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Purified Methanolic Extract of *Salacia reticulata* Wight. Ameliorates Insulin Resistance and Metabolic Alterations in Rats Fed High Fructose Diet

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Abstract: The present study was carried-out to investigate the insulin sensitizing activity of purified methanolic extract of *salacia reticulata* roots in fructose-fed rats, a well-known model of insulin resistance and hyperinsulinemia. Feeding fructose enriched diet caused a hyperinsulinemic state and increased insulin resistance index with statistically significant increase in fasting triglyceride and very low density lipoprotein-cholesterol levels. Fructose fed rats also exhibited glucose intolerance when tested by oral glucose tolerance test as indicated by increase in total area under curve of blood glucose. Dietary supplementation of purified methanolic extract of *Salacia reticulata* (0.4 g/100 g of feed) significantly reversed the fructose diet-induced metabolic alterations in rats and also improved glucose intolerance. The effect of purified methanolic extract of *Salacia reticulata* was comparable to that of rosiglitazone, a known insulin sensitizer. Acarbose, a marketed α -glucosidase inhibitor failed to reverse the metabolic alterations caused by fructose enriched diet in rats. The finding proves probable mechanism of action of *Salacia reticulata* and further validates scientific basis of its usage in the management of various metabolic disorders including diabetes.

Key words: *Salacia reticulata*, insulin resistance, acarbose, glucose intolerance, rosiglitazone

INTRODUCTION

Type 2 diabetes is a polygenic metabolic disorder, which occurs because of the inability of body to use insulin effectively. It is also a disease characterized by impaired insulin stimulated glucose uptake in skeletal muscle and adipose tissue, increased hepatic glucose production and inadequate compensation of insulin secretion from pancreatic β -cells, ultimately leading to fasting hyperglycemia, hyperinsulinemia and a state of insulin resistance (Tiwari and Rao, 2002; Jarvis and Kahn, 2000). Insulin resistance is a state in which target cells fail to respond to ordinary levels of circulating insulin. At the molecular level, it occurs due to impaired insulin-signaling results from mutations or post-translation modifications of the insulin receptor itself or any of its downstream effector molecules (Keren *et al.*, 1997). It is a complex metabolic abnormality that affects the ability of peripheral tissues to respond to insulin, it is also a prominent feature of metabolic syndrome and constitutes a major risk factor for cardiovascular disease (Chen *et al.*, 2006). The antidiabetic agents require the characteristics of insulin sensitization in peripheral tissues and/or insulinotropic action from pancreatic β -cells. Additionally, an α -glucosidase inhibitory property is another feature attributed to hypoglycemic agents to decrease post-prandial hyperglycemia (Choi *et al.*, 2006). Many traditional plant treatments for diabetes are used

throughout the world and plant drugs are frequently considered to be less toxic and free from side effects than synthetic one and WHO recommendations underline their importance in the management of diabetes (Pari and Ramalingam, 2006).

Thiazolidinedione class of drugs are known to reduce the insulin resistance in peripheral tissues such as skeletal muscles by either mimicking or enhancing insulin action without any effects on β -cell insulin secretion (Choi *et al.*, 2006). They act by binding with nuclear receptor PPAR- γ and their major site of action includes liver, skeletal muscle and adipose tissues. They also improve various lipid parameters such as High Density Lipoprotein-cholesterol (HDL-c), triglycerides (TG) and free fatty acids and thereby reduce the progression of atherosclerosis (Moller, 2001).

The plant *Salacia reticulata* (Celastraceae) is distributed widely in Southern India and in Srilankan forest. Traditionally, the roots are used as acrid, bitter, thermogenic, urinary antiseptic, astringent, anodyne and antiinflammatory. They are also useful in vitiated conditions of vata, diabetes, haemorrhoids, rheumatism, gonorrhoea and skin diseases (Warrier *et al.*, 1996; Husain and Virmani, 1992; Nadkarni, 1993).

The roots of *Salacia reticulata* are been reported to have lipolytic and antiobesity effects through inhibition of fat metabolizing enzymes (Yoshikawa *et al.*, 2002a, b). Salacinol, a unique thiosugar sulfonium sulfate is reported

to have potent α -glucosidase inhibitory activity (Yoshikawa *et al.*, 1997). The roots of *Salacia reticulata* are reported to contain mangiferin, salacinol and kotalanol, which has potent α -glucosidase inhibitory properties (Yoshikawa *et al.*, 2001; Yoshikawa *et al.*, 1998). *Salacia* extract has also reported to prevent obesity and associated metabolic disorders including the development of metabolic syndrome in TSOD (Tsumura, Suzuki, Obese, Diabetes) mice and non-obese TSNO (Tsumura Suzuki Non-Obese) mice (Akase *et al.*, 2009).

