

# *Psidium guajava* Linn. leaf extract affects hepatic glucose transporter-2 to attenuate early onset of insulin resistance consequent to high fructose intake: An experimental study

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## ABSTRACT

**Background:** Insulin resistance (IR) is amalgam of pathologies like altered glucos metabolism, dyslipidemia, impaired glucose tolerance, non-alcoholic fatty liver disease, and associated with type-II diabetes and cardiometabolic diseases. One of the reasons leading to its increased and early incidence is understood to be a high intake of processed fructose containing foods and beverages by individuals, especially, during critical developmental years. **Objective:** To investigate the preventive potential of aqueous extract of *Psidium guajava* leaves (PG) against metabolic pathologies, vis-à-vis, IR, dyslipidemia, hyperleptinemia and hypertension, due to excess fructose intake initiated during developmental years. **Materials and Methods:** Post-weaning (4 weeks old) male rats were provided fructose (15%) as drinking solution, *ad libitum*, for 8 weeks and assessed for food and water/fructose intake, body weight, fasting blood sugar, mean arterial pressure, lipid biochemistry, endocrinal (insulin, leptin), histopathological (fatty liver) and immunohistochemical (hepatic glucose transporter [GLUT2]) parameters. Parallel treatment groups were administered PG in doses of 250 and 500 mg/kg/d, po × 8 weeks and assessed for same parameters. Using extensive liquid chromatography-mass spectrometry protocols, PG was analyzed for the presence of phytoconstituents like Myrecetin, Luteolin, Kaempferol and Guavanoic acid and validated to contain Quercetin up to 9.9%w/w. **Results:** High fructose intake raised circulating levels of insulin and leptin and hepatic GLUT2 expression to promote IR, dyslipidemia, and hypertension that were favorably re-set with PG. Although PG is known for its beneficial role in diabetes mellitus, for the first time we report its potential in the management of lifelong pathologies arising from high fructose intake initiated during developmental years.

**Key words:** Fructose, glucose transporter-2, insulin resistance, leptin, *Psidium guajava*, Quercetin

## INTRODUCTION

Insulin resistance (IR), is an amalgam of metabolic alterations including hyperinsulinemia, dyslipidemia, hypertension, impaired glucose tolerance, fatty liver and pro-inflammatory state.<sup>[1]</sup> Globally, lifestyle-related epidemic of IR has assumed gigantic proportions due to the cumulative effect of lack

of physical activity on one hand, and consumption of calorie dense diet, on the other hand. It is implicated in the pathophysiology of type 2 diabetes and cardiometabolic diseases, all of which reduce the quality of life with a concomitant increase in the risk of mortality.<sup>[2-4]</sup> IR knows no boundary, can affect any age-group and both lean as well as obese individuals with equal tenacity.<sup>[5]</sup> In India, the age-standardized prevalence rates of lifestyle-related metabolic alterations are 33.5% overall, 24.9% in males and 42.3% in females.<sup>[6]</sup> Moreover, the problem is not limited to adults alone as the prevalence of, IR was observed to be 30% in Asian Indian children and adolescents.<sup>[7]</sup>

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For 1000's of years, humans consumed about 16–24 g of fructose each day, mainly as fruits and honey obtained from foraging and agricultural activity. Today, the per capita amount of fructose consumed each day is up to 100 g.<sup>[8]</sup> Most of the consumed fructose is derived from refined or processed fructose in the form of high-fructose corn syrup (HFCS) (fructose concentration up to 90%) and is part of beverages, soda, jams, jellies, desserts, pre-packaged food such as breakfast cereals, baked goods etc.<sup>[8]</sup>

Emerging epidemiological data have irrevocably evidenced that the intake of HFCS as part of beverages, etc., increases total caloric consumption and is a critical contributor to metabolic and endocrine alterations.<sup>[9,10]</sup> In fact, high fructose consumption initiated during the critical developmental stages up to adolescence has been shown to chronically skew the metabolic system such that there is an early onset of IR and weight gain and this remains a life-long feature.<sup>[2,10]</sup> In view of the clinching evidence correlating HFCS as an agent of IR, urgent strategies that reduce biological availability of this agent may help to curtail the epidemic.

Orally ingested fructose is absorbed by passive transportation across membranes of enterocytes by members of the facilitative glucose transporter (GLUT) family.<sup>[11]</sup> One of the members, GLUT2, is a low-affinity bidirectional transporter that is involved in fructose uptake in the basolateral membrane of the intestine, across the hepatic plasma membrane into the liver and renal epithelial cells.<sup>[12]</sup> It is understood that a high flux of fructose to the liver significantly enhances the rate of *de novo* lipogenesis, triglyceride synthesis and other metabolic disturbances that ultimately cause the induction of IR.<sup>[3]</sup> We hypothesize that exposure to dietary fructose during critical developmental years affects the long-term expression of hepatic GLUT that may play a key role in the early development of pathologies.

*Psidium guajava* (PG) Linn, a member of the family of Myrtaceae, is commonly known as Amrood and grown in India. It is used not only as food but also as folk medicine in subtropical areas around the world because of its pharmacologic activities. In particular, the guava leaf has been traditionally used for the treatment of diabetes in East Asia and other countries and also evaluated for anti-hyperglycemic effect in some animal models.<sup>[13]</sup> However, the effect of guava leaf extract on absorption of fructose and in curtailing the metabolic alterations leading to the onset of IR remains unclarified.

The present experimental study attempts to mimic the clinical condition of early onset of IR due to excess fructose consumption initiated during critical developmental stages. The effect of aqueous extract of leaves of *Psidium guajava* Linn. (family-Myrtaceae) on paradigms of IR has been

investigated and an underlying putative mechanistic pathway via GLUT2 is postulated.

