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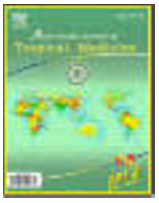

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Ameliorating effect of *Semecarpus anacardium* Linn. nut milk extract on altered glucose metabolism in high fat diet STZ induced type 2 diabetic rats

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ABSTRACT

Objective: To explore the protective effect of the drug *Semecarpus anacardium* (*S. anacardium*) on altered glucose metabolism in diabetic rats. **Methods:** Type 2 diabetes mellitus was induced by feeding rats with high fat diet followed by single intraperitoneal injection of streptozotocin (STZ) (35 mg/kg b.w.). Seven days after STZ induction, diabetic rats received nut milk extract of *S. anacardium* Linn. nut milk extract orally at a dosage of 200 mg/kg daily for 4 weeks. The effect of nut milk extract of *S. anacardium* on blood glucose, plasma insulin, glucose metabolising enzymes and GSK were studied. **Results:** Treatment with SA extract showed a significant reduction in blood glucose levels and increase in plasma insulin levels and also increase in HOMA - β and decrease in HOMA -IR. The drug significantly increased the activity of glycolytic enzymes and glucose-6-phosphate dehydrogenase activity and increased the glycogen content in liver of diabetic rats while reducing the activities of gluconeogenic enzymes. The drug also effectively ameliorated the alterations in GSK-3 mRNA expression. **Conclusions:** Overall, the present study demonstrates the possible mechanism of glucose regulation of *S. anacardium* suggestive of its therapeutic potential for the management of diabetes mellitus.

1. Introduction

Type 2 diabetes mellitus is one of the world's most common chronic metabolic disorders of multiple aetiologies and it is projected to increase to 438 million in 2030[1]. The early phenomenon of type 2 diabetes mellitus is insulin insensitivity, which not only has negative metabolic consequences but also contributes subsequent pancreas β-cell exhaustion, resulting in the onset of clinical hyperglycemia[2]. Thus, understanding the regulation of the insulin response and identifying the related mechanisms are important to early treatment and prevention of type 2 diabetes mellitus.

Lifestyle modifications are often recommended, but have been difficult to maintain over a long term. Benefits of pharmaceutical factors to treat the disease aggressively early have been recommended, but medications may have unwanted side effects. Thus, there has been a growing interest in herbal remedies that can be introduced into the general population with the least side effects and the maximal preventive outcome.

Semecarpus anacardium (S. anacardium) commonly known as bhallataka has been used in various pathologies in Indian systems of medicine found in outer Himalayas and hotter parts of central India[3]. Jeediflavanone, galluflavanone, nalluflavanone, semecarpetin, and anacardiflavanone have been identified as chief constituents in the drug[4]. HPLC and HPTLC analysis of the nut and milk extract were also carried out to confirm the presence of the above said compounds[5]. The drug is known to have anti diabetic and anti inflammatory property[6]. In experimental diabetes, enzymes of glucose metabolism are

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markedly altered and produce hyperglycemia, which leads to pathogenesis of diabetic complications. The present study was carried out test the efficacy of the drug *S. anacardium* in modulating the metabolic anomalies associated with type 2 diabetes mellitus through insulin signalling molecule.

2. Materials and methods

2.1. Animals and diet

Male Sprague dawley rats [(230±20) g] were used in this study. The animals were purchased from Central Animal House, Institute of Basic Medical Sciences, Chennai, India. Animal experimentation was conducted according to the current CPCSEA regulations (IAEC no. 02/081/07). The animals were maintained on a commercial rat feed manufactured by M/s. Pranav Agro Industries Ltd., India under the trade name "Amrut rat/mice feed".

2.2. Preparation of the drug

S. anacardium Linn. nut extract contains purified nuts of *S. anacardium* and cow's milk in the ratio as indicated in the Formulary of Siddha Medicine. 200 g of the nut was boiled with 500 mL of milk, which was repeated thrice. The decoction was stored at room temperature[7] and this was used for the study.

2.3. Drugs

Streptozotocin (STZ) was obtained from Sigma Chemicals, St. Louis, Mo., U.S.A. All other chemicals and solvents used were of analytical grade.

