriental Pharmacy and Experimental Medicine, 2012; 12: 197– 203.
51. Kunnen S, Van Eck M. Lecithin: cholesterol acyltransferase: Old friend or foe in atherosclerosis? The Journal of Lipid Research, 2012; 53: 1783–1799.
52. Rhoads GG, Gulbrandse CL and Kagen A. Serum lipoprotein and coronary artey disease in a population study of Hawaiian Japanese men. New England Journal of Medicine, 1976; 294: 293–298.

53. Tomar RS, Sisodia SS, squamosa L. Antidiabetic activity of Annona in experimental induced diabetic rats. Inter JPharm Biol Arch, 2012; 3: 1492-1495.
54. Waggiallah H and Alzohairy M The effect of oxidative stress on human red cells glutathione peroxidase, glutathione reductase level, and prevalence of anemia among diabetics. N Am J Med Sci, 2011; 3: 344–7.
55. West IC. Radicals and oxidative stress in diabetes. ã British Diabetic Association. Diabetic Medicine, 17: 171-180
56. Bagri P, Ali M, Aeri V, Bhowmik M and Sultana S. Antidiabetic effect of Punica granatum flowers: Effect on hyperlipidemia, pancreatic cells lipid peroxidation and antioxidant enzymes in experimental diabetes. Food and Chemical Toxicology, 2009; 47: 50–54.
57. Bhatia S, Shukla R, Madhu SV, Gambhir JK, Prabhu KM. Antioxidant status, lipid peroxidation and nitric oxide end products in patients of type 2 diabetes mellitus with nephropathy. Clinical Biochemistry, 2002; 36: 557–562.
58. Meister A, Anderson ME. Glutatione. Annual Review of Biochemistry, 1983; 25: 711-760.
59. Kalidindi N, Thimmaiah NV, Jagadeesh NV, Nandeep R, Swetha S and Kalidindi B. Antifungal and antioxidant activities of organic and aqueous extracts of Annona squamosa Linn. Leaves. journal of food and drug analysis, 2015; 23: 795 - 802.