## MATERIALS AND METHODS

### Plant material collection, extract preparation and analysis

Fresh leaves of *Psidium guajava* Linn (family-Myrtaceae) were locally collected in the month of August and identified and authenticated by National Bureau of Plant Generic Resources (Indian Council of Agricultural Research), New Delhi, India, and voucher was provided (PG NHCP/NBPGR/2010–33).

Leaves of *Psidium guajava* were weighed, washed, dried and powdered. Powdered leaves were then mixed with deionized water in 1:10 ratio. The mixture was boiled, refluxed for 1 h at 100°C and filtered, frozen at – 30°C and lyophilized at – 70°C. The dried powder (PG) was subjected to phytochemical analysis using standard detecting agents. It tested positive for the presence of alkaloids, terpenoids, reducing sugars, saponins, tannins, flavonoids, steroids, proteins, oils and fat.

The extract was analyzed for phytoconstituents and validated with respect to Quercetin using liquid chromatography-mass spectrometry (LC-MS).

### Chromatographic and mass spectrometry conditions

Chromatography separation was achieved using Thermo Surveyor System (Thermo Electron Corp. Waltham, MA, USA) with a quaternary pump connected to an online degasser and photodiode array detector. Chromquest software version 4.1 (Thermo Electron Corp. Waltham, MA, USA) was used to control all parameters of HPLC. For the analytical separation of the compounds Purosphere RP-18, (150 × 4.6 mm), 5 µm column (Merck Millipore, Germany) was used. The mobile phase consisted of acetonitrile (A) and formic acid aqueous solution (B) using a gradient elution of 10–100% B at 0–13 min, 100% A at 13–15 min, 100–10% B at 15–17 min, 10% B at 17–20 min. The flow rate was set at 0.5 ml/min for run time of 20 min, and the sample injection volume was 20 µL.

Tandem mass spectrometric detection of analyte and internal standard (IS) was carried out using Applied Bio Systems 400 Q TRAP triple quadrupole instrument equipped with a TurbolonSpray (ESI) source (ABS Biosystems, Foster City, CA, USA) that operated in the positive ion mode. All data acquisition and peak integration were performed using Analyst 1.5.2 software from (ABS Biosystems, Foster City, CA, USA).

Mass spectrometry was operated with optimized conditions using inbuilt algorithm of Analyst software to detect

maximum ions. Quercetin was determined in positive ionization mode and quantified by Single-Ion-Monitoring mode, monitoring the mass-to-charge ratio ( $m/z$ ) of 303.03.

#### Preparation of standard solution

Working standards of Quercetin (Sigma Aldrich, USA) were prepared from the stock solution (10 ng/mL) by dilution with mobile phase containing IS. The working standard concentration ranged from 10 to 200 ng/mL. All standard solutions were filtered using a 0.45  $\mu$ m membrane filter (Millipore, USA) and injected directly.

#### Preparation of sample solution

The prepared extract of PG was analyzed for its phytoconstituents by MS. The dried powder was accurately weighed and dissolved in mobile phase containing IS to prepare a stock concentration of 1  $\mu$ g/ml that was directly injected into LC-MS under same conditions as that of standard.

Secondly, for the purpose of quantification for Quercetin concentration in the extract, the dried powder (PG) was accurately weighed and subjected to hydrolysis with hydrochloric acid (1:10 w/v) at 70°C for 30 min. The solution was centrifuged and the supernatant collected to obtain hydrolyzed extract, that was diluted (1:50 v/v) with mobile phase containing IS, filtered through a 0.45  $\mu$ m membrane filter (Millipore, USA) and injected to LC-MS under same conditions as that of standard.

#### Animals

Protocol for the animal experiment was approved by Institutional Animals Ethics Committee (protocol no. DIPSAR/2011/24). Wistar albino rats (male, 4 weeks old, 75–77 g) were selected for the study duration of 8 weeks.

### EXPERIMENTAL DESIGN

In order to experimentally mimic the condition of high fructose intake during critical developmental stages (childhood, pre-puberty, puberty, post-puberty and early adulthood) in rodents, post-weaning 4-week-old rats were provided fructose as drinking solution (15% w/v) *ad libitum*, over a study period of 8 weeks, that is, till they were 12-week-old adult rats.

For the study, the animals ( $n = 24$ ) were randomly divided into four groups, namely, control, fructose drinking rats (FDR), PG1 and PG 2. The animals in the control group were housed in polycarbonate cages maintained under standard laboratory conditions of temperature ( $25^{\circ}\text{C} \pm 2^{\circ}\text{C}$ ), humidity, dark and light cycle (12:12 h) and provided pre-weighed standard laboratory chow (Golden Feeds, India) and drinking water, *ad libitum*. The FDR group was provided similar laboratory conditions and pre-weighed chow and 15%w/v fructose as

drinking solution (Fischer Scientific, New Delhi) *ad libitum*, in lieu of drinking water. The lyophilized extract powder (PG) was freshly re-constituted in deionized water each day, so as to administer in the dose of 250 and 500 mg/kg/d, po to the animals in test groups PG1 and 2, respectively in addition to standard pre-weighed laboratory chow and 15%w/v fructose solution, under similar laboratory conditions.

#### Measurement of food and fructose/water intake and caloric intake

Pre-measured quantity of food, water or fructose was provided *ad libitum* to the animals, and its intake over the 24 h duration was recorded throughout the study duration. Average weekly data of food and fructose consumption for each group were calculated and compared. Using the data, the total caloric intake (=kcal of metabolizable energy/g diet intake + kcal of energy/g fructose intake) of each group for the study duration was calculated.

