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Prevention of Insulin Resistance by Herbal Compound from Fruit-Pulp of *Eugenia jambolana* in *fructose fed rats*

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ABSTRACT

Aim: Present study was undertaken to investigate the preventive effect of active principle (FIIc) isolated from Eugenia jambolana on the development of insulin resistance induced by fructose in rats. **Methodology:** Crude aqueous extract of fruit-pulp of Eugenia jambolana was subjected to purification via Ion-exchange column chromatography that yielded FII which on further purification resulted FIIc. The purity of FIIc was tested by HPLC. FIIc was administered orally at a dose of 15 mg/kg b.wt. for 60 days to experimental rats. Body weight, blood glucose, serum triglyceride, total cholesterol, LDL-C, HDL-C, liver and muscle glycogen levels, TNF α, serum insulin, insulin resistance and insulinogenic index were estimated at the interval of 30 days up to a period of 60 days. **Results:** Fructose feeding for 60 days resulted significant ($p<0.001$) increase in serum biochemical parameters and decreased liver and skeletal muscle glycogen levels in untreated control. Administration of FIIc showed significant ($p<0.001$) reduction in blood glucose when compared with untreated control. Body weight, lipid profile and liver and skeletal muscle glycogen levels were significantly ($p<0.001$) improved after treatment with FIIc. Levels of serum TNF α and insulin were found to near normal following treatment with FIIc. Insulin resistance and insulinogenic index were significantly improved compared to untreated control. **Conclusion:** Our study showed that treatment with FIIc has a significant effect on fructose induced insulin resistance.

Keywords: Eugenia Jambolana, Insulin Resistance, Tumor Necrosis Factor α and Fructose.

INTRODUCTION

The last 25 years have witnessed a marked increase in total per capita fructose intake as a sweetener in the food industry, primarily in the form of sucrose and high fructose corn syrup (HFCS) [1,2]. Concern has arisen because of

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the realization that fructose at elevated concentrations, can promote metabolic changes that are actually or potentially deleterious e.g., hyperlipidemia, hyperinsulinemia, insulin resistance, hyperuricemia, hypertension, glucose intolerance and non-enzymatic fructosylation of proteins [3-5].

Metabolism of fructose involves its phosphorylation to form fructose-1-phosphate. The reaction is catalyzed by the enzyme fructokinase. Fructose-1-phosphate is split by aldolase B into glyceraldehyde and dihydroxyacetone phosphate. Both can be converted to glyceraldehydes-3-phosphate. Thus, the fructose molecule is metabolized into two triose phosphates that bypass the main rate-controlling step in glycolysis, 6-phosphofructokinase. In contrast, hepatic glucose metabolism is limited by the capacity to store glucose as glycogen and more importantly, by the inhibition of glycolysis and further glucose uptake resulting from the effects of citrate and ATP to inhibit phosphofructokinase. Because fructose uptake by the liver is not inhibited at the level of phosphofructokinase, fructose consumption results in larger increases of circulating lactate than does consumption of a comparable amount of glucose [6]. When much larger amounts of fructose are consumed (e.g, in sucrose- and HFCS- sweetened beverages), fructose continues to enter the glycolytic pathway distal to phosphofructokinase and hepatic triglyceride production is facilitated. Unlike glucose metabolism, in which the uptake of glucose is negatively regulated at the level of phosphofructokinase, high concentrations of fructose, can serve as a relatively unregulated source of acetyl-Co A. So, fructose is more lipogenic than glucose [7,8]. The classic relation between insulin resistance, increased fasting plasma insulin concentration and glucose intolerance has been hypothesized to be mediated by changes in ambient non-esterified fatty acid concentrations. An increased supply of non-esterified fatty acids in the liver also leads to an increase in the production of VLDL triacylglycerol [9]. Fructose consumption has been shown to induce hypertriacylglycerolemia. Rats fed with high fructose diet form a model of diet induced insulin resistance, associated with hyperglycemia, hypertriglyceridemia and hyperinsulinemia [10-15] and therefore, can serve as a suitable model for the study of human diabetes.

