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Efficacy of *Annona squamosa* (L.) extracts in controlling Diabetes Mellitus in Mice diabetic models

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Abstract: *Diabetes mellitus (DM) is a metabolic disorder of multiple etiologies characterized by absolute or relative deficiency of insulin secretion with or without varying degree of insulin resistance. Sedentary life style and obesity are two major epidemiological determinants of diabetes mellitus. The results clearly indicated that the aqueous extract of leaves of Annona squamosa presented significantly lowered body weight (P<0.001) when compared with the normal control (NC) mice in diabetic mice (DC). A significant body weight gain was observed in the treated groups of diabetic mice (DT150 and DT250) as compared to the DC ones. The DT150 and DT250 group showed an increase of 18% and 25% in body weight respectively after 15 days of treatment. Contrary to this DTRGZ group of mice showed an increase of 30% body weight after 15 days of treatment.*
The DC mice showed significantly (P<0.001) higher level of glucose (+279%) when compared with their normal control counterparts. The diabetic mice of both DT150 and DT250 groups showed a reduction in plasma glucose levels when compared to the DC ones; nevertheless, the reduction was particularly evident in the DT250 mice (-44%; P<0.001). When compared, the glucose levels of the DT250 versus the DC group mice during the four week treatment program, a significant lower value in the first was also found (-45%; P<0.001). Nevertheless, this drop in the glucose levels was more evident in the DT150 mice (-38%) than in the DT250 mice. In contrast to this, DTRGZ group of mice showed almost 67% drop in glucose level after four weeks of treatment program.
It is evident that the FINS and HOMA-IR of the diabetic control were much higher than those of the normal control group (P<0.01). When the mice had been administered with vehicle or experimental compounds respectively for 28 days, the FINS levels of A. squamosa extract at 150 mg/kg body weight/day and 250 mg/kg body weight/day doses and rosiglitazone groups were significantly lower as compared to diabetic control (P<0.01), as were the FBG levels (P<0.01). The aqueous leaf extract of A. squamosa at 150 mg/kg body weight/day and 250 mg/kg body weight/day caused lowering of HOMA-IR in treated mice groups as compared to diabetic control (P<0.01). The results clearly indicated that the aqueous extract of Annona squamosa is antidiabetic in nature due to the presence of different types of active phytochemicals.
Key words*: Diabetes mellitus, Annona squamosa, Streptozotocin, Albino mice*

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I. Introduction

Diabetes mellitus (DM) is the third leading disease, after heart attack and cancer affecting almost every organ in the human body [1] is also called silent killer. This is a metabolic disorder of multiple etiologies [2] (Mohler et al., 2009) characterized by absolute or relative deficiency of insulin secretion with or without varying degree of insulin resistance [3, 1].

Diabetes mellitus is characterized by recurrent or persistent hyperoglycemia with an elevated fasting (>110mg/dL of blood) and post prandial (> 130mg/dL of blood) plasma glucose level. According to WHO (2006) [4] diagnosis a fasting plasma sugar of >126mg/dL and post prandial plasma sugar value of > 200mg/dL is considered as diabetes mellitus. There are two major forms of diabetes mellitus namely Type-1, characterized by diminished production of insulin due to degeneration of pancreatic B- cells, and Type-2, the multifactorial syndrome characterized by either hypo secretion of insulin or insulin insensitivity or sometimes both. Sedentary life style and obesity are two major epidemiological determinants of diabetes mellitus. The current therapy of this disorder includes exogenous insulin administration (particularly in case of Type-1 diabetes mellitus), and oral hypoglycemic agents (for Type-2DM) which includes Metformin, Pioglitazone, Sulphonylurea etc. which may have adverse effects in diabetic subjects. Multiple risk factors for diabetes have been identified (WHO, 2006) (4). The greatest risk is impaired glucose tolerance, a precursor of diabetes. Thus, a number of type 2

diabetes prevention trials have included subjects with impaired glucose tolerance. These trials compared intensive lifestyle modifications (e.g., diet, exercise and weight loss), OHAs and placebo controls [5, 6]. Ayurvedic treatment known as *Apatarpana* (balanced diet with restricted calories) and *Santarpana* (highly nutritious, high-calorie diet intended to increase weight) are recommended for patients with type 2 and type 1 diabetes, respectively [7].