2.4. Induction of experimental diabetes

Diabetes was induced in rats by feeding them with a high fat diet consisting of 10% lard, 20% sucrose, 2.5% cholesterol, 1.0% cholate for 2 weeks[8]. After 2 weeks the animals were kept in an overnight fast and injected with STZ (35 mg/kg body weight in 0.1 M citrate buffer pH-4.5)[9]. Animals had free access to food and water after the STZ injection. Rats with blood glucose level $\geqslant$126 mg /dL at 0 min or $\geqslant$199.8 mg/dL at 120 min were considered to be diabetic and used for the study. Both STZ injected and non-injected animals continued on their original diet throughout the study. The authors declare that the experiments conducted during these studies comply with the current laws of their country.

2.5. Experimental design

The animals were randomly divided into five groups of six animals each. Group Ⅰ served as a control receiving olive oil as vehicle. Group Ⅱ diabetic rats served as diabetic control rats. Group Ⅲ diabetic rats were treated with *S. anacardium* at a dosage of 200 mg/kg.b.wt for four weeks, Group Ⅳ diabetic rats were treated with the standard drug metformin at a dosage of 500 mg/kg.b.wt for four weeks[10], Group Ⅴ control rats were given *S. anacardium* alone at different dosage of 200 mg/kg.b.wt for four weeks and they served as drug control group.

2.6. Biochemical analysis

After 30 days of treatment, the animals were sacrificed under chloroform anesthesia. The blood was collected and serum was separated and used for biochemical estimations. Liver and skeletal muscle tissues were quickly excised off, a portion of liver and skeletal muscle tissues were washed with saline and their homogenates were prepared using 0.1 M phosphate buffer, pH 7.4. The homogenates were centrifuged and the supernatant was used for the study. The levels of blood glucose were done according to Trinder[11], HOMA-IR and HOMA-β by the method of Mathews *et al*[12]. Insulin was assayed by the solid phase enzyme linked immunosorbent assay.

Hexokinase was assayed by the method of Banstrup *et al*[13]. Phosphogluco-isomerase was assayed by the method of Horrocks *et al*[14]. Aldolase was estimated by the method of King[15]. Glucose-6-phosphatase was assayed according to the method of Gancedo and Gancedo[16]. Fructose1, 6-biphosphatase was assayed by the method of Gancedo and Gancedo[16].Glucose 6 phosphate dehydrogenase activity was assayed by the method of Beutler[17]. Glycogen phosphorylase activity was determined by the method of Shull *et al*[18]. Glycogen content in liver and muscle was estimated by the method of Morales *et al*[19].

2.7. Total RNA Isolation and RT-PCR

Total RNA was purified from freshly liver tissue using 1 mL of the TRI reagent by the method of Chomczynski and Sacchi[20]. The RNA purity and concentration were determined spectrophotometrically at A260/A280 nm. The purity of RNA obtained was 1.8-1.9. 1 g of total RNA were reverse transcribed by RT-PCR kit (INVITROGEN) according to the manufacturer's instructions and further amplified by PCR.

GSK-3-TGGTCCGAGGAGAGCCCAATGT/
TGCCTCTGGTGGAGTTCGGGG-35/55 ℃-320-NM032080.1
GAPDH- GCTCTCTGCTCCTCCCTGTTCTAGA/

ACAAACATGGGGGCATCAGCGG-35/55 ℃-461-NM_017008.3.

The PCR products were resolved by electrophoresis through a 2% agarose gel and stained with ethidium bromide. The densities of PCR products in the agarose gel were scanned with a Gel Doc image scanner (Bio-Rad, USA), and quantified by Quantity One Software (Bio-Rad, USA).

2.8. Statistical analysis

All the values were expressed as mean±SD of 6 rats from each group and statistically evaluated by one way analysis of variance followed by Duncan's Multiple Range Test for multiple comparisons. A value of $P<0.05$ was considered statistically significant.

3. Results

3.1. Effect of S. anacardium Linn. nut milk extract on blood glucose, insulin, HOMA -IR and HOMA β

Table 1 depicts the levels of blood glucose, plasma insulin, HOMA-IR and HOMA β of control and experimental groups of rats. The diabetic rats showed a significant ($P<0.05$) increase in the levels of glucose and alterations in HOMA -IR and HOMA β. *S. anacardium* treatment resulted in decrease in the blood glucose level when compared to diabetic rats. No changes were compared with drug alone treated groups. No significant alterations were observed in insulin level between the diabetic rats when compared to control rats. And also *S. anacardium* treatment resulted in the improvement in the aberrations in HOMA-IR and HOMA-β.

