Raphanus sativus (Linn.) fresh juice priming moderates sucrose-induced postprandial glycemia as well as postprandial glycemic excursion in rats

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Consumption of sugar sweetened beverages increase individual’s susceptibility towards weight gain, development of Type 2 diabetes mellitus, hypertension, cardiovascular disorders and number of chronic illnesses. Extended postprandial hyperglycemic rise and larger postprandial glycemic spikes following sugar ingestion in diabetic as well as non-diabetic individuals relate with the onset of cardiovascular complications and development of atherosclerosis. Oxidative stress induced due to prolonged postprandial hyperglycemia further aggravates development of diabetic complications. Influence of Raphanus sativus leaf and root juice on sucrose induced postprandial glycemic rise and postprandial glycemic excursions in rats was evaluated in this research. It was observed that priming rats with R. sativus leaf and root juice assuaged sucrose induced postprandial glycemic rise and postprandial glycemic excursions. The juice of R. sativus leaf was found superior than root juice in achieving these results. Similarly, the juice of leaves was more potent (p<0.01) in decreasing sucrose induced postprandial glycemic load than the juice of root. Acidic in nature, juice of R. sativus leaf and root was rich source of polyphenol, flavonoid and displayed potent free radical scavenging activity. Additionally, juices also mitigated formation of advance glycation end-products and glycation of hemoglobin under hyperglycemic environment.

Keywords: Antioxidant activity, α-Glucosidase inhibition, Postprandial glycemic excursion, Postprandial hyperglycemia, Raphanus sativus
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A century ago, Bang1 introduced method of blood sugar estimation for clinical application and Jacobsen2 prepared sketch of time dependent blood glucose concentration curve following different types of food in normal, diabetic and gravid individuals. Since then, postprandial blood glucose concentration curve has become an important determinant under different settings to investigate and monitor efficiency of glucose regulating mechanisms in body3. Despite the fact that several conditions videlicet hepatic disorders, inflammatory conditions, stress, uremia, hypertension, schizophrenia, alcoholism, gastric or duodenal ulcers, manic disorders, neoplastic disease, phaeochromocytoma and gout etc, unfavorably affect carbohydrate metabolism and influence postprandial blood glucose curve3, it has been extensively used as a diagnostic criterion in diabetes mellitus, endocrine disorders, carcinoma and arthritis etc4.

The rapid rise and large increase in blood glucose levels following a meal in diabetic as well as non-diabetic individuals have found in relevance with the onset of cardiovascular complications and has been recognized as an important contributing factor towards development of atherosclerosis5. The progressive depreciation in pancreatic β-cells to produce insulin and ability of cells and tissues to properly utilize glucose, deteriorates glucose homeostasis and hence accentuate postprandial hyperglycemia. Furthermore, in postprandial state, the height of glycemic excursion following a meal plays an important role in exacerbating disease conditions6,7. In addition, prolonged postprandial hyperglycemic condition in diabetic and pre-diabetic individuals induces oxidative stress8. The transient postprandial hyperglycemic spikes coupled with oxidative stress in dysglycemic individuals represent independent risk factor aggravating endothelial dysfunction and development of diabetic vascular complications8. Therefore,
monitoring of postprandial glycemia and glycemic excursion has become an important step to manage glycemic control\textsuperscript{7–9}.

The dietetics has been referred to as the primary strategy for treatment of postprandial hyperglycemia\textsuperscript{7}. Consumption of green vegetables has been shown to be a promising dietary milieu in maintaining glucose homeostasis both in experimental animals\textsuperscript{10} and clinical studies\textsuperscript{11, 12}. Raphanus sativus (Linn.) has been consumed by mankind as vegetable for eons. Apart from its use as a food material, the traditional Indian medical classics have described use of R. sativus (Mūlaka) beneficial in conditions of diarrhea, gastro-enteritis, piles, jaundice, anemia, leprosy, erysipelas, edema, dysuria, oxidative stress, rheumatoid arthritis, tumor, chronic coryza, asthma, hiccough, cough and urticaria\textsuperscript{13}. Recently, the multifactorial antidiabetic properties in R. sativus have been discussed\textsuperscript{14}. However, the evidence of its benefits in modulating postprandial hyperglycemia and postprandial hyperglycemic excursions remains to be examined in experimental animal studies and clinical cases.

