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Hypocholesterolemic Efficacy of *Annosa Squamosa* (L.) Extract In Mice Diabetic Models

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Abtract: *The majority of Type-2 DM patients suffer from visceral obesity and they often have high circulating levels of lipids including cholesterol, triglycerides, low levels of high density cholesterol, which also contribute to the development of vascular complications. In the present study the efficacy of methanol extract of leaves of Annona squamosa on serum lipid profile of diabetic mice was studied. The results clearly indicated that Diabetic mice treated with A. squamosa extract (DT_{150}) showed significantly lower values of serum TC (-26.5%; p<0.001) and TGs (-55.6%; p<0.001), when compared with the DC counterparts. The DT_{250} treatment showed superior lowering effects compared with the DC counterparts as well as DT_{150} group mice by (-34.7%; p<0.001) on serum TC levels and (-50%; p<0.001) on TGs levels. Contrarily, treatment with rosiglitazone (DT_{RGZ}) showed (-33.2%; p<0.001) on TC levels and (-09.6%; p<0.001) on TGs levels compared with diabetic control mice.*

Key Words: *Annona squamosa, Lipid profile, Streptozotocin, Mice*

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I. Introduction

Type-2 Diabetes Mellitus (DM) is a heterogeneous disease with both genetic and environmental causative factors. Gerich (1996) [1] has summarized the pathophysiology of Type-2 DM as follows. Initially the pancreatic beta cells are not able to respond with appropriate insulin secretion to glucose challenge. At the same time, an increased demand for insulin due to environmentally induced insulin resistance has also act. At this juncture, a compensatory increase of the insulin secretion is still sufficient to maintain a normal glucose level. By gradual decrease of insulin secretion and increase of insulin resistance, a reduced suppression of hepatic glucose output and impaired glucose tolerance appear. With further increase in insulin resistance, an absolute increase in hepatic glucose output occurs which leads to fasting hyperglycemia. At the same time, pancreas fail to compensate for the increased demand of insulin any further and hyperglycemia sets in. If untreated, hyperglycemia and insulin resistance in Type-2 DM increase the risk of several macro and micro vascular complications such as, hypertension, coronary vascular disease, cardiomyopathy, stroke and retinopathy, neuropathy, nephropathy [2, 3] (Kannel and McGee, 1979; Garcia, 1974). The majority of Type-2 DM patients suffer from visceral obesity and they often have high circulating levels of lipids including cholesterol, triglycerides, low levels of high density cholesterol, which also contribute to the development of vascular complications. Independent of coronary artery complications, complex changes in the mechanical and electrical properties of the heart may contribute to diabetic cardiopathy. The devastating consequences of these complications include lower-limb amputation, end stage renal failure, loss of vision and myocardial infarction.

It is clear that the contributory abnormalities in Type-2DM are insulin deficiency, insulin resistance and increased hepatic glucose output. The current therapies used to treat patients with these complications are aimed at correcting one or more of these physiological abnormalities. The diabetes control and complications study [4] (DCCT, 1996) and the United Kingdom Prospective Diabetes Study [5] (UKPDS, 1998) demonstrated that good metabolic control through intensive drug therapy and strict lifestyle management could reduce the risk of developing diabetic complications.

Phytochemicals have played an important role in the development of chemotherapeutic agents. Phytochemicals have multiple beneficial activities including manipulation of carbohydrate metabolism by various mechanisms, preventing and restoring integrity and functioning of β-cells, insulin releasing activity, improving glucose uptake and utilization, and antioxidant properties. Furthermore, natural products are widely viewed as templates for optimization programs with the goal of creating new drugs.

Annona squamosa (L.), commonly known as custard apple tree belongs to family Annonacea is a native tree of West Indies. The plants range in height from 3 to 6 m and bear crown of irregular branches with deciduous leaves. Leaves are lanceolate to oblong with blunt tip and arranged alternately no short hairy petioles.