In this article, we have investigated the effect of purified active principle (FIIc) on insulin resistance in a non-genetic, pre-diabetic model of the rodents developed by feeding fructose rich diet. In our lab we purified active compound from fruit-pulp of Eugenia jambolana (Tanwar et al, 2016) [16] and received the patents for isolation of FIIc (US Patent No. 6,428,825 August 2002; Indian Process Patent No. 188759 May 2003; Indian Product Patent granted No. 230753, February 2009). In our earlier published studies, we showed FIIc has significant antihyperglycemic and antihyperlipidemic activity (Tanwar et al, 2016) [16] and showed significant effect on different diabetic complications like nephropathy (Tanwar et al, 2010) [17] and atherosclerosis (Tanwar et al, 2011) [18]. In this study we are reporting the first-time effect of FIIc on fructose induced hyperglycemia, hypertriglyceridemia and hyperinsulinemia. Insulin resistance is calculated by Homeostasis Model Assessment along with TNF α and glycogen levels (liver and skeletal muscle) are also estimated, which is not reported earlier in the literature.

MATERIALS AND METHODS

Plant material

The fruits of E. jambolana were sourced from the Azadpur Mandi herbal market in Delhi, India. Their identity was confirmed by a botanist using taxonomic methods (voucher specimen no: P-96/7). The specimen is preserved for future reference at the Botanical Garden in Kolkata, India.

Preparation of crude aqueous extract

Fresh fruits of E. jambolana were purchased from the local market and washed thoroughly. The seeds were separated from fruit pulp of E. jambolana. The pulp was ground for 10 minutes in a mixer along with distilled water (500 mL). It was allowed to stand overnight at 4oC. Pulp was then filtered through 5-6 layers of muslin cloth. The whole procedure was carried out in a cold room at 4oC. The filtrate was first centrifuged for 15 minutes in a refrigerated centrifuge at 10,000 rpm at 4oC and then lyophilized to store it for longer duration. The yield of lyophilized water extract was about 10 g from 650 g of fruit pulp, obtained from 1kg fruits of E. jambolana (Tanwar et al, 2016) [16].

Isolation and Chemical characterization of active compound FIIc

Lyophilized pulp extract was purified using DEAE-52 ion exchange chromatography. Fractions were eluted with 0.1 M phosphate buffer (pH 6.0). Fraction (FII) showed Potent anti-hyperglycemic activity and was further purified to FIIc. (Patents have already been granted for the isolation of active

principle) [16,18]. The purity was tested by HPLC, showing a single peak. It was eluted with a water:methanol:acetonitrile (70:15:15) mobile phase and monitored by PDA detector at wavelength 220 nm. Elemental analysis revealed the presence of C, H, O, and N. Spectral data (UV, NMR and IR) identified FIIc as α-hydroxy succinamic acid (C4H7O4N) and more details are described in Tanwar et al. 2016 [16].

Experimental Design

Male Wistar albino rats (weighing 160-200 g) were procured from Central Animal House of University College of Medical Sciences (UCMS), Delhi, India. The animals were housed in standard conditions of temperature (22 ± 2oC) & at 12 h light-dark cycle. After 3 days of acclimatization, chow control rats were provided with standard laboratory diet and experimental rats were placed on a fructose rich diet containing 66 % fructose, 12 % fat and 22 % protein (Sleder et al, 1981) [14] for 60 days. The experimental protocol for the present study was approved by Institutional Animal Ethical Committee (IAEC) of University College of Medical Sciences, Delhi, India.

The experiment was carried out on the following groups (n = 7).

Group 1 - Chow control (receiving only normal chow diet)

Group 2 - Fructose control (receiving fructose rich diet)

Group 3 - Receiving fructose diet + FIIc at the dose of 15 mg/kg b.wt.

Control rats (groups 1 & 2) received vehicle i.e. distilled water. The treated group received the FIIc at the above-mentioned dose dissolved in 1 ml of distilled water. The treatment was given daily for a period of 60 days using standard orogastric cannula at a fixed time interval on each day. FBG were estimated at day 0 and after each 15 days till the entire experimental period. All other biochemical parameters were determined at day 0, day 30 and day 60.