3.2. Effect of S. anacardium Linn. nut milk extract on glycogen metabolism

The changes in the level of glycogen content and alterations in the activity of glycogen phosphorylase in liver of control and experimental group of rats have been shown in Table 2. The level of glycogen content in liver tissue was reduced significantly ($P<0.05$) in diabetic control group of rats. Treatment with the drug *S. anacardium* resulted in significant increase in glycogen content.

3.3. Effect of S. anacardium Linn. nut milk extract on glycolytic enzymes and gluconeogenic enzymes

The alterations in the activities of glycolytic enzymes and gluconeogenic enzymes were depicted in Table 3 and 4, respectively. Significant decrease ($P<0.05$) in glycolytic enzymes and a significant increase ($P<0.05$) in the gluconeogenic enzymes were observed in diabetic rats when compared with control rats. Upon treatment with the drug *S. anacardium* and metformin the activities of these enzymes were restored.

3.4. Effect of S. anacardium Linn. nut milk extract on Gsk -3

Figure 1 shows the mRNA expression of GSK -3 in the liver of control and experimental group of animals. The expression of GSK -3 was increased in the diabetic group of rats. But upon treatment with the drug *S. anacardium* its expression was restored.

Table 1

Effect of *S. anacardium* nut milk extract on fasting glucose, insulin, HOMA-IR and HOMA-β of control and experimental animals.

Groups	Glucose	Insulin	HOMA-IR	HOMA-β
Group Ⅰ	103.00±4.20	23.46±0.95	4.52±0.32	461.69±32.45
Group Ⅱ	265.00±15.32 [a]	17.00±1.35 [a]	8.91±0.73[a]	347.12±24.67[a]
Group Ⅲ	125.00±11.46 [ab]	29.00±1.15[ab]	5.28±0.42[ab]	431.02±38.57 [ab]
Group Ⅳ	133.00±18.65 [ab]	18.00±1.65[ab]	6.73±0.45[ab]	130.80±11.57[ab]
Group Ⅴ	105.00±4.88	21.60±1.15	4.20±0.34	378.60±38.04

Values are expressed as mean ±SD for 6 animals.
a: Group Ⅰ *vs.* other groups $P<0.05$, b: group Ⅱ *vs.* Ⅲ, Ⅳ and Ⅴ $P<0.05$.

Table 2

Effect of *S. anacardium* nut milk extract on glycogen metabolism of control and experimental animals.

Groups	Glycogen phosphorylase	Glycogen synthase	Glycogen
Group Ⅰ	11.56 ± 1.04	45.62 ±3.12	79.08 ± 5.75
Group Ⅱ	25.88 ± 2.19 [a]	25.32 ± 1.80 [a]	40.19 ± 3.56 [a]
Group Ⅲ	11.87 ± 0.98 [ab]	40.00 ±3.10[ab]	75.57 ± 6.01 [ab]
Group Ⅳ	16.93 ± 1.27 [ab]	35.00 ±1.67[ab]	65.28 ± 4.71 [ab]
Group Ⅴ	11.62 ± 0.85	46.00 ±1.25	79.12 ± 6.24

Units: Glycogen phosphorylase- μ mol of inorganic phosphorous liberated/hr/mg protein; Glycogen synthase μ mol of uridine diphosphate liberated/hr/mg protein; Glycogen-mg glucose/g tissue.
Values are expressed as mean ±SD for 6 animals.
a: Group Ⅰ *vs.* other groups $P<0.05$, b: group Ⅱ *vs.* Ⅲ, Ⅳ and Ⅴ $P<0.05$.

Table 3
Effect of *S. anacardium* nut milk extract on glycolytic enzymes in liver of control and experimental animals.

Groups	Glycokinase	Phosphogluco isomerase isomerase	Aldolase
Group Ⅰ	18.87 ± 1.56	14.69 ± 1.20	13.80 ± 0.97
Group Ⅱ	10.05 ± 0.92[a]	8.21 ± 0.64 [a]	6.56 ± 0.42 [a]
Group Ⅲ	17.47 ± 1.23 [ab]	12.56 ± 1.04[ab]	11.43 ± 0.81 [ab]
Group Ⅳ	15.61 ± 1.09 [ab]	14.50 ± 0.96[ab]	13.67 ± 1.12 [ab]
Group Ⅴ	18.83 ± 1.64	14.74 ± 1.31	13.86 ± 1.10

Units: Hexokinase- nmol of glucose-6-phosphate liberated/min/mg protein, Phosphoglucoisomerase-nmol of fructose liberated/min/mg protein, Aldolase-nmol of glyceraldehyde liberated/min/mg protein.
Values are expressed as mean ±SD for 6 animals.
a: Group Ⅰ *vs*. other groups $P<0.05$, b: group Ⅱ *vs*. Ⅲ, Ⅳ and Ⅴ $P<0.05$.