Diabetes mellitus has been classified into some other specific types:

1. **Maturity- onset diabetes of the young (MODY):** This subgroup is a relatively rare monogenic disorder characterized by non- insulin- dependent diabetes with autosomal dominant inheritance and an age at onset of 25 years or younger. Patients are nonobese, and their hyperglycemia is due to impaired glucose-induced secretion of insulin.
2. **Diabetes due to mutant insulin:** This is a very rare subtype of nonobese Type- 2 diabetes. Since affected individuals were heterozygous and possessed one normal insulin gene, diabetes was mild, and showed autosomal dominant genetic transmission.
3. **Diabetes due to mutant insulin receptors:** In more than 40 people with diabetes, defects in one of their insulin receptor gene have been observed.
4. **Diabetes mellitus associated with a mutation of mitochondrial DNA:** Diabetes due to a mutation of mitochondrial DNA that impairs the transfer of leucine or lysine into mitochondrial proteins has been described. Most patients have a mild form of diabetes that responds to oral hypoglycemic agents. Two- thirds of patients with this subtype of diabetes have a hearing loss, and a smaller proportion had a syndrome of myopathy, encephalopathy, lactic acidosis, and stroke- like episodes (MELAS).
5. **Obese Type- 2 patients**: The most common form of diabetes is secondary to extra pancreatic factors that produce insensitivity to endogenous insulin. When an associated defect of insulin production prevents adequate compensation for this insulin resistance, nonketotic mild diabetes occurs. The primary problem is a "target organ" disorder resulting in ineffective insulin action that can secondarily influence pancreatic B cell function.

II. Chronic Complications of Diabetes

Diabetes mellitus is associated with late clinical manifestations that include a number of pathologic changes that involve small and large blood vessels, cranial and peripheral nerves, and the lenses of eye. These lesions lead to hypertension, renal failure (nephropathy), blindness (retinopathy), autonomic and peripheral neuropathy, amputations of the lower extremities, myocardial infarction, and cerebrovascular accidents.

The Clinical Practice Guidelines for the Prevention and Management of Diabetes recommends a target glycosylated hemoglobin (HbA_{1c}) concentration of 7.0% or less for all patients with diabetes and, for those in whom it can be safely achieved, a target HbA_{1c} concentration in the normal range, usually $\leq 6.0\%$ (WHO, 2006) [4]. Although nonpharmacologic therapy (e.g., diet, exercise and weight loss) remains a critical component in the treatment of diabetes, pharmacologic therapy is often necessary to achieve optimal glycemic control. Orally administered antihyperglycemic agents (OHAs) can be used either alone or in combination with other OHAs or insulin. Various classes of OHAs are now available that target the different pathophysiologic factors contributing to diabetes: α-glucosidase inhibitors to delay intestinal carbohydrate absorption [8, 9, 10,] Lebovitz, 1997; Inzucchi, 2002; Bayraktar et al., 1996), biguanides to target hepatic insulin resistance [11, 12, 13, 14, 15] (Bailey and Turner, 1996; Kirpichnikov et al., 2002; Zhou et al., 2001; Holmes et al., 1999; Salpeter et al., 2004), insulin secretagogues to increase pancreatic insulin secretion [16, 17, 18, 19, 20] (Klepzig et al., 1999; Lebovitz, 2001; Strom et al., 2003; Hatorpe, 2002; McLeod, 2004), insulin sensitizers or thiazolidinediones which function as ligands for the peroxisome proliferator-activated receptor gamma (PPARγ) to target adipocyte and muscle insulin resistance [21, 22, 23, 24, 25, 26, 27, 28] (Lister et al., 1999; Finegood et al., 2001; Bell, 2003; Bakris et al., 2003; Herz et al., 2003; Nesto et al., 2003; Kelly et al., 1999; Lee et al., 2003), and intestinal lipase inhibitor or orlistat to inhibit fat absorption and promote weight loss in obese patients [29, 30, 31, 32] (Guerciolini, 1997; Hollander et al., 1998; Hanefeld and Sachse, 2002; Kelley et al., 2002).

Despite excellent potencies, these synthetic antidiabetic drugs had presented unwanted therapeutic profiles, marked by fluid retention, hypoglycemia at higher doses, liver problems, lactic acidosis, weight gain and potential cardiac hypertrophy. There is also evidence that hyperglycaemia per se has deleterious effects on beta cell function and insulin action (glucotoxicity). Thus, a concerted effort to search more effective drugs for T2DM has become the need of the time in terms of efficacy as well as safety due to the undesirable side effects of synthetic drugs.

Over the past 25 years, 50% of prescription drugs have been developed from natural products and their derivatives. These medicines have emerged as unique, safe, effective, and relatively inexpensive remedies producing minimal or no side effects with tall claims of efficacy as add on therapy [33]. Herbal drugs with antidiabetic activity can be classified into four categories according to their mode of action The first group has

insulinomimetic effect and includes plant like *Momordica charantia* (bitter gourd) [34]. Second group acts on the β-cells to increase the production of insulin and include plants like *Allium cepa* (onion) and *Pterocarpus marsupium* (Vijaysaar) [35]. The third one enhances glucose utilization in diabetic patients and includes plants like *Gingiber officinale* (ginger), *Cyamospsis tetragonalobus* (Gower plant) and *Grewia asiatica* (phalsa). They increase the viscosity of gastrointestinal contents, slow gastric emptying and act as a barrier to diffusion [35]. Fourth group act by miscellaneous mechanisms and include plants like *Euphorbia prostrata*, *Fumaria parvia*, *Panax ginseng* and *Phyllanthus embelica*. They may alter the fiber content and thereby altering the rate and speed of absorption of glucose from the gut [35]. The isolation and formulation of active constituents from these plants along with their pharmacological and toxicological evaluation are the need of the modern therapeutics.