Blood samples were collected in vials containing anticoagulants (sodium fluoride and potassium oxalate) for plasma separation to estimate blood glucose. The blood collected in plain vials was allowed to clot for separation of serum. Serum was used to estimate serum lipid profile, insulin and TNF α levels. Thereafter, the animals were euthanized by CO2 inhalation. The liver and skeletal muscles were excised immediately. Tissues were rinsed in ice-chilled normal saline and stored at -700C for estimation of glycogen content.

Analytical methods

Blood glucose was determined by the method based on the glucose oxidase method [19]. Triglycerides (TG) were measured by the method of Fossati and Lorenzo, 1982 [20]. Total cholesterol (TC) was assayed as per the method of Allain et al. 1974 [21]. High-density lipoprotein (HDL) was determined as per method describe by Burstein et al., 1970 [22]. Low- density lipoprotein (LDL) and very low-density lipoprotein (VLDL) were calculated by using the formula of Friedwald et al., 1972 [23]. TNF α and Insulin levels were estimated by Enzyme linked Immuno Sorbant assay (ELISA) using commercially available kits from Diaclone France and Mercodia, Sweden respectively. Tissue glycogen was assayed as described by Carroll et al., 1956 [24].

Assessment of Insulin Resistance

This was done by homeostasis model assessment (HOMA) [25] using fasting glucose and insulin values as follows:

HOMA IR = Fasting Insulin (μU/ml) X FBG (mmol/L)/ 22.5

Acute toxicity study: For toxicity studies of FIIc, three groups of fasted healthy rats (five animals per group) were administered orally graded doses of FIIc (up to a dosage of 5, 10 and 15 times of effective dose) and one group is taken as control. The rats were observed during the first 4h and then after every 24 h up to 30 days for any gross behavioral changes or mortality, if happens. Liver function tests such as serum glutamate phosphotransferase (SGPT) and alkaline phosphatase (ALP) as well as kidney function tests such as urea and creatinine were performed in serum at the end of the study using standard kits. Body weights were also recorded prior to treatment and then post-treatment.

Statistical analysis

The results are presented as mean ± S.E.M. The data was analyzed by repeated measure analysis of variance (ANOVA) followed by Dunnett's multiple comparison test for more than one-time points studies. One way analysis of variance followed by Tukey's multiple comparison test for one-time studies using SPSS software version 17.0. A value of $P<0.05$ was considered significant.

RESULTS

Glycemic Control

The basal value of blood glucose was comparable in fructose control, chow control and FIIc treated groups indicating no significant inter-group variation. However, fructose rich diet resulted in significant hyperglycemia as compared with controls starting from day 15 and continued till the end (60 days). FIIc resulted in significant (P<0.001) improvement in fasting blood glucose after 60 days of treatment. The effect of FIIc in fructose fed rats is shown in Figure1.