Table 4
Effect of *S. anacardium* nut milk extract on gluconeogenic enzymes in liver of control and experimental animals.

Groups	Glucose 6 phosphatase	Fructose 1,6 bisphosphatase	Glucose 6 phosphate dehydrogenase
Group Ⅰ	13.01 ± 0.96	17.56 ± 1.32	5.45 ± 0.38
Group Ⅱ	7.71 ± 0.52[a]	8.89 ± 0.69 [a]	2.77 ± 0.16 [a]
Group Ⅲ	12.90 ± 0.90 [ab]	14.71 ± 1.14[ab]	5.26 ± 0.31 [ab]
Group Ⅳ	11.64 ± 1.01 [ab]	14.50 ± 0.96[ab]	4.08 ± 0.24 [ab]
Group Ⅴ	12.95 ± 1.21	17.60 ± 1.50	5.49 ± 0.18

Units: Glucose-6-phosphatase&Fructose1, 6-bisphosphatase-nmol of inorganic phosphorous liberated/min/mg protein, Glucose 6-phosphate dehydrogenase-Units/mg protein.
Values are expressed as mean ±SD for 6 animals.
a: Group Ⅰ *vs*. other groups $P<0.05$, b: group Ⅱ *vs*. Ⅲ, Ⅳ and Ⅴ $P<0.05$.

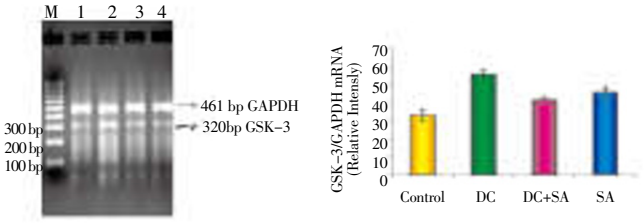

Figure 1. Effect of *S. anacardium* nut milk extract on gene expression of GSK-3 in liver control and experimental group of rats.
M: 100 bp marker; L1: Group Ⅰ (Control); L2: Group Ⅱ (DC: Diabetic control); L3: Group Ⅲ (DC+SA: Diabetes induced and SA: *S. anacardium* treated); L4: Group Ⅴ (Control and SA: *S. anacardium* alone).
a: Group Ⅰ *vs*. other groups $P<0.05$, b: group Ⅱ *vs*. Ⅲ, $P<0.05$.

4. Discussion

Type 2 diabetes mellitus is the most common form of the metabolic disorder which is caused by impaired insulin secretion paralleled by a progressive decline in β cell function and chronic insulin resistance[21]. Nowadays there has been a growing interest in herbal remedies that can be introduced into the general population with the least side effects and maximum preventive outcome[22]. Herbal medicines can only be effective as an alternative to oral hypoglycaemic agents, in type 2 diabetes where pancreatic islets are not totally destroyed. Hence, we used a type 2 diabetic rat model to study the anti diabetic effect of the drug.

Insulin resistance and β cell dysfunction are the important characteristics of type 2 diabetes mellitus. Therefore, we studied the effect of Semecarpus anacardium on blood glucose and insulin levels and also performed HOMA-IR and HOMA-β to check the degree of insulin resistance and beta cell function. The drug *S. anacardium* effectively reduced the blood glucose levels thereby establishing its hypoglycaemic effect[23]. A significant decrease in HOMA-IR and an increase in both insulin concentration and HOMA-β level by treatment with the drug *S. anacardium* suggests that improvement in the glucose homeostasis is plausibly due to an improvement in insulin action and β - cell function. Flavonoids present in the drug might have contributed to this effect.

The synthesis and degradation of glycogen in the liver and skeletal muscle tissues are the important mechanisms involved in the control of blood glucose homeostasis and alterations in glycogen metabolism have been observed in diabetic rats consistent with other studies[24]. The activities of the enzymes, glycogen synthase and glycogen phosphorylase were considerably altered in diabetic rats thereby indicating insulin resistance. Oral administration of *S. anacardium* to diabetic rats resulted in an increase in glycogen content in liver and also increased the activity of glycogen synthase enzyme while decreasing the activity of glycogen phosphorylase. There by indicating the possible effect of the drug on sensitizing these tissues for glucose uptake and storage.