#### Measurement of body weight and visceral weight

Weekly body weight chart of each animal was recorded using sensitive digital balance and average body weight for each group was analyzed and compared. At the completion of study, the animals were euthanized by exposure to carbon dioxide and their organs (heart, kidney, liver) were surgically removed, washed with normal saline (0.9%), dabbed dry on blotting paper and weighed digitally.

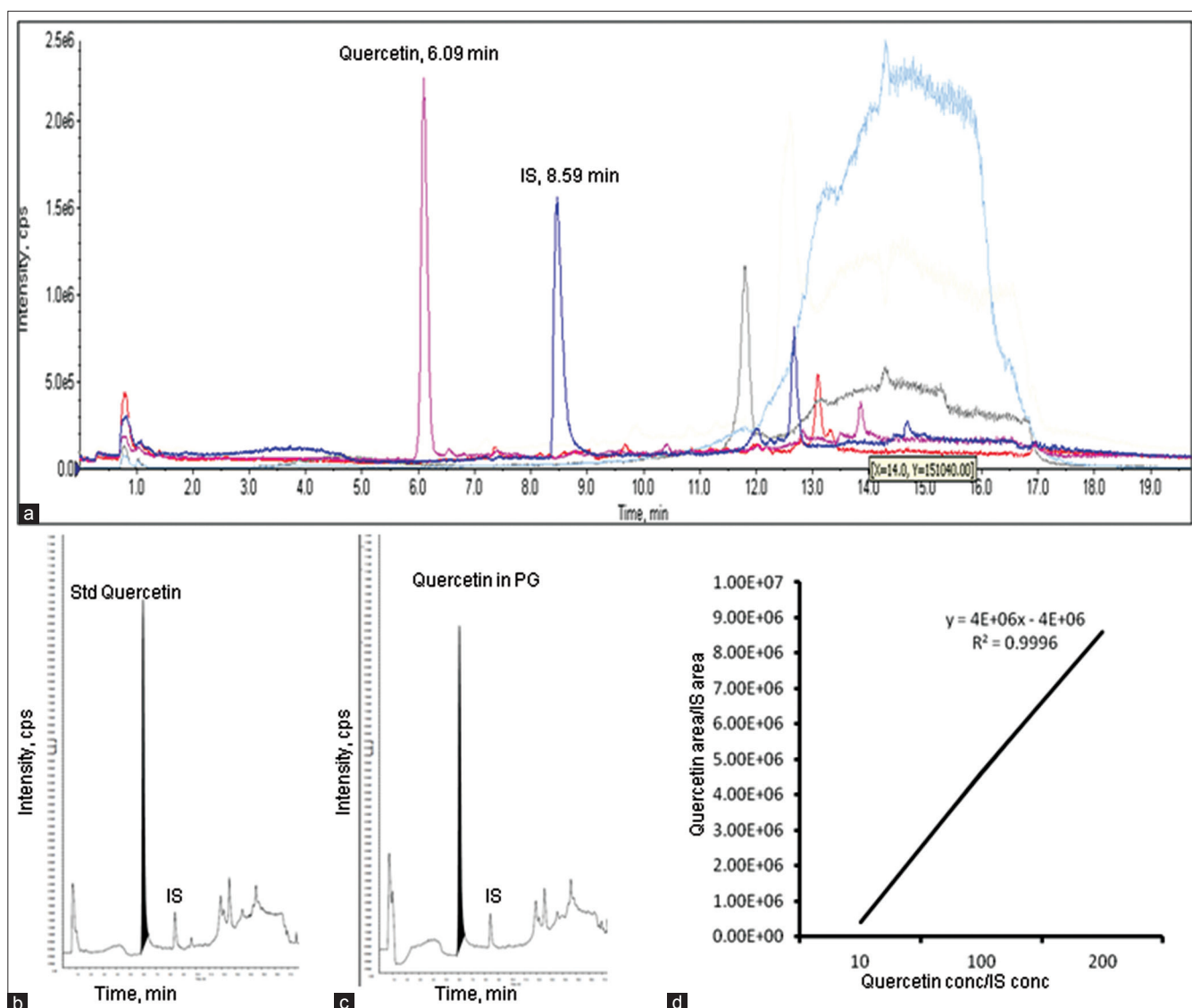
#### Measurement of fasting blood glucose and oral glucose tolerance test

Weekly fasting blood glucose (FBG) level of each animal was measured. Using fresh pricking stillite, few drops of blood were withdrawn from the tail of 12 h-fasted animal and FBG measured using a glucometer (ACCU-CHECK<sup>®</sup> GO, Hoffmann-La Roche Ltd). Homeostasis Model Assessment (HOMA)-index of IR was calculated using the formula:  $\text{HOMA IR} = 0.062 \times \text{glucose level (mg/dl)} \times \text{insulin (ng/mL)}$ .

At the end of 8-week study duration, whole body insulin sensitivity was assessed by oral glucose tolerance test (OGTT), according to the standard method.<sup>[14]</sup> Following 16 h fasting with free access to drinking water, the animals were fed glucose (2 g/kg, po). Using glucometer, blood glucose levels were estimated in blood sample drawn from tail vein of the animals at different time points-0, 15, 30, 60, 90, 120 min post glucose feeding and the average area under the curve (AUC) was calculated for each group.

#### Measurement of mean arterial pressure

Using non-invasive-rat-tail-cuff automatic blood pressure analyzer (CODA-6, Kent Scientific System, USA), mean arterial pressure (MAP) of the animals in all the groups



**Figure 1:** (a) Extracted ion-chromatogram at 303.040 Da of *Psidium guajava* (PG)+ internal standard (1 µg/ml). Single ion monitoring chromatogram of Quercetin in (b) reference standard (100 ng/mL), (c) hydrolyzed aqueous extract of leaves of PG. (d) Calibration curve of Quercetin showing the linearity in the concentration range of 10–200 ng/ml

were measured, at the start and end of the study. Before the actual measurement cycle, the animals were subjected to acclimatization cycles for 15 min to avoid variations due to stress and other confounding factors.