Our previous studies with purified fraction of methanolic extract of the roots of *Salacia reticulata* (SRM-methanolic) showed inhibitory activity on different enzymes involved in carbohydrate digestion such as α -amylase, sucrase and maltases to a varying extent and the potency was comparable to acarbose, a standard α -glucosidase inhibitor (Rafiq and Mitra, 2009).

Study to Prevent Non-Insulin Dependent Diabetes Mellitus (STOP-NIDDM) trial (a large multicentric trial) revealed that, acarbose; a prototype of α -glucosidase inhibitor, reduced the incidence of cardiovascular accidents and also brings about the reduction of hypertension in Impaired Glucose Tolerant (IGT) patients (Chiassen *et al.*, 2003). Further more, a meta analysis of seven long-term studies has also shown that acarbose prevented myocardial infarction and Cardiovascular Diseases (CVD) in patients with type 2 diabetes (Nakamura *et al.*, 2005).

Keeping these things in mind and available information in the literature, present study was designed and carried out to investigate whether administration of purified methanolic extract of *Salacia reticulata* has any beneficial effect like improvement in insulin sensitivity and other metabolic parameters in fructose-fed rats, a well-known model of insulin-resistance and hyperinsulinemia (Hwang *et al.*, 1983).

MATERIALS AND METHODS

Collection of plant material: Plant samples were collected from Attappadi Estates, Mannarghat, Palakkad district of Kerala, India, during January 2004 and the collected plants were identified with the local floras (Gamble, 1997). Root pieces were dried and stored as authentic source for the comparison and authentication of market sample. The specimen of same was deposited in the herbarium of Research and Development Center, The Himalaya Drug Company, Bangalore, India.

Chemicals: All the reagents and chemicals used in the present experiment are from reputed brands (analytical and laboratory grades) and supplied by approved vendors.

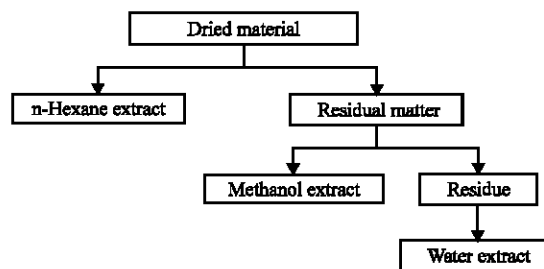


Fig. 1: Extraction chart

Extraction procedure: The shade-dried raw materials were coarsely powdered with the help of pulverizer. The powdered materials were packed in 5 L capacity soxhlet (continuous extraction) apparatus and the extraction was carried out as per the Fig. 1. The temperature for solvent (n-Hexane and methanol) extraction was set at 60 and 70°C, respectively. The extraction was monitored continuously and the completion of extraction at each stage was confirmed by color, optical density and thin layer chromatography. The extracts were then filtered through Whatman filter paper No.1 and concentrated to dryness on rotary evaporator. The residual material was subjected for percolation with double distilled water for 48 h. The extract was filtered through filter pads and concentrated to dryness on water bath at 100°C. The methanolic extract of *Salacia reticulata* obtained was weighed to calculate the yield.

The methanolic extract obtained was further subjected for sequential solvent fractionation using solvents of increased polarity like chloroform and ethyl acetate to obtain a SRM-methanolic. The SRM-methanolic was used in the present experiment.

Phytochemical investigation of SRM-methanolic: The selected methanolic fractions of SRM-methanolic was subjected to thin layer chromatography over precoated Thin Layer Chromatography (TLC) plates (e-Merck) by the method of Wagner and Bladt (1995). The sample was dissolved in respective solvent at the concentration of 100 mg mL⁻¹ and filtered through Whatman No. 1 filter paper. Approximately, 50 μ L of sample was spotted on plates. The TLC plates were run in the above mobile phase(s) and air-dried for 30 min. Each plate was sprayed with the following chemical reagents to identify the nature of phytochemical constituents present in the extracts and fractions.