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

Type- 2 diabetes is the commonest form of diabetes constituting 90% of the diabetic population. The global prevalence of diabetes is estimated to increase to 5.4% by the year2025. [39] The World Health Organization has predicted that the major burden will occur in the developing countries. The countries with the largest number of diabetic people are, and will be India, China and United States in 2025. .Epidemiological studies among migrant Asian Indians showed higher prevalence of Type- 2 diabetes compared with the host populations and other migrant ethnic groups. Studies conducted in India have highlighted that not only is the prevalence of Type- 2 diabetes high, but also that it is increasing rapidly in the urban population [36,37, 38] (Mohan et al., 2001; Raman Kutty et al., 2000; Iyer et al., 2000). The burden of Type- 2 diabetes and its complications related to Indian scenario has been illustrated by Ramachandran et al., 2002 [39].

The genetics of Type- 2 diabetes has been reviewed by Torben Hansen, 2002 [40]. Beck- Nielson and Groop (1994) [41] have suggested that the diabetic phenotype is the result of interaction of both genetic component and an important non- genetic component.

The coexistence of obesity, glucose intolerance, dyslipidemia, and hypertension, is termed as insulin resistance syndrome (IRS). Gerald Reaven (1988) [42] initially proposed that resistance to insulin- mediated glucose disposal is the pathophysiological interface for several complex metabolic alterations and disease. Insulin resistance syndrome in Asian Indians has been reviewed by Anoop Misra and Naval k. Bikram (2002) [43].

Regarding chromosomal abnormalities deletion syndrome due to chromosome 22q11.2 has been investigated by Elder et al., 2001[44].

Transient neonatal diabetes mellitus (TNDM) is a rare condition which presents with intrauterine growth retardation, dehydration, and failure to thrive. The condition spontaneously resolves before 1 year of age but predisposes patients to Type- 2 diabetes later in life. Rebecca J Gardner et al., (1999) [45] have previously shown that, in some cases, TNDM is associated with paternal uniparental disomy (UPD) of chromosome 6 and suggested that an imprinted gene responsible for TNDM lies within a region of chromosome 6q. By analyzing three families, two with duplications (family A and patient C) and one with several affected subjects with normal karyotypes (family B), Elder et al., (2001) [44] have further defined the TNDM critical region. In patient A, polymorphic microsatellite repeat analysis identified a duplicated region of chromosome 6, flanked by markers D6S472 and D6S311. This region was identified on the Sanger Centre's chromosome 6 radiation hybrid map (http://www.sanger.ac.uk/HGP/Chr6) and spanned approximately 60 cR3000. Using markers within the region, 418 unique P1 derived artificial chromosomes (PACs) have been isolated and used to localize the distal breakpoints of the two duplications. Linkage analysis of the familial case with a normal karyotype identified a recombination within the critical region. This recombination has been identified on the radiation hybrid map and defines the proximal end of the region of interest. They therefore propose that an imprinted gene for TNDM lies within an 18.72 cR3000 (~5.4 Mb) interval on chromosome 6q24.1-q24.3 between markers D6S1699 and D6S1010.

Vladimir K. Bakalov et al., (2009) [46] have investigated that Turner syndrome (TS) is caused by the absence or fragmentation of the second sex chromosome, which is associated with increased risk of diabetes mellitus (DM), but the specific phenotype and genetic etiology of this trait are unknown. Cytogenetic factors related with diabetes have been largely reviewed by [47, 48, 49, 50, 51, 52, 53] Bakalov et al., 2004: Salgin et al., 2006; Song et al., 2008; Neve et al., 2005; Snee et al., 2006; Zhao et al., 2005; Mokhtari et al., 2008 etc. Suheir Assady (2009) [54] has suggested the stem cell based therapy of diabetes mellitus.

Despite excellent potencies, these synthetic antidiabetic drugs had presented unwanted therapeutic profiles ,marked by fluid retention, hypoglycemia at higher doses, liver problems, lactic acidosis, weight gain and potential cardiac hypertrophy. There is also evidence that hyperglycaemia per se has deleterious effects on beta cell function and insulin action (glucotoxicity). Thus, a concerted effort to search more effective drugs for T2DM has become the need of the time in terms of efficacy as well as safety due to the undesirable side effects of synthetic drugs.