### Biochemical parameters

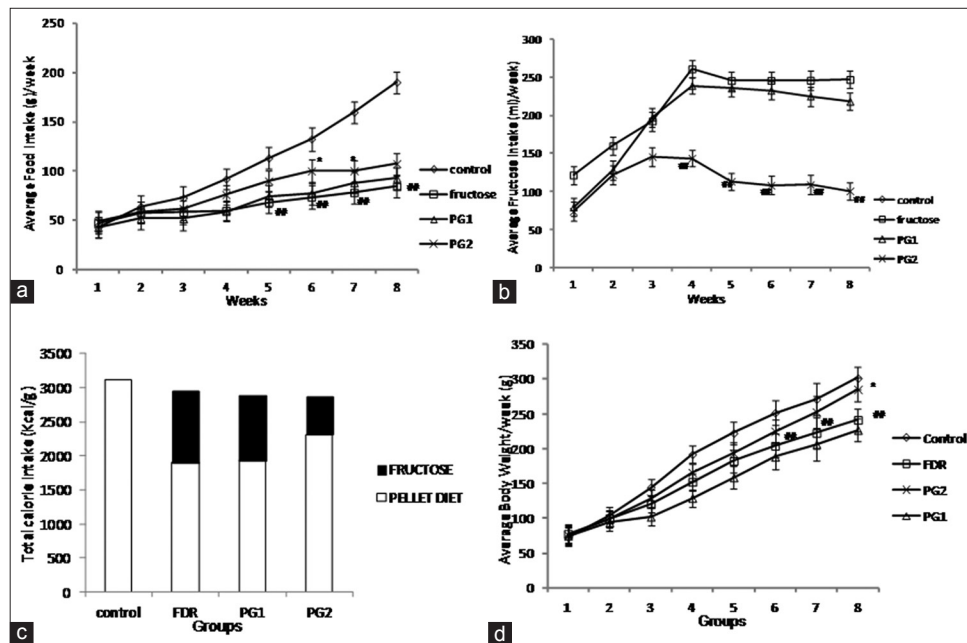
Upon completion of the study, small volumes of blood (up to 1 mL) were collected by cardiac puncture under xylazine anesthesia. The blood samples were clearly labeled, and plasma and serum were separated using standard protocols. All samples were preserved at -80°C till estimations were made. Following blood collection, the animals were euthanized using carbon dioxide and their liver isolated, sectioned and fixed (10% formalin) for histopathological processing and evaluation.

Biochemical estimation of fasting serum insulin and plasma leptin concentrations, (SPI bio, Germany), were conducted in the collected samples using respective Rat ELISA kits based on indirect sandwich enzyme immunoassay, in accordance with the protocol detailed by the manufacturer. In addition, lipid profile (Grenier Diagnostic, Germany) including serum total cholesterol, serum triglycerides, serum high density lipid (HDL), serum low density lipid (LDL) and serum very low density lipid (VLDL) were measured using protocols in accordance with the manufacturer's instructions.

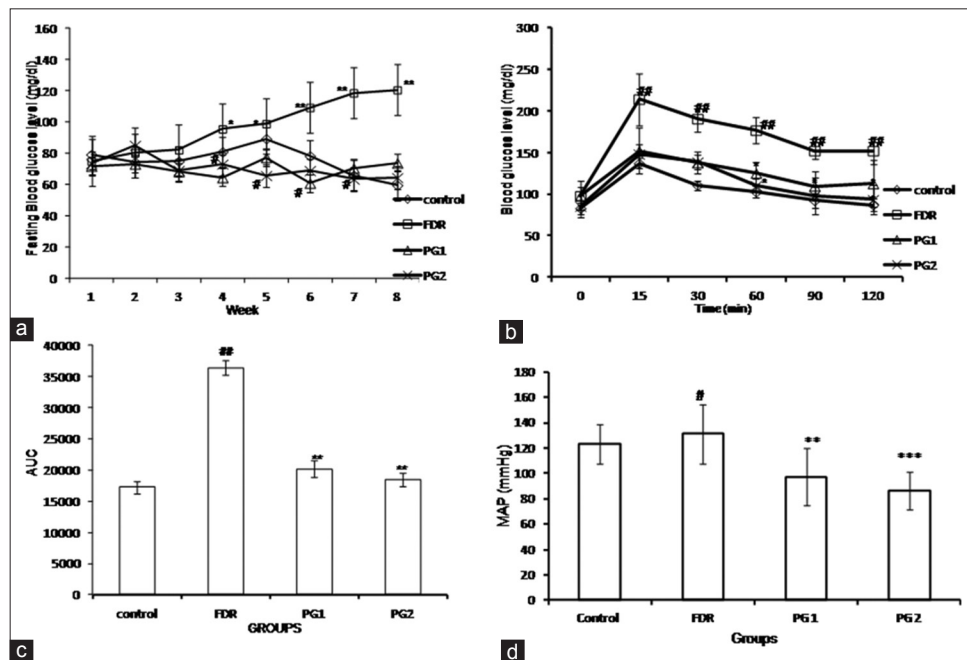
### Liver histopathology

Rat liver sections were fixed in fresh 10% neutral buffered formalin overnight at room temperature and





**Figure 2:** *Psidium guajava* (PG) ameliorates preferential intake of fructose over pellet food in rodents. Over 8 week study period, (a) average weekly food intake was significantly reduced in fructose drinking rats (FDR) group as compared to control, (b) average weekly fructose intake was significantly reduced in PG2 group as compared to FDR, (c) total calorie consumption in the various groups was not significantly different, but the fraction of calories preferentially derived from fructose was highest in FDR and lowest in PG2 and (d) average body weight in the various groups was not significantly different at the start of the study but at the end of the study, but was significantly lower in FDR as compared to control which was reverted in PG2. Each value represents mean  $\pm$  standard deviation for 6 rats in each group. \* $P < 0.05$  versus FDR, \*\* $P < 0.001$  versus control