Animals: The rats were housed under standard laboratory conditions, air-conditioned with adequate fresh air supply (12-15 air changes h⁻¹), temperature 19-25°C, relative humidity 30-70%, with a 12 h light and 12 h dark cycle. They were housed in groups of 4 cage⁻¹ in standard

polypropylene hanging cages (size approximately: L 410×B 220×H140 mm), with stainless steel top grill having facilities for food and drinking water in bottle, the water was provided *ad libitum*. The study was approved by local Institutional Animal Ethics Committee (IAEA) of Committee for the Purpose of Supervision and Control of Experiments on Animals (CPCSEA), Government of India.

Fructose enriched diet (FRU): A special diet was prepared in such that, the fructose content provided 60% of total calories in the diet (Hwang *et al.*, 1983; Nandhini *et al.*, 2005). The diet was prepared in laboratory with the following composition (g kg⁻¹) Casein, high protein 207.0; DL-methionine 3.0; fructose 600.0; animal fat (bovine) 50.0; cellulose 79.81; mineral mix 50.0; zinc carbonate 0.04 and vitamin mixture 10.

Composition of salt mixture (g): Calcium carbonate 16.67, Calcium phosphate 47.3, Copper sulphate 0.017, Ferric citrate 0.333, Magnesium sulphate 5.0, Manganese sulphate 0.417, Potassium chloride 11.67, Potassium iodide 0.017, Sodium chloride 6.67, Sodium phosphate (dibasic) 11.67 and Zinc carbonate 0.217.

The vitamin mixture has following composition (g): Vitamin A acetate 1.8, Vitamin D₂ 0.125, DL- α -tocopherol 22; Ascorbic acid 45; Inositol 5.0; Choline chloride 75; Menadione 2.25; Niacin 4.25; Pyridoxine HCl 1.00; Thiamine 1.00; Calcium pantothenate 3.00; Biotin 0.02 and Glucose q.s. to 1 kg.

Experimental design: The male Wistar rats (40 in number) of 240 to 280 g in body weight were randomized into five groups of eight each. The animals were coded and each animal was identified by their unique accession number and by picric acid body marking.

- **Group I (Normal control):** The animals were provided with standard laboratory feed Tetragon Chemie, Vetcare)
- **Group II (Positive control):** The animals were fed with high fructose diet (FRU) and maintained as untreated control
- **Group III:** The animals were fed with fructose enriched diet, containing 0.4 g of drug SRM-methanolic 100⁻¹ g of feed. The drug concentration was fixed in such that each animal received approximately around 100 mg of drug day⁻¹
- **Group IV:** The animals were fed with fructose enriched diet containing 5 mg of rosiglitazone 100⁻¹ g of feed, which approximately corresponds to 5 mg kg⁻¹, body weight day⁻¹ of rosiglitazone

- **Group V:** The animals were fed with fructose-enriched diet containing 40 mg of acarbose 100 g of feed (10 mg of acarbose/day/animal)

Group-II to group-V animals were fed with fructose enriched diet, supplemented with respective drugs in a suitable concentration as mentioned above. The concentration of purified SRM-methanolic in feed was adjusted based on the daily individual feed consumption of rats. The previous experimental studies with SRM-methanolic showed optimum blood glucose lowering activity in selected concentration. The dose of standard drugs were fixed based on the available information in the literature, the dose of rosiglitazone (a standard insulin sensitizer) and acarbose (a standard α -glucosidase inhibitor) was approximately around 5 mg kg⁻¹, body weight day⁻¹ and 10 mg animal⁻¹ day⁻¹, respectively (Seda *et al.*, 2002; Nakanura *et al.*, 2005).

Animals were maintained on these dietary regimens for 4 weeks and body weight measurements were carried out weekly. At the end of the 4-weeks of dietary intervention, the rats were fasted overnight and blood of around 2-3 mL was collected under light ether anesthesia from retro-orbital plexus of animals belonging to all the groups. Blood samples were immediately centrifuged (3000 g for 20 min) and serum separated and analysed using HITACHI 704 (Boehringer Mannheim, Mannheim, Germany) Automatic Clinical Analyser for the following parameters except for sodium and potassium, which were analysed by Ilyte (Instrumentation Laboratory, Italy) electrolyte analyser (Monika *et al.*, 2006).

All clinical chemistry parameters were analyzed using Diasys Diagnostic system kits manufactured by GmbH Germany, except for glucose (Spinreact, Spain) and sodium and potassium (Instrumentation Laboratory, Italy).