Pallab Das Gupta and Amartya De (2012) [55] have compiled some herbal plants and their active ingredients which play an important role in the management of diabetes mellitus. The antidiabetic properties of Bitter melon (*Momordica chantrantia*), Fiery costus (*Costus igneus*), Dendelium (*Taraxacum officinale*), French Lilac (*Golega officinalis*), Termeric (*Cutcuma longa*), Bael (*Aegle marmelus*), Amla (*Emblica officinalis*), Fenugreek (*Trigonella foenum- gracum*), Ginseng (*Panax ginseng*), Nayantara (*Catharanthus roseus*), Neem (*Azadirachta indica*), Cinnamon (*Cinnamomum zeylanicum*), black berry (*Rubus fruticosus*) etc. have been greatly illustrated [56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68] (Shukla et al., 2000; Wadkar et al., 2008; Rakesh et al., 2009; Zhizun Song et al., 1997; Mostak Ahmad et al., 2009; M Upendra Rao et al., 2010; G B KattiShanker et al., 2011; Kumar et al., 2012; Manisha Modak et al., 2007; Atul Kumar et al., 2014; Santwana Rani and Baidyanath Kumar, 2015; Shilpi Jaya and Baidyanath Kumar, 2017; Pratima Kumari et al,. 2018 etc.

Annona squamosa is traditionally used for the treatment of epilepsy, dysentery, cardiac problems, worm infestation, constipation, hemorrhage, bacterial infection, dysurea, fever, thirst and ulcer [69] (Raj et al., 2009). The flavonoid of *A. squamosa* possesses anti-oxidative [70] (Saija et al., 1995). Gupta et al., (2005) [71] 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 aqueous extract of leaves of *A. squamosa* in controlling diabetes mellitus in streptozotocin induced mice diabetic models.

III. Materials and Methods

Aqueous extract of *Annona squamosa* was used for assaying hypoglycemic activities in Streptozotocin induced mice diabetic models. The leafy twigs of *Annona squamosa* was collected from Sanjay Gandhi Biological Park, Patna (Bihar) and identified and botanically authenticated to the relevant monographs of Indian Pharmacopoeia (2012; 2014) [72, 73].

Freshly harvested leaves were washed under running tap water, blotted with filter paper and was dried in the shade at room temperature. The dried plant sample weighted 940 gm after grinding was kept in the separate peculator (made of glass) and filled up with about 3 liters commercial alcohol (95% ethanol and 5% water) and left it for 24 hours. After 24 hours the whole dissolved solution (phytochemicals of *Annona squamosa*) is drained out in a 500 ml of conical flask. About 50 ml of dissolved solution was then taken in 300 ml round bottle flask and evaporated under reduced pressure and low temperature (60^0C) in Rotavapour (popular in India). In rotavapour, the commercial alcohol was vaporized and the extract remained in the round bottle flask was collected and kept in a plastic jar. The commercial alcohol was again filled up in percolator and left it for 24 hours. The process was repeated four times for complete extraction of leaf extract.

Significant insights into the etiology of diabetes in human have been gained from the study of animal models. The albino mouse is an excellent model for study of human diabetes. Therefore all mice used in this study were in the albino genetic background. Adult albino mice weighing around 17–20 gram with 6.5 ± 0.5 cm length are selected for experiments. The mice were housed in shoe-box type cages under good hygienic conditions in the departmental animal house during experimental period. 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).

The mice were 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).

In each cage one pellet of feed per mice was given. The diet was palatable to the animal as evidenced by feeding success. It has been observed that an adult mice normally intakes 4 to 5 gram of diet per day. The daily food consumption of the mice varied depending upon the physiological and health status of the mice as well as the environmental temperature. The consumption of food increased considerably when the mice were pregnant or at lactating stage and decreased considerably with the dose-duration and increased temperature in summer.

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. Data were statically analyzed by mean ± S.E and by one-way ANOVA.

The results related to body weight change and blood glucose level in mmol/l in mice during present course of investigation have been presented in Table-1, 2 and 3; Fig- 1, 2 and 3.