**Figure 3:** *Psidium guajava* (PG) ameliorates excess fructose intake induced insulin resistance in rodents. As compared to control, fructose ingestion in fructose drinking rats (FDR) skewed (a) fasting blood glucose level, (b) blood glucose levels in response to oral glucose loading during oral glucose tolerance test (OGTT) (c) total area under the curves for blood glucose in OGTT, and (d) mean arterial blood pressure. In treated PG 1 and 2 groups, the paradigms were reverted toward control in a dose-dependent manner. Each value represents mean  $\pm$  standard deviation for 6 rats in each group. # $P < 0.05$  versus control, ## $P < 0.01$  versus control, \* $P < 0.05$  versus FDR, \*\* $P < 0.01$  versus FDR, \*\*\* $P < 0.001$  versus FDR

then embedded in paraffin. Section (4  $\mu$ m) were cut using a rotary microtome and stained in hematoxylin and eosin (H and E) stain for histopathological study.

### Hepatic immunohistochemistry

Paraffin embedded liver sections were subjected to heat-induced antigen retrieval as per standard protocol. The

sections were rinsed in PBS and blocked in 1% normal goat serum for 1 h at room temperature. The sections were then incubated in rabbit anti-rat GLUT2 (1:100 dilution) for 16 h at 4°C, washed in PBS, blocked in 1% normal mouse serum and incubated for 16 h at 4°C in mouse anti-rat GR. Appropriate secondary antibody was applied for 1 h at room temperature.

### Statistical analysis

Results are shown as mean  $\pm$  standard deviation (SD) of  $n = 6$  values. To analyze difference in variables, Student's *t*-test was used. Comparison between different treated groups was done using one-way ANOVA.  $P \leq 0.05$  was considered as statistically significant.

## RESULTS

### Validation of extract using liquid chromatography–mass spectrometry

In the unhydrolyzed extract, the presence of Myrecetin, Luteolin, Kaempferol and Guavanoic acid could be confirmed as protonated molecular ions  $[M + H]^+$  at 319.03, 287.04, 287.04 and 531.36, respectively and representative extracted chromatogram at 303.040 Da is presented in Figure 1a. Following hydrolysis of the extract, the presence of Quercetin could also be confirmed.

The matching of the retention times of mass spectra of the standard Quercetin [Figure 1b] with that from hydrolyzed sample of extract [Figure 1c], provided unequivocal confirmation of the identification of Quercetin in the extract. Following optimization of MS conditions the  $m/z$  303.03 precursor ion to the  $m/z$  153.08 product ion was used for quantification of Quercetin. The calibration curve was linear ( $R^2 = 0.9996$ ) in the 10–200 ng/mL range and by external calibration method, the concentration of Quercetin in the unhydrolyzed extract was calculated to be 9.9%w/w [Figure 1d].

### Effect of PG on food, fructose intake and total calorie consumption

The average weekly food intake was lower in FDR as compared to control group, and a significant difference ( $P < 0.01$ ) was recorded after 4 weeks into the study. The consumption of pellet diet in treatment groups PG2, was significantly greater than FDR [Figure 2a]. The average weekly fructose intake was highest in FDR group. The fructose intake in PG2 group was significantly lower than FDR group following 3 weeks of treatment [Figure 2b].

Total calorie intake of the animals over study duration in control, FDR, PG1 and PG2 groups was 3124.79, 2960.19, 2895.326 and 2879.706 Kcal/g, respectively. The fraction of calories derived from fructose in control, FDR, PG1 and PG2 groups was 0, 35.7, 33.04 and 19.57%, respectively [Figure 2c].

### Effect of PG on body weight, visceral weight and lipid profile

The initial body weight of the animals in the various groups was not different statistically. Over the study, there was a rise in the weekly average body weight of each group [Figure 2d]. Upon study completion, the average body weight gain of animals in control, FDR, PG1 and PG2 groups was 228.32, 163.72, 169, and 122.05 g, respectively.

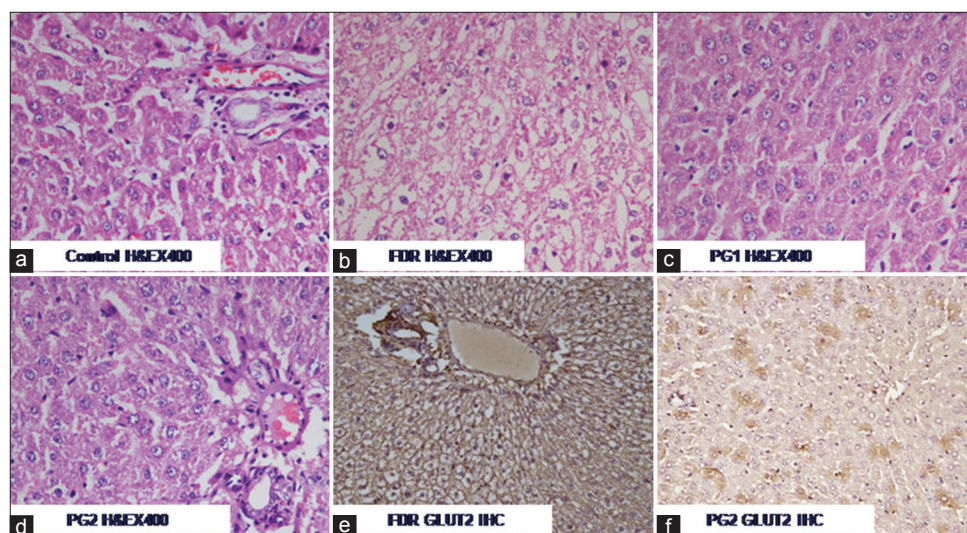
Mean wet weight of liver in FDR group was significantly ( $P < 0.05$ ) greater than that of the control group by over 1.87 g. Average wet weight of liver in PG1 and 2 groups was lower by 5.62 and 4.5 g, respectively, as compared to FDR group ( $P < 0.001$ ) [Table 1].