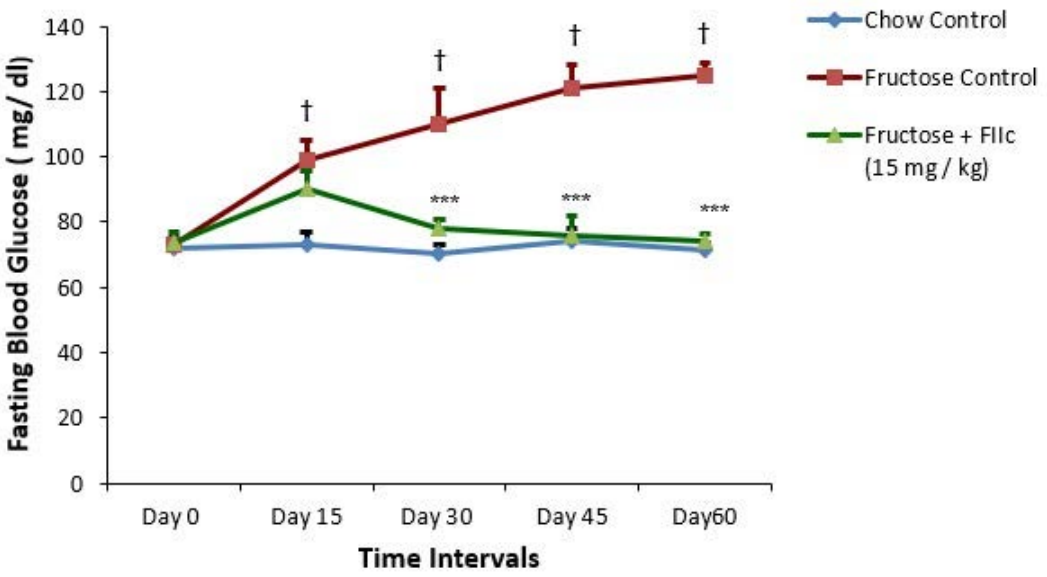

Figure 1. Antihyperglycemic effect of active compound (FIIc) on fasting blood glucose in fructose fed rats.

Values are given as mean ± S.E.M (n = 7).

†P< 0.001 Vs chow control; ***P< 0.001 Vs fructose control.

Lipidemic Control

As shown in table1, fructose feeding for 60 days significantly (p<0.001) increased serum triglyceride levels compared to chow control group. The supplementation of FIIc along with fructose feeding for 60 days significantly (p<0.001) improved the serum triglyceride levels when compared to fructose control group.

Table 1. Effect of FIIc on serum triglyceride in fructose fed rats after 60 days of treatment

Groups	Dose	Serum triglyceride (mg/dl)		
		Initial levels	After treatment	
		Day 0	Day 30	Day 60
Chow control	Vehicle	65.6 ± 2.14	64.6 ± 3.4	65.8 ± 3.3
Fructose control	Vehicle	66.6 ± 2.12	91 ± 2.6†	127 ± 15.2†
Fructose + FIIc	15 mg/ kg	67 ± 3.2	80.0 ± 4.2†	65.6 ± 3.0***

All values are given as mean ± S.E.M for a group of seven animals each.

†P< 0.001 Vs chow control; ***P< 0.001 Vs fructose control.

Insulin and TNF α

Fructose feeding significantly (p<0.001) increased serum insulin and TNF α when compared to normal control. However, treatment with FIIc significantly reduced the serum insulin (table 2) and TNF α levels (table 3). Insulin resistance was calculated by HOMA, showed significant improvement after treatment with FIIc (Figure 2).

Table 2. Effect of FIIc on serum insulin levels in fructose fed rats after 60 days of treatment

Groups	Dose	Serum insulin levels (µU / ml)		
		Initial levels Day 0	After treatment	
			Day 30	Day 60
Chow control	Vehicle	28 ± 1.5	29 ± 1.52	29.2 ± 2.3
Fructose control	Vehicle	29 ± 2.4	47.4 ± 3.0†	63.4 ± 3.4†
Fructose + FIIc	15 mg/ kg	28.2 ± 1.8	35.6 ± 3.4†	31 ± 2.6***

All values are given as mean ± S.E.M for a group of seven animals each.

†P< 0.001 Vs chow control; ***P< 0.001 Vs fructose control.

Table 3. Effect of FIIc on serum TNF α levels in fructose fed rats after 60 days of treatment

Groups	Dose	Serum TNF α levels (pg / ml)		
		Initial levels Day 0	After treatment	
			Day 30	Day 60
Chow control	Vehicle	6.2 ± 0.6	7 ± 0.2	7.2 ± 0.3
Fructose control	Vehicle	6.3 ± 0.4	22.4 ± 3.9†	35.6 ± 5.9†
Fructose + FIIc	15 mg/ kg	6.6 ± 0.3	13.8 ± 3.1†	11.4 ± 2.0***

All values are given as mean ± S.E.M for a group of seven animals each.

†P< 0.001 Vs chow control; ***P< 0.001 Vs fructose control.