Table- 1: Body weight changes in Mice in gm before and after treatment with aqueous extract of A. squamosa

Mice group		Day 0	Day 7	Day 15
Normal (non-diabetic)	Normal control (NC)	17.90 ± 2.74	19.84 ± 2.45	22.78 ± 2.21
Diabetic	Diabetic Control (DC)	11.72 ± 1.04	9.50 ± 0.85	9.31 ± 1.31
	A. squamosa extract (150 mg/kg) (DT_{150})	11.71 ± 2.02*	12.08 ± 1.65*	13.78 ± 1.51*
	A. squamosa extract (250 mg/kg) (DT_{250})	10.88 ± 1.61*	13.72 ±1.27*	14.74 ±2.265*
	Rosiglitazone (2 mg/kg) (DT_{RGZ})	10.60 ± 2.76*	13.82 ± 2.89*	15.02 ± 1.04*

Values expressed as Mean ± SE, n = 6 in each group; * Significant as compared to control

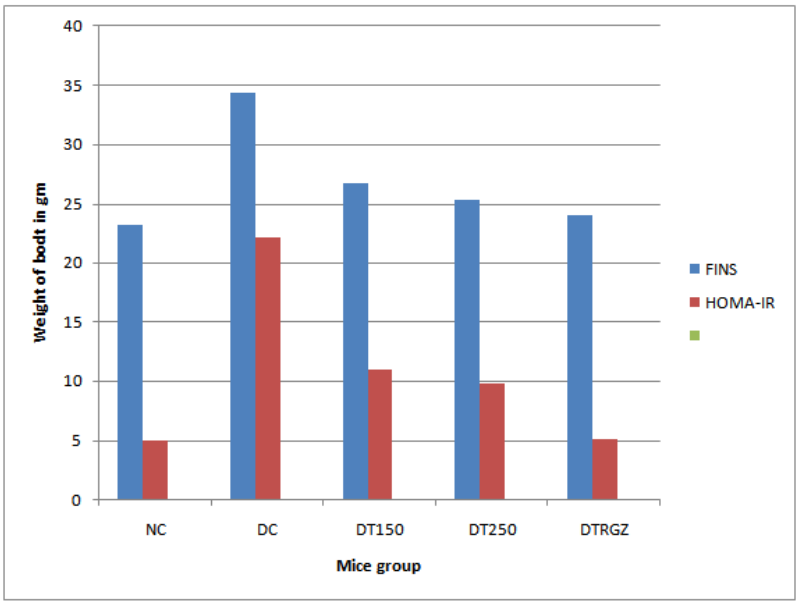

Fig-1: Body weight changes in Mice in gm before and after treatment with aqueous extract of A. squamosa

Table-2: Showing effects of different doses of A. squamosa extract and rosiglitazone on blood glucose levels in mice

Mice Groups	Blood glucose levels in (mmol/l) in four different weeks				
	Pretreatment	Post-treatment			
	0	1	2	3	4
Normal control (NC)	3.98±0.11**	4.07±0.18**	4.06±0.21**	4.05±0.14**	3.99±0.16**
Diabetic control (DC)	14.94±1.53*	14.91±1.43*	14.78±1.54*	14.99±1.47*	14.94±1.49*
A. squamosa extract (150mg/Kg) DT_{150}	14.90±1.48	13.09±1.18*	10.65±2.01**	9.68±1.24**	9.24±1.78**
A. squamosa extract (250mg/Kg) DT_{250}	14.60±1.50	11.83±1.30**	9.68±1.23**	8.26±1.73**	8.17±1.21**
Rosiglitazone (2mg/Kg) DT_{RGZ}	15.01±1.44	9.88±1.42**	5.56±1.24**	4.95±1.31**	4.96±0.94**