At the study end-point, significant elevation in plasma LDL, triglycerides, VLDL, total cholesterol, and fall in HDL levels were recorded in FDR group as compared to control [Table 1]. In PG treated groups, decrease in

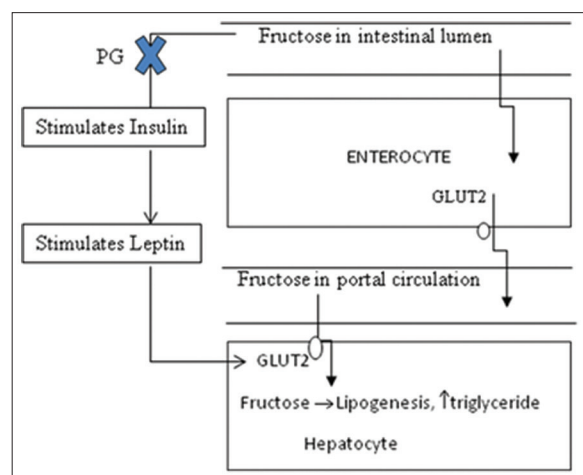
**Table 1: Effect of 8 weeks treatment with PG 1–2 on biochemical parameters and visceral weight in rodents provided with fructose as drinking solution during developmental stages.**

Parameter	Control	FDR	PG1	PG2
Liver Wt	11.31 $\pm$ 0.668	13.18 $\pm$ 0.624*	7.552 $\pm$ 0.746***	8.682 $\pm$ 0.419***
Left Kinney wt	1.1590 $\pm$ 0.01321	1.270 $\pm$ 0.105	0.8678 $\pm$ 0.0402***	0.9967 $\pm$ 0.0449*
Right Kidney wt	1.154 $\pm$ 0.0357	1.290 $\pm$ 0.0684	0.8483 $\pm$ 0.0322***	0.9317 $\pm$ 0.03558***
Heart wt	1.221 $\pm$ 0.07852	1.148 $\pm$ 0.07453	0.6193 $\pm$ 0.05435***	0.8437 $\pm$ 0.03960***
HDL (mg/dl)	25 $\pm$ 0.12	14 $\pm$ 0.18*	21 $\pm$ 0.22***	28 $\pm$ 0.11***
LDL (mg/dl)	27 $\pm$ 0.15	111.6 $\pm$ 0.957***	34. $\pm$ 2 0.22*****	26.0 $\pm$ 0.12***
TG (mg/dl)	140 $\pm$ 1.22	192 $\pm$ 0.957*	94 $\pm$ 0.8164***	105 $\pm$ 1.34***
VLDL (mg/dl)	28 $\pm$ 0.111	38.4 $\pm$ 0.878***	18.8 $\pm$ 1.77***	21 $\pm$ 0.343***
Total Cholesterol	80	164 $\pm$ 0.816***	74 $\pm$ 0.91***	75 $\pm$ 0.556***
OGTT-AUC	17307 $\pm$ 1011.1	36444 $\pm$ 1110.01	20208 $\pm$ 1023.6***	18486 $\pm$ 1005.45
Insulin (ng/ml)	0.482 $\pm$ 0.151	0.9711 $\pm$ 0.054**	1.619 $\pm$ 0.1677**	0.733 $\pm$ 0.289*
Leptin (pg/ml)	330 $\pm$ 75.11	544.6 $\pm$ 63.656*	258.3 $\pm$ 61.41**	129.8 $\pm$ 31.08***
HOMA-IR	2522 $\pm$ 0481	6.014 $\pm$ 0.435**	3.65 $\pm$ 0.554**	283 $\pm$ 0.373**

Each value is the mean  $\pm$  SEM of six rats. # $P < 0.05$  versus control, \* $P < 0.05$  versus FDR, \*\* $P < 0.01$  versus FDR, \*\*\* $P < 0.001$  versus FDR. FDR: Fructose drinking rats, SEM: Standard error of mean



**Figure 4:** *Psidium guajava* (PG) ameliorates fructose induced fatty liver and hepatic expression of glucose transporter (GLUT 2). Photomicrograph (H and E,  $\times 400$ ) of sections of liver showing (a) normal liver and hepatocyte trabeculae in control group; (b) parenchyma with macrovesicular fatty change in fructose drinking rats (FDR) group; (c) occasional hepatocyte with fat accumulation in PG 1 group; (d) absence of any fatty change in PG 2 group; photomicrograph ( $\times 200$ ) of immunohistochemical staining for GLUT 2 protein in liver sections of FDR (e) and PG 2 groups (f) showing abundant expression in former reduced to patchy appearance following treatment with aqueous leaf extract of PG (500 mg/kg/d)



**Figure 5:** Putative mechanism of ameliorative effect of *Psidium guajava* (PG) on the onset of insulin resistance (IR). Circulating insulin and leptin modulates hepatic expression of glucose transporter (GLUT 2) that in turn mediates the flux of fructose into liver, where it is taken up by biochemical pathways leading to synthesis of triglycerides and fatty acids. Treatment with PG favorably re-sets the insulin, leptin and GLUT2 talk to ameliorate the metabolic paradigms of IR

plasma triglycerides, total cholesterol, LDL and VLDL was recorded. In contrast, HDL level was increased in both the treated groups as compared to FDR group [Table 1].