HOMAIR = Fasting Insulin (µU/ml) X Fasting Blood Glucose (mmol/L) / 22.5

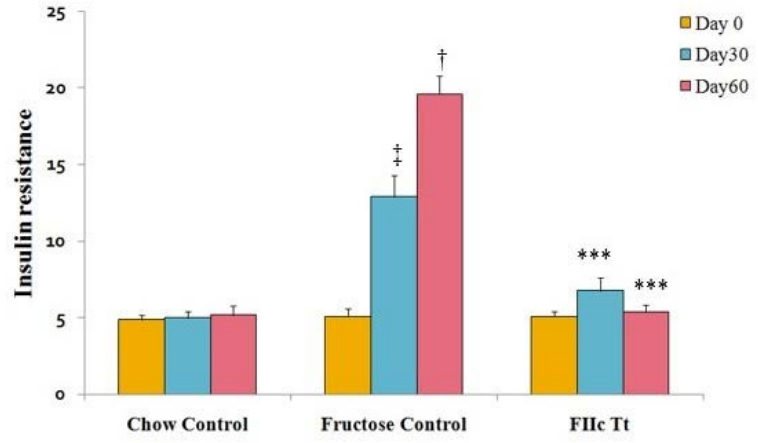

Figure 2. Effect of FIIc on insulin resistance in fructose fed rats.

Values are given as mean ± S.E.M (n = 7).

‡P< 0.01 Vs chow control; †P< 0.001 Vs chow control; ***P< 0.001 Vs fructose control

Liver and Skeletal Muscle Glycogen Levels

As summarized in table 4 and 5, the initial value of liver and skeletal muscle glycogen was comparable in all the groups. Feeding with fructose resulted in a significant reduction in liver and muscle glycogen levels compared to chow control group. However, treatment with FIIc results in significant ($p<0.001$) improvement in the liver and muscle glycogen levels.

Table 4. Effect of FIIc on liver glycogen levels in fructose fed rats after 60 days of treatment

Groups	Dose	Liver glycogen (mg/gm tissue)		
		Initial levels Day 0	After treatment	
			Day 30	Day 60
Chow control	Vehicle	23.3 ± 0.87	23.8 ± 0.68	23.2 ± 0.6
Fructose control	Vehicle	23.15 ± 0.98	18.5 ± 2.6‡	11.3 ± 1.24†
Fructose + FIIc	15 mg/ kg	23.07 ± 1.0	19.05 ± 1.23	21.7 ± 2.3***

All values are given as mean ± S.E.M for a group of seven animals each.

‡P< 0.01 Vs chow control; †P< 0.001 Vs chow control; ***P< 0.001 Vs fructose control.

Table 5. Effect of FIIc on skeletal muscle glycogen levels in fructose fed rats after 60 days of treatment

Groups	Dose	Skeletal muscle glycogen (mg/gm tissue)		
		Initial levels Day 0	After treatment	
			Day 30	Day 60
Chow control	Vehicle	9.18 ± 0.33	9.0 ± 0.38	9.0 ± 0.15
Fructose control	Vehicle	9.26 ± 0.21	5.9 ± 0.26‡	4.9 ± 0.24†
Fructose + FIIc	15 mg/ kg	9.24 ± 0.27	6.1 ± 0.16	6.9 ± 0.45***

All values are given as mean ± S.E.M for a group of seven animals each.

‡P< 0.01 Vs chow control; †P< 0.001 Vs chow control; ***P< 0.001 Vs fructose control

DISCUSSION

Hyperinsulinemia affects the development and clinical course of at least three major related diseases: namely non-insulin dependent diabetes mellitus, essential hypertension and coronary artery disease [26]. In addition, it is a common metabolic abnormality in obesity and dyslipidemia [27]. Lowering endogenous insulin levels is a key step to successful therapy directed at insulin resistance disease [28].