Oral Glucose Tolerance Test (OGTT): At the end of 3-week period, the animals were fasted overnight and all the groups of animals were subjected for oral glucose tolerance test as per the standard procedure. In short, the fasted animals were orally challenged with 2 g kg⁻¹ of glucose and around 0.3 mL of the blood was collected from the retro-orbital plexus at different time intervals (0, 30, 60 and 120 min) and the serum separated and subjected for glucose estimation (Mitra *et al.*, 1996). By using the blood glucose values (mg dL⁻¹) at different time intervals, the total area under curve of blood glucose (AUC_{glucose}) were calculated.

Estimation of serum biochemical parameters: The various serum biochemical parameters estimated include glucose, lipid profiles such as cholesterol, triglycerides (TG), Low Density Lipoprotein-cholesterol (LDL-c),

Very Low Density Lipoprotein-cholesterol (VLDL-c), High Density Lipoprotein-cholesterol (HDL-c), creatinine, uric acid, albumin and total proteins. The enzymatic markers such as Serum Glutamic Oxaloacetic Transaminase (SGOT) and Serum Glutamic Pyruvic Transaminase (SGPT), the electrolytes, sodium and potassium were also estimated. The serum insulin was estimated by standard radioimmunoassay technique using kit (RIAK-1, supplied by Board of Radiation and Isotope Technology, Bhabha Atomic Research Centre, Mumbai, India).

The degree of insulin resistance was estimated by using Homeostasis Model Assessment (HOMA) as an index of insulin resistance (Mlinar *et al.*, 2007):

$$\text{HOMA Index} = \frac{\text{Insulin } (\mu\text{U L}^{-1}) \times \text{Glucose } (\text{mmol L}^{-1})}{22.5}$$

Estimation of urinary biochemical parameters: The urine collection for all animals were carried out in the last week of treatment and subjected for measurement of parameters such as pH, sodium, creatinine, uric acid etc.

Statistical analysis: All the values were expressed as Mean±SEM. The results were analyzed statistically using One-Way ANOVA to find out the level of significance followed by Dunnet's post comparison test. The minimum level of significance was fixed at p<0.05. The analysis was carried out using GraphPad Prism, Version 4.01.

RESULTS

At the end of the study period the serum samples obtained were subjected for analysis of various biochemical parameters (Table 1). Serum insulin levels were significantly elevated in animals fed with fructose-enriched diet, indicating hyperinsulinemic state. The insulin resistance index (HOMA) was also found to be significantly increased. Triglycerides and VLDL levels were also significantly elevated in fructose fed rats compared to animals, which received normal feed. The above elevation of insulin, insulin resistance index, TG and VLDL were significantly reversed in animals which received SRM-methanolic and rosiglitazone along with fructose enriched diet. But notably, there was no statistically significant differences in values were observed in case of acarbose treated group, when compared to rats fed with only fructose-enriched diet. During the entire study period, there was no statistically significant change in body weight was observed in any of the groups.

The urine collected during the last week (26th day) of the study from all the groups were subjected for estimation various biochemical parameters such as pH, uric acid, creatinine sodium and potassium etc. The results (Table 2) indicate, there was no statistically significant difference between any of the groups.

Table 1: Body weight and serum biochemical parameters of the rats in different experimental groups

Parameter	G I (Normal control)	G II (Positive untreated control-FRU)	G III (FRU+ SRM-methanolic)	G IV (FRU+ Rosiglitazone)	G V (FRU+Acarbose)
Body weight gain (g)	10.800±7.01	21.57±3.90	5.400±6.03	15.000±4.78	17.400±7.30
Glucose (mg dL ⁻¹)	98.700±6.34	113.00±7.63	122.500±9.68	104.100±4.64	110.900±5.27
Triglyceride (mg dL ⁻¹)	33.140±3.20	105.70±12.2 [†]	60.080±4.73 [#]	41.110±2.00 [#]	121.900±20.50
Cholesterol (mg dL ⁻¹)	68.910±3.54	56.34±7.96	57.470±4.57	48.050±4.31	46.120±3.70
Insulin (μU mL ⁻¹)	21.140±1.11	61.33±12.00 [†]	32.750±8.57 [*]	23.290±1.49 [#]	54.800±19.37
HOMA	5.050±0.52	17.10±4.23 [†]	8.580±1.52 [*]	5.740±0.463 [#]	15.520±5.88
HDL-c (mg dL ⁻¹)	38.500±2.26	40.70±1.87	33.600±2.21	32.080±2.93	32.640±2.92
VLDL-c (mg dL ⁻¹)	7.120±0.71	20.80±2.10 [†]	13.100±1.40 [*]	9.120±1.04 [#]	24.400±4.07
Uric acid (mg dL ⁻¹)	0.600±0.04	1.05±0.05	0.680±0.17	1.000±0.10	1.100±0.17
Urea (mg dL ⁻¹)	30.560±1.82	34.16±1.55	31.170±1.84	30.400±2.40	26.400±1.33
Creatinine (mg dL ⁻¹)	0.590±0.04	0.55±0.02	0.610±0.07	0.560±0.03	0.530±0.01
SGOT (U L ⁻¹)	175.500±7.33	166.70±8.95	148.100±9.03	179.000±9.48	187.900±27.46
SGPT (U L ⁻¹)	43.530±1.15	41.99±3.36	40.200±3.16	41.010±1.87	55.420±8.75
Sodium (mmol L ⁻¹)	125.700±3.17	124.20±3.64	121.500±3.23	136.000±6.62	136.100±4.03
Potassium (mmol L ⁻¹)	5.085±0.17	5.094±0.23	4.582±0.15	5.228±0.09	5.738±0.34
Protein (g dL ⁻¹)	6.800±0.105	7.24±0.089	7.410±0.137	6.730±0.212	6.750±0.246
Albumin (g dL ⁻¹)	2.060±0.02	2.26±0.04	2.170±0.05	2.050±0.09	2.050±0.13