#### Effect of PG on fasting blood glucose and oral glucose tolerance test

At week 0, there was no difference in the FBG level of the animals in the various groups. The FBG of FDR group ( $120.666 \pm 16.20$  mg/dl) was significantly greater ( $P < 0.001$ ) than that of control group ( $59.833 \pm 3.53$  mg/dl) at study end-point [Figure 3a].

Concomitantly, after 8 weeks of treatment with PG at the dose of 250 and 500 mg/kg/d, the FBG was recorded as  $73.833 \pm 5.6$  and  $64.5 \pm 7.43$  mg/dl that was significantly lower ( $P < 0.01$ ) as compared to FDR.

In the test for glucose tolerance, the peak blood glucose level was attained at 15 min post oral feeding. At this time-point, the glucose concentration in FDR was  $214.2 \pm 31.27$  mg/dl, as compared to  $137 \pm 6.02$  mg/dl in the control group. At 120 min, post oral glucose feeding, the glucose levels were still raised at  $152.2 \pm 16.22$  mg/dl in FDR. The glucose levels at 120 min in control and PG2 were at  $87 \pm 8.12$  and  $93.4 \pm 18.19$  mg/dl, respectively and marginally high at  $112.6 \pm 28.61$  mg/dl in PG1 group [Figure 3b].

The AUC OGTT-in control, FDR, PG1 and 2 groups was  $17307 \pm 1011.1$ ,  $36444 \pm 1110.01$ ,  $20208 \pm 1023.6$  and  $18486 \pm 1005.45$ , respectively. The AUC was significantly lower ( $P < 0.001$ ) in PG1 and 2 groups as compared to FDR [Figure 3c].

#### Effect of PG on mean arterial blood pressure

At the 8 week time point, the average MAP in FDR group ( $131.1833 \pm 23.061$  mmHg), was significantly greater ( $P < 0.05$ ) than the control group ( $123.33 \pm 15.275$  mmHg). In PG 2 group, MAP ( $86.515 \pm 14.82$  mmHg) was significantly ( $P < 0.01$ ) lower as compared to FDR group [Figure 3d].

#### Effect of PG on serum insulin, homeostasis model assessment-insulin resistance and plasma leptin levels

At 8-week time point, serum insulin concentration of the FDR group ( $0.971 \pm 0.054$  ng/mL) was



significantly increased ( $P < 0.001$ ) as compared to control group ( $0.4282 \pm 0.151$  ng/mL) [Table 1]. Significant reduction ( $P < 0.05$ ) in serum insulin concentration ( $0.733 \pm 0.289$  ng/mL) in PG2 group was recorded [Table 1]. Fructose drinking led to over 3-fold increase in HOMA-IR value of FDR group as compared to control group. Significant ( $P < 0.01$ ) reduction in HOMA-IR was recorded in PG2 ( $2.83 \pm 0.373$ ), as compared to FDR group ( $6.014 \pm 0.435$ ).

At the end of the 8<sup>th</sup> week of the study, fasting plasma leptin levels in control group was  $330.4 \pm 75.11$  pg/mL, that was significantly ( $P < 0.05$ ) lower than FDR group ( $544.4 \pm 63.65$  pg/mL). In PG2 group, a significant decrease ( $P < 0.001$ ) in leptin concentration was recorded as compared to FDR group.

### Effect of PG on liver histopathology

Histopathological study was carried out to observe any change in liver at cellular level. At the end of the 8 week study, the histology of sections of normal liver from control group showed normal hepatocyte trabeculae [Figure 4a], whereas FDR group shows macro vesicular fatty changes [Figure 4b]. Administration of aqueous leaf extract of PG in the dose of 250 mg/kg/d reduced fatty change to focal presence [Figure 4c]. Further, in PG2 group there was the absence of any fatty change [Figure 4d].

### Effect of PG on liver immunohistochemistry

Expression of GLUT 2 in the hepatocytes of FDR was observed to be evenly and abundantly distributed as determined by immunohistochemistry [Figure 4e]. Treatment with aqueous leaf extract of PG in the dose of 500 mg/kg/po reduced the hepatic GLUT 2 protein abundance to patchy appearance [Figure 4f].

## DISCUSSION

The lifestyle associated pathological states like IR are gaining demonic proportions and are a serious cause for concern as they affect productive years and translate into lifelong cost burden due to health related issues. It has been suggested that one of the main culprit behind the onset of these morbidities is ingestion of excess fructose in the form of processed foods and beverages starting from critical developmental stages (i.e., childhood to early adulthood).<sup>[9]</sup> Besides lifestyle modifications, there is a paucity of prevention and treatment modalities for lifestyle associated diseases and the present study addresses this lacuna.

The study design involves providing fructose (15% w/v) as drinking solution to animals starting at post-weaning infant age of 4 weeks up to when they were 12-week-old

adults. The total study span of 8-week can be delineated into two halves, where, the first 4 weeks (age span of animals ~ 28–56 days) of the study represent critical developmental stages of pre-puberty, puberty and adolescence and the latter 4 weeks (age span of animals ~ 56–84 days) represent early adulthood. Using this simulation, the present study reports, the ameliorative effect of leaf extract of the common guava tree (*Psidium guajava* Linn, Myrtaceae) against pathologies induced by fructose intake initiated during developmental years in rodents.