In this research, Wistar rats were first primed with fresh juice of leaves and roots of R. sativus and then fed with sucrose solution. The sucrose induced postprandial glycemia and glycemic excursions in juice-primed and unprimed rats were examined. Simultaneously, antioxidative potentials of these juices were also evaluated in various in vitro experimental models.

**Material and Methods**

**Material**

Green leaved white root R. sativus Linn. (fam. Brassicaceae; En. Radish, Hin. Mooli and San. Mūlaka) was procured from local vegetable vendors (Hyderabad). Healthy green leaves were separated from white root and washed properly under running water. Leaves and root were chopped into small pieces separately. Fine smoothie was made out of chopped leaves and root separately in food grade grinder. Smoothie was placed on clean muslin cloth and squeezed to get juice. Juice of leaves and root were centrifuged at 7500 rpm for about 10 min at 20°C and clear supernatant was used for phytochemicals analysis and in vitro experiments. The pH of juices was measured with pH meter (Eutech Instruments, Singapore) Fresh juice was prepared each time for experimental purpose.

**Animal experiment**

Wistar rats were procured from National Institute of Nutrition, Hyderabad and were housed in animal house facility of Shri Vishnu College of Pharmacy, Bhimavaram (Reg. No. 439/PO/RE/S/01/CPCSEA). Animal experiment was performed using adult Wistar rats (200±20 g body weight). Institutional Animal Ethical Committee approval for experiment was obtained. Animal experimentation was performed as per the procedure and dosage described earlier\textsuperscript{15}. Experiments with live animals were conducted in compliance with the relevant laws and institutional guidelines. Rats were kept for overnight fasting. Next day forenoon, blood was collected from retro orbital plexus in EDTA containing tubes. Fasting plasma glucose levels (“0” h) were measured by glucose-oxidase determination method using semi-auto analyzer (Biochemical Systems International, Arezzo - Italia). Rats were divided into three groups (four rats in each group) as follows:

1. Control (Sucrose treated) group

   In this group of rats, normal saline solution (7.5 mL/kg body weight) was administered orally 15 min before oral administration of sucrose (2 g/kg body weight) solution (28%) prepared in drinking water.

2. Leaf juice group

   In this group, rats were primed orally with freshly prepared juice of R. sativus leaves (muslin cloth squeezed juice, 7.5 mL/kg body weight) 15 min before oral administration of sucrose solution as above.

3. Root juice group

   Rats of this group were given freshly prepared R. sativus root juice (muslin cloth squeezed juice) orally 15 min before oral feeding of sucrose solution in the dosage described above.

In all the groups, oral feeding of test samples was completed before 10:30 AM. Rats did not show any sign of discomfort after feeding of R. sativus juice. Blood samples were collected at intervals of 30, 60, 90 and 120th minute post-sucrose feeding. Plasma was separated out for glucose estimation as described above. Plasma glucose concentration curve was plotted against each time point. The postprandial glycemic load (AUC\textsubscript{0–120} min mg/dL/hr) was calculated following trapezoidal rules\textsuperscript{16}. Postprandial glycemic excursion values (delta, Δ-glucose, mg/dL) at 30, 60 and 90 mins were calculated by subtracting plasma glucose values from their respective ‘0’ min values.