In type 2 diabetes mellitus, hyperglycemia results from both lack of suppression of hepatic glucose production and also results from decreased glucose uptake by peripheral tissue skeletal muscle since it accounts for 80% of glucose disposal[25]. Hence enzymes that regulate glucose

metabolism are potential targets for controlling hepatic glucose balance and thereby blood glucose levels in type 2 diabetes. A significant decrease in the activities of glycolytic enzymes hexokinase, phosphoglucoisomerase and aldolase were observed in diabetic rats consistent with that of other studies[26]. The restoration of the activities of the glycolytic enzymes by the drug might be due to the presence of flavonoids and polyphenolic compounds in the drug, since several researchers have shown that polyphenols have the capacity to modulate carbohydrate metabolizing enzymes in liver and skeletal muscle by improving pancreatic β cell function as well as exerting insulinomimetic action[27].

In type 2 diabetes, activation of gluconeogenic enzymes is due to the state of insulin resistance the predominant cause of the elevated EGP. The activities of glucose 6 phosphatase and fructose 1, 6 bisphosphatase were significantly altered in liver of diabetic rats when compared with normal control rats. This is mainly due to insulin resistance resulting in the activation of the gluconeogenic enzymes contributing to hepatic glucose production, since under normal conditions, insulin functions as a suppressor of gluconeogenic enzymes[28]. Treatment of diabetic rats with the drug *S. anacardium* and metformin resulted in a decrease in the activities of gluconeogenic enzymes. *S. anacardium* might have brought this effect by increasing the insulin levels.

Decreased activity of glucose-6-phosphate dehydrogenase enzyme the rate-limiting step of the HMP shunt pathway was observed in type 2 diabetic rats in agreement with other studies[29-31]. The restoration in the activity of this enzyme upon treatment with the drug might be due to its anti oxidant effect and also its protective effect of the drug by increasing insulin levels. Diabetic rats treated with *S.anacardium* showed significantly increased liver glucose-6-phosphate dehydrogenase activity and this resulted in an increased production of the reducing agent, NAD, with concomitant decrease in oxidative stress.

Glycogen synthase kinase-3 (GSK-3) a serine/threonine kinase plays an important role in the regulation of glycogen synthesis and it suppresses the function of two key targets of insulin action namely, glycogen synthase and insulin receptor substrate-1and its activity is higher in diabetic tissues[32]. Oxidative stress is known to induce insulin resistance through reduced insulin-mediated suppression of the active form of GSK-3 in type 2 diabetic rats[33]. The inhibition of GSK-3 leads to an increase in glycogen synthesis, which promotes insulin sensitivity. Activation of GSK-3 leads to reduction in glycogen synthesis and decrease in insulin sensitivity. Hence, we studied the effect of the drug *S. anacardium* in modulating this insulin signalling molecule. Increased expression of GSK -3 seen in diabetic animals was decreased in *S. anacardium* treated animals. The decrease in the mRNA expression of GSK-3 in our study is correlated with an increase in glycogen content in both liver and skeletal muscle. This shows the activity of the drug in improving the insulin sensitivity. Flavonoids and plant derived products have been shown to inhibit the expression of GSK-3[34].

In recent times, attention has been shifted to herbal drugs as potential agents for treatment of type 2 diabetes. Identifying targets of action of these preparations is an essential aspect of therapeutic drug development in combating metabolic alterations and molecular lesions characteristic of insulin resistance and type 2 diabetes. The drug effectively restored the glycolytic and suppressed the gluconeogenic pathway thereby improving or restoring the carbohydrate metabolism. The present study effectively shows that high fat diet STZ induced type 2 diabetes is characterized by disordered carbohydrate metabolism and also impaired insulin signalling. *S. anacardium* is adequately competent to counteract these effects of diabetic manifestations. This is mainly due to the presence of flavonoids in the drug, since they are known to have beneficial effect on the carbohydrate metabolism by up regulating the expression of glycolytic enzymes and decreasing the expression of gluconeogenic enzymes. However, the future prospective use of *S. anacardium* in the treatment of type 2 diabetes warrants exhaustive experimental and clinical studies to determine the exact nature of active principle and its mechanism of action for development as an alternative therapeutic agent against type 2 diabetes mellitus s.

Conflict of interest

The authors declare that there are no conflicts of interest.

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