**p<0.05 as compared with normal control. **p<0.001 as compared with diabetic control.*

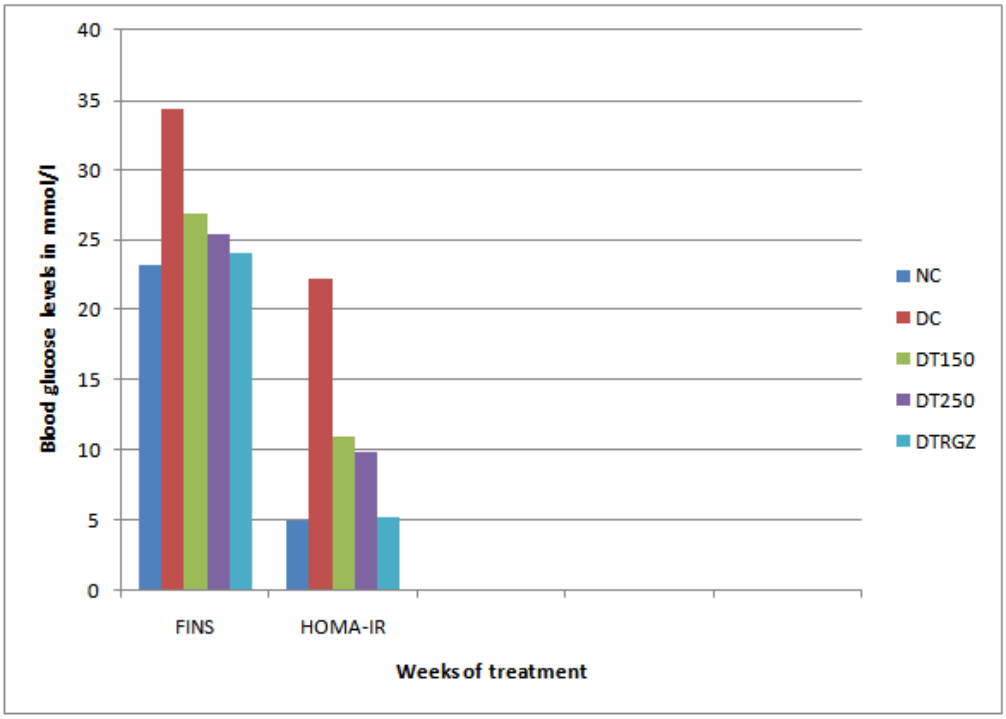

Fig- 2: Effects of different doses of *Annona squamosa* extract and rosiglitazone on blood glucose levels in mice

Table- 3: Effect of different doses of A. squamosa extract on FINS and HOMA-IR (mean ± S D)

Mice groups	FINS (mIU/L)	HOMA-IR
Normal Control (NC)	23.21 ± 2.11**	4.93 ± 1.11**
Diabetic control (DC)	34.46 ± 3.15*	22.21 ± 2.69*
A. squamosa extract (150 mg/kg) (DT 150)	26.84 ± 1.74**	10.96 ± 1.84**
A. squamosa extract (250 mg/kg) (DT 250)	25.38 ± 1.58**	9.82 ± 1.66**
Rosiglitazone (2 mg/kg) (RGZ)	24.04 ± 2.41**	5.11 ± 1.21**

P<0.05 as compared with normal control
****P<0.01 as compared with diabetic control***
FINS = Fasting insulin
HOMA-IR = Homeostasis model index of insulin resistance;