The development of insulin resistance in fructose fed rats is well documented in the literature [3,4]. Chronic fructose feeding to experimental animals is reported to produce glucose intolerance [10,11,13] associated with hyperinsulinemia and loss of normal in vivo sensitivity to insulin [12-14]. Results of the present study, which is in accordance with previous studies, showed high fructose feeding in rats for 60 days leads to fasting hyperglycemia, hypertriglyceridemia and hyperinsulinemia. Further, fructose induced insulin resistant animal model has been recommended for assessment of the therapeutic efficacy of insulin sensitizers and drugs that are likely to have effect on insulin sensitivity [29]. Therefore, this animal model was selected to study the efficacy of FIIc in preventing insulin resistance.

A variety of orally active hypoglycemic agents are frequently used to manage the glucose intolerance of NIDDM patients. But the effectiveness of these drugs is limited and suffers from a variety of side effects including hypoglycemia [30]. Many patients develop failure to oral anti-hyperglycemic agents and consequently need insulin therapy, which was disadvantages of its own (accurate dosing, danger of hypoglycemia, parenteral therapy and short shelf life). All these factors together reduce compliance. As Eugenia jambolana is commonly used fruit

in India and employed as a household remedy for diabetes. Earlier reports have suggested an anti-hyperglycemic effect of Eugenia jambolana [31-37]. Since the efficacy of this plant, more precisely active compound (FIIc) purified from it, has not been evaluated in models of insulin resistance. So, this study was undertaken.

The hyperglycemia, hypertriglyceridemia and hyperinsulinemia observed in fructose control group at 30 days were further intensified by 60 days of fructose feeding. Hypertriglyceridemia after fructose feeding results from the enhanced rate of hepatic VLDL-triglyceride synthesis [3,38] and a decrease in peripheral triglyceride clearance [39]. The increased gene expression of several lipogenic enzymes, including acetyl coenzyme-A carboxylase, fatty acid synthase and malic enzyme [40] is also responsible for the enhanced synthesis of triglyceride in the liver of fructose fed rats. Increased delivery of triglycerides to the muscle interferes with the utilization of glucose [41] impairing insulin action leading to hyperglycemia and hyperinsulinemia. As insulin resistance and reduced insulin binding have been reported in hypertriglyceridemic persons [42], this may be one mechanism by which fructose diets promote insulin resistance. FIIc treatment significantly improved fructose-induced hyperglycemia and hyperinsulinemia. These positive effects can be attributed to prevention of hypertriglyceridemia in these rats.

The ability of insulin to stimulate glucose disposal is markedly impaired as evidenced by increased insulin resistance in fructose fed rats indicating decline in insulin sensitivity in peripheral tissues. FIIc administration in fructose fed rats showed significant improvement in insulin resistance, indicating the ability of FIIc in promoting insulin sensitivity. However, the mechanism by which FIIc prevented insulin resistance by endogenous substances will be subject of future investigation.

Oral administration of FIIc for 60 days to the fructose fed rats significantly improved the blood glucose, insulin and TNF α levels which agrees with the previous reported studies [43,44]. High fructose diet also resulted in a significant reduction in the liver and muscle glycogen levels in rats [43]. FIIc significantly improved liver and skeletal muscle glycogen levels after 60 days of treatment. Further, improvement in insulin resistance following supplementation with FIIc can be explained by elevation of tissue glycogen levels which might be due to enhanced insulin sensitivity in peripheral tissues.

Oral administration of FIIc at a dose of 15 mg / kg lowers blood glucose, insulin, TNF α, insulin resistance and improved hypertriglyceridemia in fructose fed rats. If these results are extrapolated to humans, then FIIc might prove useful in the treatment and/or prevention of insulin resistance in non-diabetic states such as obesity and impaired glucose tolerance.

CONCLUSIONS

FIIc significantly prevents fructose induced hyperinsulinemia, hyperglycemia & hypertriglyceridemia. FIIc administration showed a significant effect on TNFα and significantly improved the altered levels of tissue glycogen. The administration of FIIc also resulted significant improvement in insulin resistance as calculated via HOMA assessment model. Our study showed that treatment with FIIc has a significant effect on fructose induced insulin resistance.

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DECLARATION OF INTEREST STATEMENT

All the authors state that no competing financial interests exist.

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