The leaves of *Annona squamosa* contain steroids, alkaloids, saponins, terpenes, tannins, phenolic substances, carbohydrates, volatile oil, flavonoids, diazepine and squamoline. Six other aporphine alkaloids have been isolated from the leaves and stems viz. corydine, roemerine, norcorydine, norisocarydine, isocorydine and glaucine. *Annona squamosa* is used medicinally, and known to possesses cardiotonic, anti-ulcer, anti-lipidemic, antioxidant, anti-spasmodic, anti-ovulatory, insecticidal, anti-hyperthyroidism, abortifacience, anti-septic, anti-pyretic, anti-diabetic, anti-inflammatory, antimicrobial and hepatoprotective activities

Annona squamosa is traditionally used for the treatment of epilepsy, dysentery, cardiac problems, worm infestation, constipation, hemorrhage, bacterial infection, dysurea, fever, thirst and ulcer [6] (Raj *et al*., 2009). The flavonoid of *A. squamosa* possesses anti-oxidative [7] (Saija *et al.,* 1995). Gupta *et al*., (2005) [8]] reported that ethanolic extract from leaves of North Indian *A. squamosa* could reduce blood glucose levels of normal and streptozotocin induced diabetic rats as well as normal and alloxan-induced diabetic rabbits. The isolation and formulation of active constituents from *Annona squamosa* along with their pharmacological evaluations are the present need for of the modern therapeutics. Therefore the present investigation has been undertaken to evaluate the efficacy of methanol extract of leaves of *A. squamosa* in controlling hypercholesterolemia in streptozotocin induced mice diabetic models.

II. Materials and Methods

Methanol extract of *Annona squamosa* Linn. (Annonaceae) was used for assaying the serum lipid profile in Streptozotocin induced mice diabetic models. The leafy twigs of *Annona squamosa* were washed under running tap water, blotted with filter paper dried in the shade at room temperature. The dried plant sample (2.6 kg) was then soaked with absolute methanol under reflux condition for the methanolic extract preparation. The sample was then homogenized with extraction buffer and the supernatant collected after three rounds of extraction. The solvent was evaporated under reduced pressure in a rotary evaporator at 40 ^{0}C. To this thick paste colloidal silicon dioxide was added and dried in vacuum tube dryer. The obtained methanol extract was stored in deep freezer at $^{-}20^0$ C until further test.

The mice were allowed to acclimatize for 15 days in an environmentally controlled room under standard environmental conditions (21±2°C, 55±5%Relative humidity, 12 hr Light: Dark cycle) and fed on diet consisted of wheat grains-1Kg, Choker wheat-250gm, Gram grains-250gm, Maize grains-250gm, Soybean grains-250gm, Sundrop oil-50gm, Milk powder-2 table spoon and Jaggery-50gm. This diet provided carbohydrate 48.3%, crude protein 23.5%,crude fat5.9% crude ash5.9% and crude fibre 3.9% (W/W).

The animal model for the present study was based on multiple administration of low dose of freshly prepared streptozotocin (STZ). For induction of diabetes, initially the normal mice were kept 24 hours without food and water. The weight of normal mice was determined. Diabetes was induced by multiple intra-peritoneal injection of freshly prepared STZ solution in 0.05 M sodium citrate (pH 4.5) at the dose of 35 mg/kg body weight followed by an hour of fasting. The mice were then allowed to access the respective food and water *ad libitum*. Mice with fasting blood glucose level of 200 mg/dl (7.8 mmol/l) or higher were considered to be diabetic and were used in the study. A parallel set of control mice (non-diabetic) were injected with citrate buffer only.

The mice were grouped into five categories viz., Normal control (NC), Diabetic Control (DC), Diabetic Treated (DT_{150}), Diabetic Treated$_{250}$) and Diabetic Treated (DT_{RZG}). NC received only citrate buffer solution. DC group was STZ induced which received citrate buffer only. DT_{150} and DT_{250} received 150mg/Kg and 250mg/Kg body weight of methanol extract respectively. DT_{RGZ} received rosiglitazone at a dose of 2mg/Kg of body weight. All the mice were fed with common pellet diets for 2 weeks after arrival, and then randomly divided into two groups. One group continued to receive common pellet diets and constituted the normal group; the other was fed with diets high in fat and fructose, in order to induce type-2 diabetes. All the mice had free access to food and water.