$$\text{HOMA-IR} = \frac{\text{Insulin (in mU/l) X Glucose (in mmol/l)}}{22.5}$$

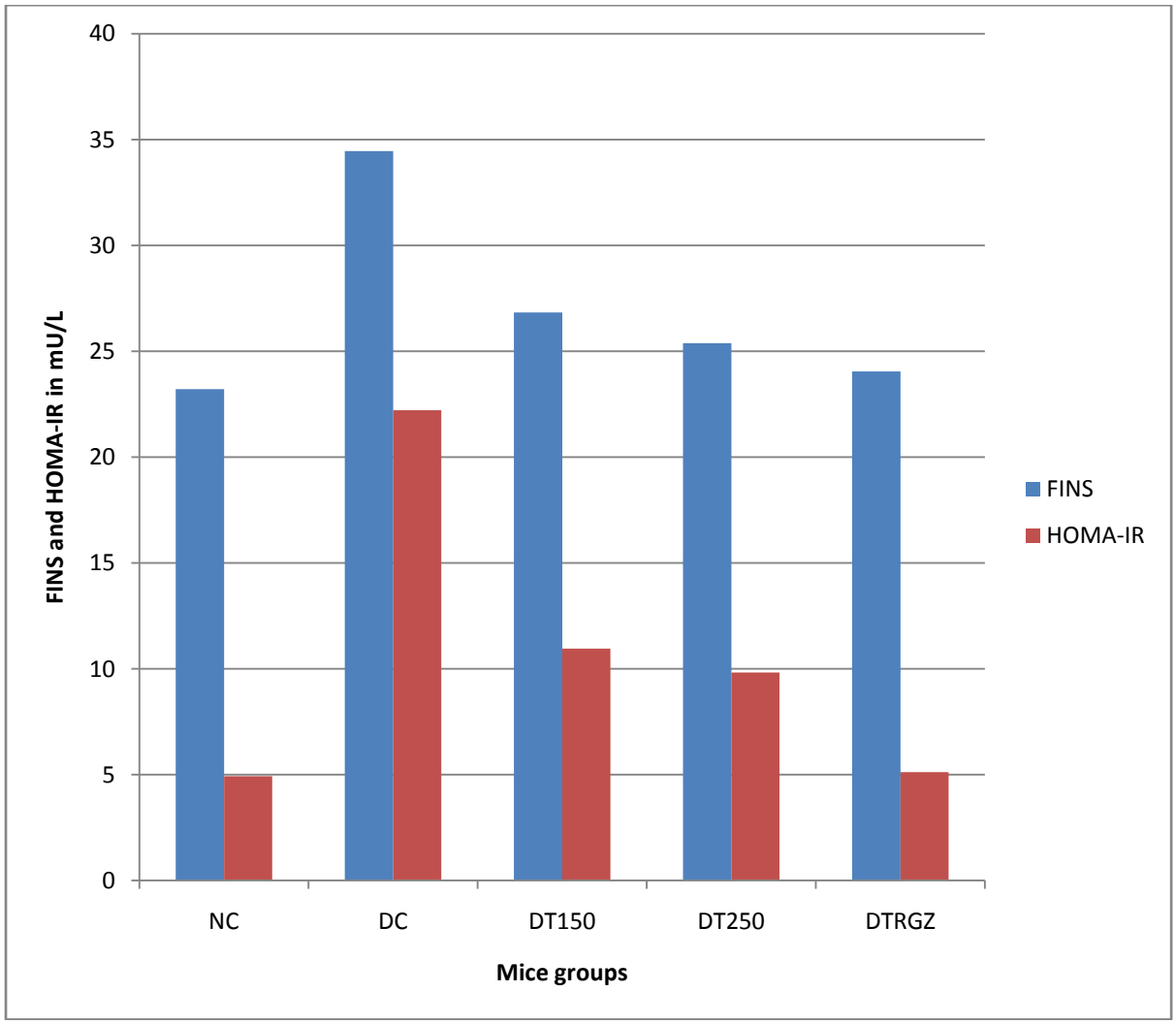

Fig-3: Effect of different doses of A. squamosa extract on FINS and HOMA-IR

IV. Results

The aqueous leaf extract of *Annona squamosa* has been reported to be effective in the alleviation of diabetes through its insulin-potentiating activities [74] (Okoli et al., 2010). From the results (Table- 1; Fig-1) it is evident that the diabetic control mice (DC) presented significantly lowered body weight ($P<0.001$) when compared with the normal control (NC) mice. A significant body weight gain was observed in the treated groups of diabetic mice (DT150 and DT250) as compared to the DC ones. The DT150 and DT250 group showed an increase of 18% and 25% in body weight respectively after 15 days of treatment. Contrary to this DTRGZ group of mice showed an increase of 30% body weight after 15 days of treatment (Table-1; Fig-1).

The changes in the blood glucose levels before and after receiving treatment in normal and diabetic mice are presented in Table-2 and Fig-2. The DC mice showed significantly (*$P<0.001$*) higher level of glucose (+279%) when compared with their normal control counterparts. The diabetic mice of both DT150 and DT250 groups showed a reduction in plasma glucose levels when compared to the DC ones; nevertheless, the reduction was particularly evident in the DT250 mice (-44%; $P<0.001$). When compared, the glucose levels of the DT250 versus the DC group mice during the four week treatment program, a significant lower value in the first was also found (-45%; $P<0.001$) (Table-2; Fig-2). Nevertheless, this drop in the glucose levels was more evident in the DT150 mice (-38%) than in the DT250 mice. In contrast to this, DTRGZ group of mice showed almost 67% drop in glucose level after four weeks of treatment program (Table-2; Fig-2).

Effect of different doses of *A. squamosa* extract on FINS and HOMA-IR is presented in Table-3 and Fig-3. From the results it is evident that the FINS and HOMA-IR of the diabetic control were much higher than those of the normal control group ($P<0.01$). When the mice had been administered with vehicle or experimental compounds respectively for 28 days, the FINS levels of *A. squamosa* extract at 150 mg/kg body weight/day and 250 mg/kg body weight/day doses and rosiglitazone groups were significantly lower as compared to diabetic control ($P<0.01$), as were the FBG levels ($P<0.01$). The aqueous leaf extract of *A. squamosa* at 150 mg/kg body

weight/day and 250 mg/kg body weight/day caused lowering of HOMA-IR in treated mice groups as compared to diabetic control ($P<0.01$) (Table-3;Fig-3).

V. Discussion

Diabetes has a significant impact on the health, quality of life and life expectancy of patients as well as healthcare expenditure. With increasing incidence and mortality from its complications, prompt and adequate glycemic control in diabetes is paramount if management can meaningfully improve the quality of life and increase life expectancy [1] (Nyenwe et al. 2011).

Several studies to test new drugs with potential antidiabetic activity were used in animal models of streptozotocin (STZ)-induced diabetes [75] (Fröde and Madeiros, 2008). Although none of the chemically-induced diabetic models can reproduce the complexity of the human disease, they can be helpful to understand at least some aspects of the potential bioactivities of natural or synthetic products. We used diabetic albino mice induced by STZ (35 mg/kg body wt.), which was sufficient to induce a stable state of diabetic condition in this animal species.

Induction of diabetes with streptozotocin is associated with a characteristic loss of body weight, which is probably due to muscle wasting. In our study there was a significant weight loss in the vehicle treated diabetic mice, where as treatment with the *A. squamosa* extract at two doses showed improvement in their body weight, indicating that the aqueous extract had beneficial effect in preventing loss of body weight of diabetic mice. The probable mechanism of this benefit is due to its effect in controlling muscle wasting, i.e., by reversal of antagonism. The metabolic disturbances were corrected after the plant extract was administered at the two different dose of 150 and 250 mg/kg body weight for four weeks as shown by a reduction in biochemical parameters in diabetic mice treated with plant extract. This result is in accordance with Lenzen S. (2008) [76] and Chung et al. (2003) [77]. They have found that multiple low dose of STZ sufficiently induce stabilized acute diabetes in which there is a progressive deterioration in the glucose tolerance and insulin secretion after the STZ injection. It ultimately causes increased oxidative stress, which play an important role in the pathogenesis of various complications.