The values indicate Mean±SEM, n = 6-8 rats group⁻¹, [†]p<0.01 significantly different from normal control., ^{*}p<0.05 significantly different from positive control (fructose fed rats), [#]p<0.01 significantly different from positive control (fructose fed rats)

Table 2: Urinary biochemical profile of the rats in different experimental groups

Parameter	G I (Normal control)	G II positive untreated control (FRU)	G III (FRU+SRM-methanolic)	G IV (FRU+Rosiglitazone)	G V (FRU+Acarbose)
Urinary pH	8.06±0.11	8.25±0.09	8.25±0.11	8.00±0.09	8.20±0.12
Uric acid (mg dL ⁻¹)	1.81±0.54	2.85±1.05	2.88±0.58	1.96±0.40	2.76±0.55
Creatinine (mg dL ⁻¹)	8.48±2.24	13.35±2.12	14.26±2.41	8.88±2.03	10.74±2.28
Sodium (mmol L ⁻¹)	116.10±32.90	111.70±29.58	98.48±17.50	84.01±15.90	107.50±20.60
Potassium (mmol L ⁻¹)	102.00±42.70	90.86±19.60	84.87±15.50	89.28±22.50	90.62±12.60

The values indicate Mean±SEM; n = 6-8 rats per group

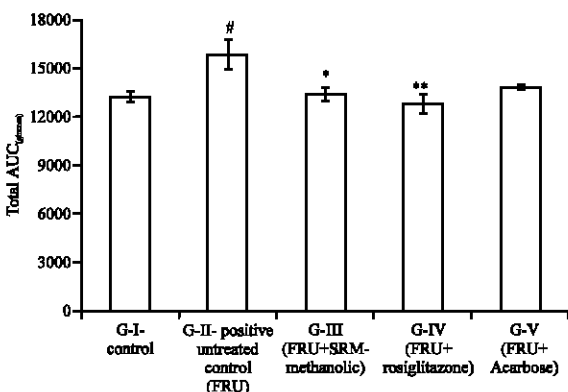


Fig. 2: Effect of supplementation of SRM-methanolic on OGTT of fructose fed rats. # $p < 0.05$ compared to normal fed control; * $p < 0.05$ compared to positive (Fru fed) control; ** $p < 0.01$ compared to positive (Fru fed) control

Oral Glucose Tolerance Test (OGTT) was conducted for animals of all the groups during the third week of the study. The results obtained (Fig. 2) showed the animals which received fructose enriched diet was significantly glucose intolerant when compared to normal control. Supplementation of SRM-methanolic and rosiglitazone with fructose feed significantly decreased $AUC_{glucose}$. The $AUC_{glucose}$ values of the acarbose supplemented groups did not statistically differ from animals treated only with fructose.