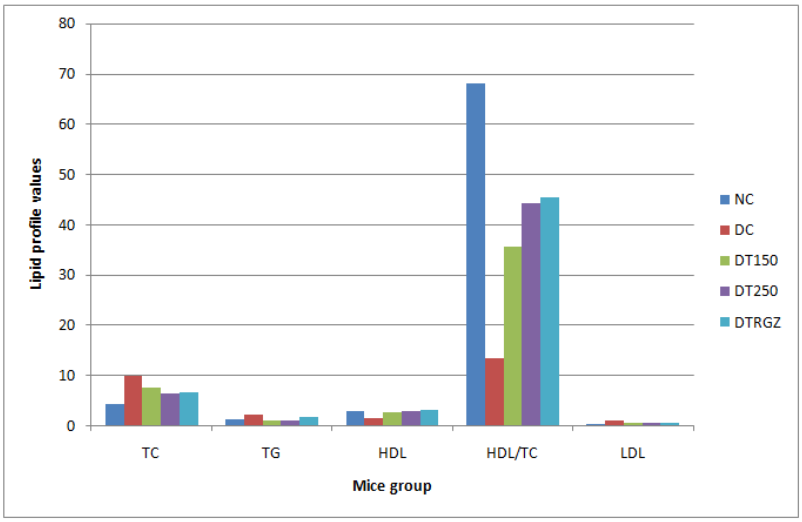
For the experiment, the mice were divided into five groups having six mice in each group: DC group (diabetic control mice), NC group (non-diabetic control mice) and three DT group (diabetic mice treated with two different doses of extract as well as rosiglitazone/ kg body weight). Body weights were recorded weekly during the experimental period. Treatment with extracts was started after one week of STZ treatment, which was considered as the 1st day of treatment. Blood samples were taken after 8 hrs fasting from the retro-orbital sinus vein prior to the administration of test substances or the buffer and 4 weeks after the treatment under mild ether anesthesia and allowed to clot for 30 minutes at room temperature. Blood samples were centrifuged at 3000 rpm for 20 minutes. Serum was separated and stored at -20°C until biochemical estimations were carried out.

The total cholesterol, total triglyceride, Cholesterol-HDL, Cholesterol- LDL and HDL/TC values were assayed and the results obtained have been presented in Table-1. Data were statically analyzed by mean ± S.E and by one-way ANOVA.

Table-1: Showing effects of Different doses of *Annona squamosa* extract and rosiglitazone on serum lipid profile in STZ induced mice diabetic model

Groups	TC (mmol/lit)	TG (mmol/lit)	HDL (mmol/lit)	HDL/TC (%)	LDL (mmol/lit
Normal control (NC)	4.25±0.86**	1.16±0.09**	2.87±0.29**	68.25±4.66**	0.28±0.04**
Diabetic control (DC)	9.95±1.56*	2.15±0.29*	1.35±0.58*	13.45±1.97*	0.98±0.16*
A. squamosa extract (150mg/kg) (DT_{150})	7.55±0.44**	0.95±0.08**	2.65±0.36**	35.65±3.37**	0.51±0.06**
A. squamosa extract (250mg/kg) (DT_{250})	6.35±0.64**	0.99±0.17**	2.78±0.46**	44.40±4.26**	0.45±0.07**
Rosiglitazone 2mg/kg (DT_{RGZ})	6.60±1.35**	1.75±0.17**	2.95±0.55**	45.45±5.56**	0.42±0.09**

P<0.05 as compared with normal control. **p<0.01 as compared with diabetic control. TC=Total cholesterol; TG= Triglycerides; HDL= High density lipoprotein; LDL= Low density lipoprotein



TC, TG, HDL and LDL in mmol/lit. HDL/TC in %

NC = Normal control mice; DC = Diabetic control mice; DT150 = Diabetic mice receiving 150 mg/kg weight of *A. squamosa* methanol extract; DT250 = Diabetic mice receiving 250 mg/kg weight of *A. squamosa* methanol extract; DTRGZ = Diabetic mice receiving rosiglitazone 2 mg/kg.

III. Results and Discussion

From the results it is evident that the diabetic mice had higher total cholesterol (TC) (+137%; $p<0.001$) and TGs (+72%; $p<0.001$) values in comparison to normal control (Table-1 and Fig-1).These changes in biochemical parameters are as expected, as when the uncontrolled diabetic status progresses, substantial changes in total cholesterol and triglycerides values are predictable. Diabetic mice treated with lower dose of *A. squamosa* extract (DT_{150}) showed significantly lower values of serum TC (-26.5%; $p<0.001$) and TGs (-55.6%; $p<0.001$), when compared with the DC counterparts. The DT_{250} treatment showed superior lowering effects compared with the DC counterparts as well as DT_{150} group mice by (-34.7%; $p<0.001$) on serum TC levels and (-50%; $p<0.001$) on TGs levels. Contrarily, treatment with rosiglitazone (DT_{RGZ}) showed (-33.2%; $p<0.001$) on TC levels and (-09.6%; $p<0.001$) on TGs levels compared with diabetic control mice (Table-1 and Fig-1)