At present, several drugs are available for the management of hyperglycemia but they are expensive and possess side effects also. Therefore, search for a suitable alternative is continued. For the developing countries herbal plants may be the most attractive target for their availability, low cost and better safety margin. The hypoglycemic activity of *Annona squamosa* has attracted many researchers to prove it scientifically and to investigate its mechanisms of actions. Hence, in the present study, the two defined doses of aqueous extract of leaves of *A. squamosa* have been investigated for their antidiabetic potential.

Chronic hyperglycaemia in diabetes is a risk factor constantly fuelled by postprandial elevation of blood glucose. Control of postprandial hyperglycemia in diabetes is of great importance due to its close relation to the risk of micro and macro-vascular complications and death [1] (Nyenwe et al. 2011). In this study, experimental evaluation of the hypoglycemic potentials of aqueous extract of *A. squamosa* has shown that the higher dose of extract (250 mg/kg body wt.) suppress postprandial rise in blood glucose levels more effectively than lower dose of extract (150 mg/kg body wt.) which is the index of effectual glycemic control. It may be due to alteration in the fiber content as well as phytochemical interactions which thereby altering the rate and speed of absorption of glucose from the gut. These favorable effects of *A. squamosa* leaf extract may be attributed to higher affinity and synergistic action of their phytochemicals on multiple targets including PPAR-γ activation and DPP-IV inhibition which may therefore regulate the hyperglycemia, lipogenesis and hypertriglyceridemia associated with diabetes [78, 79] (Shimizu *et al.*, 2003; Barnett, 2006).

In the hypoglycemic activity studies of methanol extract, daily oral administration of the extract for 28 days produced a gradual but sustained reduction in blood glucose levels in diabetic treated mice. Streptozotocin causes hyperglycaemia and glucose intolerance or syndromes similar to either type 1 or type 2 diabetes [76, 75] (Lenzen et al., 2008;Frode and Medeiros, 2008). Effective and sustained reduction in blood glucose levels of treated diabetic mice by the extract indicates that it may be useful in overt cases of diabetes. Treatment with the two doses of extract also reduced mortality of diabetic mice from hyperglycaemia and prolonged their survival. In this study, some of the diabetic non-treated control animals all died on day 10 post-induction of diabetes whereas the extract-treated group survived beyond the period of the experiment. Effective control of blood glucose level is a key step in preventing and reversing diabetic complications, and improving the quality of life of diabetic patients [80] (Bavarva and Narasimhacharya, 2008). Hence, chronic administration of the extract may cause a progressively sustained reduction in hyperglycaemia known to reduce the risk of complications associated with the disease.

After the administration of aqueous leaf extract of *A. squamosa*, at two doses there was a significant improvement in their body weight, indicating that the leaf aqueous extract had beneficial effect in preventing loss of body weight of diabetic mice (Table-1; Fig-1). This might be due to its positive effect in controlling

muscle wasting i.e., by reversal of antagonism and gluconeogenesis, by reviving and protecting β-cells and stimulating them to produce sufficient insulin.

The metabolic disturbances were corrected after the plant extract was administered at the dose of 150 mg/kg body weight for two weeks. In the present investigation, a significant and abrupt increase in Fasting Blood Glucose level of albino mice was noticed during the course of experiment in diabetic control mice (DC).The FBG reached up to 14.99 mmol/L on 4th week when subjected to STZ leading to Hyperglycemic condition. There was also increase in FINS and HOMA-IR. The present findings are in accordance with Lenzen (2008) [76] who found that multiple low dose of STZ induces stabilized acute diabetes in which there is a progressive deterioration in the glucose tolerance and insulin secretion after the STZ injection. It ultimately causes increased oxidative stress, which play an important role in the pathogenesis of various complications.

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. The result 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. This observation is in collaboration with the findings of Ramesh et al., 2002 who has earlier reported the hypoglycemic activity of methanolic extract of *A. squamosa* in albino mice. 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 [81] (Pandey et al., 2009).

In the present investigation a significant decrease ($P<0.01$) in Fasting insulin (FINS) and HOMA-IR was also recorded, thereby improving IR on treatment with *A. squamosa* leaf extract.

VI. Conclusion

From the results it can be concluded that the aqueous extract of leaves of *Annona squamosa* is antidiabetic 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 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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