In traditional folk medicine, the leaf extract of *Psidium* is advocated as a remedy for diabetes and pharmacologically established for anti-inflammatory, antioxidant, anti-microbial activities.<sup>[13,15-17]</sup> Phytochemical analysis of aqueous leaf extract of *Psidium guajava* demonstrated the presence of alkaloids, terpenoids, reducing sugars, saponins, tannins, flavonoids, steroids, proteins, oils and fat. It is reported that Quercetin, a flavonoid, is present in the leaves as flavanol-glycoside and attributed with the clinical effects of the plant.<sup>[18]</sup> Using LC-MS derived external calibration method, Quercetin was calculated to be present in a concentration of 9.9%w/w. Quercetin is naturally present as glycoside and extracted as such in an aqueous extract. Upon hydrolysis, the aglycone moiety-Quercetin could be detected, analyzed and quantified.

It is widely accepted that sweetness is a preferred taste as well as acquired one that may be enhanced by repeated exposure to sweet diet. It is reported that the consumption of sweetener is more when it is in beverage form than when it is in solid form.<sup>[19]</sup> Further, in presence of calorically sweetened solution, there may be a reduction in the intake of solid food.<sup>[9]</sup> We also report here preferential ingestion of fructose over pellet diet by the animals. The total caloric intake (fructose + pellet diet) of the animals in the FDR group is about 6% more than those in the control group. However, animals in FDR group preferred fructose over pellet diet and a large portion of the calories (~35%) were derived from it. Although the total body weight in control was greater than FDR group, the liver weight in latter was greater. Thus, in accordance with the literature, this study also evidences that metabolic milieu and phenotype is more critical marker for IR than weight gain/obesity.<sup>[5]</sup> On the other hand, PG (250 and 500 mg/kg) treated animals showed greater preference for pellet diet than fructose that contributed diminishing fractions of 25.9 and 18.71%, respectively of total calories, and a marked less weight gain than FDR or control group.

Here, we report elevation in circulating levels of fasting plasma insulin levels in FDR group as compared to control group. Blakely *et al.* showed a significant increase in fasting serum insulin concentration in rats that were provided 15% fructose.<sup>[20]</sup>



Circulating insulin concentration along with HOMA-IR values indicate the development of IR, wherein to achieve euglycemic condition, greater concentration of insulin is required. HOMA-IR, a fasting IR index, helps to designate IR as normal, borderline high and high when values range as  $< 2.60$ , between  $2.60$  and  $3.80$  and  $> 3.80$ , respectively.<sup>[21]</sup> By this scale, in the present study, fructose intake clearly led to the development of IR in FDR group (HOMA-IR- $6.014 \pm 0.435$ ). Eight weeks of administration of aqueous extract of leaves of *Psidium guajava* (500 mg/kg/day) favorably attenuated the HOMA-IR value to 2.83 that may be categorized as “borderline high”. Treatment with PG showed a significant decrease in plasma insulin concentration, HOMA-IR values and fasting blood sugar than FDR group. So, initiation of fructose in the diet during developmental years led to the manifestation of IR at early adulthood, but PG could mitigate the pathology.

The mechanisms of absorption and metabolism of orally ingested fructose are not well understood. Recently, it has been shown that oral load of fructose, rapidly induces release of gastric leptin that enters the intestine at a concentration compatible with the activation of leptin receptors. It has been proposed that a positive regulatory control loop exists in which fructose triggers release of gastric leptin that, in turn, up-regulates GLUTs and concurrently modulates metabolic functions in the liver.<sup>[22]</sup> In the present study, FDR group recorded hyperleptinemia, but treatment with PG favorably lowered leptin levels toward control [Figure 5].

The liver plays a major role in metabolic milieu and phenotype determination as it is a site for substrate metabolism and primary target of insulin action. Following release in response to glucose load, insulin binds to the hepatic insulin receptor to elicit key gene transcriptions that lead to the expression of GLUTs. Preliminary evidence suggests that fructose is passively absorbed in small intestine via facilitated transport involving GLUT. Fructose is then transported out of the enterocytes and into the portal circulation, across basolateral membrane by low-affinity, bidirectional transporter, GLUT2. From portal circulation, fructose is transported across hepatic plasma membrane into the liver via GLUT2 transporter where it undergoes metabolism pathways initiated by fructokinase, the net effect of which is, fatty acid, TG and VLDL biosynthesis and *de novo* lipogenesis.<sup>[20]</sup> In condition of IR enhanced hepatic *de novo* lipogenesis (excess TG, low HDL-cholesterol, elevated LDL particles, intrahepatic accumulation of FFA and lipids) is recorded.

Here, we demonstrate that the hepatic expression of GLUT2 in animals treated with PG was markedly lower than that in FDR group indicating a lower flux of fructose from the portal circulation to the liver. Further, above-mentioned markers of

hepatic *de novo* lipogenesis were in affirmative in FDR group but not in control, PG1 or PG2 groups, clearly indicating genesis of hepatic IR in former but not in latter. In addition, at study end-point, rise in MAP was recorded in FDR, but was favorably attenuated by PG in a dose-dependent manner.

Taken together, for the first time, we propose a molecular mechanistic pathway by which beneficial action of PG in retarding IR and weight gain due to fructose intake may be explained. It may be hypothesized that on one hand, PG reduces preferential intake of fructose and on the other hand, interferes with its hepatic uptake. The mechanism underlying change in preference of PG treated animals is worth exploring. In order to address the query if animals underwent taste aversion, a small study was undertaken (data not presented here) as per reported protocol.<sup>[23]</sup> In comparison to standard 5-HT (20 mg/kg, ip), where a pronounced rejection of sweet beverage was recorded in fluid deprived animals, there was no such behavior exhibited despite repeated administration of PG up to dose of 1000 mg/kg, po.

## CONCLUSION

The results of this study indicate an early development of insulin insensitivity, leptin resistance, hyperinsulinemia, weight gain, hypertension and hypertriglyceridemia in 8 week period when post-weaning developing rats were fed 15% fructose solution *ad libitum* and aqueous extract of leaves of *Psidium guajava* conferred protection from the same.

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