Relative to normal control, the diabetic mice had higher value of low density lipoprotein (LDL) (+256%; $p<0.001$) while diminished value of high density lipoprotein (HDL) (-54%; $p<0.001$).This is because when the

unrestrained diabetic condition advances, considerable changes in these biochemical parameters are as expected and predictable. Diabetic mice treated with lower dose of *A. squamosa* extract (DT_{150}) showed significantly lower values of serum LDL (-51%; $p<0.001$) and higher value of HDL (-48.4%; $p<0.001$), when compared with the DC counterparts. All over again, the DT_{250} treatment showed even better lowering effects on LDL (-54%; $p<0.001$) compared with the DC counterparts and improved level of HDL (+53%; $p<0.001$). In contrast, treatment with rosiglitazone (DT_{RGZ}) showed a considerable diminished level of LDL (-57%; $p<0.001$) while improved level of HDL (+55%; $p<0.001$) compared with diabetic control mice.

Chronic oral administration of the extract also reduced total cholesterol and triglyceride levels in diabetic and normoglycaemic albino mice consistent with the hypolipidemic effect earlier reported [9] (Khanna *et al*., 2002). Diabetic dyslipidemia is marked by elevated triglycerides, cholesterol and low density lipoprotein (LDL) particles of altered composition and decreased high density lipoprotein (HDL), and constitutes an important cardiovascular risk factor in diabetics [10] (Agrawal *et al*., 2006). Reduction in total cholesterol and triglycerides through dietary or drug therapy has been found beneficial in preventing diabetic complications as well as improving lipid metabolism in diabetic patients [11, 12] (Brown et al., 1993; Ahmed et al., 2001). Experimentally, streptozotocin-induced diabetic hyperglycemia is accompanied by increase in serum cholesterol and triglyceride levels [12] (Ahmed *et al*., 2001) and mimics overt diabetes disease. Thus, in addition to glycaemic control, extract of this plant may further reduce mortality from complications of the disease by ameliorating diabetes induced dyslipidemia.

Sanjay Kumar Sharma *et al*., (2018) [13] investigated that after administration of *A. squamosa* aqueous extract, STZ induced diabetic mice showed significant decrease in Fasting Blood Glucose (FBG) which was almost similar to rosaglitazone. They clearly demonstrated that *A. squamosa* extract in the dose of 150 mg/kg body weight/day and 250 mg/kg body body weight/day caused a significant reduction of FBG and a significant improvement in oral glucose tolerance in STZ induced type 2 diabetic mice. The extract caused decrease in the FBG in STZ induced diabetic mice up to 8.26 mmol/L on 250 mg/kg body weight dose in post treatment which was almost near approach to Rosiglitazone dose of 2 mg/kg. Decrease in FBG after treatment with extract indicates the effectiveness of active phytochemicals to resume normal functional status of pancreas. Glucose lowering effect of *A. squamosa* extract might be due to stimulation of surviving β-cells of Islets of Langerhans leading to the increase in secretion of insulin. The extract contains phenolic compounds that acted on ATP sensitive K+ channel and regulated blood glucose level [14] (Pandey *et al*., 2009).

The hypolipidemic efficacy of Annona squamosa extract may be due to activation of lipoprotein lipase (LPL) and stimulation of β-cells to secrete sufficient insulin to clear triglycerides from plasma. Therefore, alkaloids and phenolic compounds show high hypoglycemic and hypolipidemic potential for the treatment of Type-2D. Alkaloids from annona extract has also been reported to have significant antidiabetic activity and acts by stimulation of insulin production from pancreas, extrapancreatic action and enhancement of glycolytic enzymes. Sellamuthu *et al*., 2009, Saxena *et al*., (1993) and Basnet *et al*., (1994) [15, 16 and 17] reported that Annona extract contain alkaloids and phenolic compounds which are strong hypoglycemic in nature. In addition, Annona marin from swertia extract has also been reported as a potent lipid lowering agent comparable to the clinical drug atorvastatin which may also contribute to its cardioprotective ant anti-atherosclrerotic role (Vaidya *et al*., 2009; Woo *et al*., 2008) [18, 19].

From the results it can be concluded that the methanol extract of leaves of *Annona squamosa* is antidiabetic as well as hypolipidemic in nature due to the presence of different types of active phytochemicals, which may have different mechanism of action. The combination of these phytochemicals, therefore, might be beneficial as hypoglycemic and lipid lowering agents. The *Annona squamosa* extract might be considered as a safe supplementary therapy for long- term and effective management of diabetic patients.

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