DISCUSSION

Insulin resistance, a complex metabolic abnormality that affects the ability of peripheral tissues to respond to insulin, is a prominent feature of metabolic syndrome and type-2 diabetes and also constitutes a major risk factor for cardiovascular disease (Chen *et al.*, 2006). The insulin resistant state is commonly associated with lipoprotein abnormalities that are risk factors for atherosclerosis especially hypertriglyceridemia, high VLDL cholesterol and low levels of HDL cholesterol (Harati *et al.*, 2003). Recently, the STOP-NIDDM trial (a large multicentric trial) revealed that, acarbose; a prototype of α -glucosidase inhibitor reduced the incidence of cardiovascular accidents and also brings about the reduction of hypertension in IGT patients (Chiassen *et al.*, 2003). Further more, a meta analysis of seven long-term studies has also shown that acarbose prevents myocardial infarction and CVD in patients with type-2 diabetes (Nakamura *et al.*, 2005). Rats fed with a high-fructose diet (>60% of total calories) provide a useful animal model of insulin resistance and hyperinsulinemia. The sites of

fructose-induced insulin resistance are documented to be the liver, skeletal muscle and adipose tissue. These rats also develop a cluster of abnormalities, which include hypertension, hypertriglyceridaemia, oxidative stress and glucose intolerance in addition to hyperinsulinaemia and insulin resistance etc. (Hwang *et al.*, 1983; Nandhini *et al.*, 2005). Abnormalities in lipid profile are one of the major common complications in diabetes mellitus. High levels of total cholesterol and more importantly LDL-cholesterol in blood are major coronary risk factors. Insulin deficiency and resistance causes an increase in free fatty acid mobilization from adipose tissue, which results in increase in increased production of cholesterol rich LDL-C particle and dyslipidemia (Latha and Daisy, 2010).

Our findings revealed that supplementation of SRM-methanolic with fructose feed to rat significantly reversed the hyperinsulinemia and insulin resistance index as measured by HOMA, TG and VLDL-cholesterol levels. In other words, SRM-methanolic and rosiglitazone ameliorated the insulin resistance and caused marked improvement of the insulin sensitivity in fructose fed rats. The spectrum of activity observed was similar to rosiglitazone.

But notably, there was no statistically significant differences in values observed in case of acarbose treated group, when compared to rats fed with only fructose-enriched diet. Our findings were in contrary to the earlier findings (Nakamura *et al.*, 2005), where it was reported that acarbose significantly improves insulin sensitivity in fructose fed rats at the same dose level that we have selected. This could be because of the fact that acarbose is pure α -glucosidase inhibitor (Relph, 1999). α -glucosidases are specific intestinal brush border enzymes, which catalyzes the final step in the digestion of carbohydrates, these enzymes hydrolyze the disaccharides (derived from starch digestion and ingested disaccharides) to absorbable monosaccharides (Sou *et al.*, 2001). Fructose being a readily absorbable monosaccharide, acarbose may have very little effect on its absorption, which may lead to development of abnormalities like hyperinsulinemia observed in acarbose supplemented groups. It is also important to note that, oral bioavailability of acarbose is very poor and its therapeutic benefit is mainly due its local effect on α -glucosidases of gastrointestinal tract (Clissold and Edwards, 1988). There are also reports available indicating that acarbose lacks direct stimulatory action on insulin secretion (Breuer, 2003).

In case of Oral Glucose Tolerance Test (OGTT), the changes in blood glucose concentration, which result from an oral carbohydrate load is theoretically dependent on the rate at which carbohydrate enters the small

intestine, the rate of digestion and intestinal absorption of glucose and the rate of insulin-driven metabolism. The relationship between plasma levels of glucose and insulin after an external load of glucose can be studied using OGTT (Srinivasan and Karundevi, 2005). The OGTT conducted for animals of all the groups during the third week of the study, indicate that animals which received fructose-enriched diet were significantly glucose intolerant, when compared to normal control and our findings are in consistent with reports of other investigators (Nandhini *et al.*, 2005). We found that supplementation of drugs SRM-methanolic significantly decreased the AUC_{glucose} values indicating the better glucose disposal rate. The effect observed could directly be related to insulin sensitizing activity of SRM-methanolic as described above and the activity was comparable to that of rosiglitazone.

The detail phytochemical investigation revealed presence of steroidal compounds, terpenoids, glycosides, phenolic compounds and amino acids in SRM-methanolic but, the absence of alkaloids. The present study indicate that the antidiabetic effect of *Salacia reticulata* goes beyond the α -glucosidase inhibition and could be due to combination of several mechanisms such as insulin sensitization, effect on fat metabolism etc. Further studies are in progress in our laboratory to elucidate the mechanisms by which *Salacia reticulata* exhibits its beneficial effect on various parameters associated with metabolic disorders and complications associated with diabetes.

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