**Phytochemicals analysis and in vitro biological activities evaluation**

**Chemicals and reagents**

Aluminium chloride hexahydrate (AlCl\textsubscript{3}.6H\textsubscript{2}O), bovine serum albumin (BSA), crude rat intestinal
acetone powder, gallic acid, gentamycin, β-nicotinamide adenine dinucleotide hydrate (NADH), nitro-blue tetrazolium (NBT), p-nitrophenol α-D glucopyranoside, phenazine methosulphate (PMS), trichloro acetic acid (TCA), tris-HCl, and rutin were purchased from Sigma-Aldrich, St. Louis (USA). Folin–Ciocalteu reagent was obtained from Merck Specialties Pvt. Ltd Mumbai (India). Other fine chemicals of analytical grade were acquired from Indian manufacturers.

**Total polyphenol**

The reaction mixture containing 25 μL juices, 2.5 mL distill water, 250 μL of 1N Folin-Ciocalteu reagent and 250 μL Na₂CO₃ (20% w/v) in 5 mL vial were incubated for 60 min in dark. The blue chromophore constituted by phosphotungstic-phosphomolybdenum complex was measured at 765 nm spectrophotometrically (BioTek synergy4, BioTek Instruments Inc, Winooski, VT, USA) 15. Different concentrations of gallic acid were prepared and processed accordingly. Total polyphenol content in juices were calculated fitting their absorbance values in regression curve formula of gallic acid. All the experiments were carried out in triplicate. Total polyphenol content in juices of leaf and root was expressed as gallic acid equivalent.

**Total flavonoids**

Total flavonoids concentration in juice was estimated by reacting 125 μL of juices with equal volume of AlCl₃.H₂O (2% w/v) prepared in methanol as described earlier 15. Absorbance was read at 415 nm spectrophotometrically. Various concentrations of flavonoid rutin was prepared and processed accordingly to prepare standard curve. Flavonoid content in juice was expressed as rutin equivalent. All the experiments were carried out in triplicate.

**Super oxide radical (O₂⁻) scavenging**

Protection of NBT reduction by O₂⁻ radical generated due to oxidation of NADH in presence of PMS under aerobic condition by juices was assayed following method of Lee et al. (2011) 17 with suitable modifications. Reaction mixture constituted 1mL tris-Buffer (16 mM, pH 8.0), 100 μL NBT (50 μM), 100 μL NADH (78 μM) and 70 μL test sample into Eppendorf tubes. Reaction was started by addition 100 μL of PMS (1M) to the mixture and incubated at 25°C for 5 min. Absorbance was measured spectrophotometrically at 560 nm. Percentage of O₂⁻ radical scavenging activity by juices was calculated as follows: [(Ac - Aj)/Ac] x100 where, Ac represents absorbance of control samples without juice and Aj absorbance in presence of juice.

**Advanced glycation end-products (AGEs)**

200 μL BSA (10 mg/mL in 67 mM sodium phosphate buffer containing 3 mM sodium azide, pH 7.4) was incubated with 200 μL glucose solution (50 mg/mL in sodium phosphate buffer, pH 7.4) in presence of 200 μL respective juices. Incubation was carried out for 7 days at 37°C. Intrinsic fluorescence of juices was balanced by individual blank maintained for each sample. After incubation, 60 μL of 100% TCA was added to each reaction mixture and centrifuged at 15000 rpm for 4 min at 4°C. Pellet was collected and washed with 600 μL of 10% TCA. Washed pellet was dissolved in 572 μL of alkaline phosphate buffer (pH 10.6). Fluorescence of vespertysine-like (λexc 370 nm; λem 440 nm) and pentosidine-like (λexc 335 nm; λem 385 nm) AGEs was measured spectrophotofluorometrically 18. Percentage inhibition of AGEs formation in presence of juices was calculated as follows: [(fc - fj)/ fc] x100 where fc represented fluorescence of control samples and fj was fluorescence of test sample.

**Hemoglobin glycation**

Blood from Wistar rats was collected into tubes containing EDTA and centrifuged at 1000 rpm for 20 min at 4°C. Pellet was suspended in phosphate buffer saline (pH 7.4). Pellet containing RBC’s were lysed using 2 volumes of cell lysis buffer (1:2, cell volume: lysis buffer) and centrifuged at 1000 rpm for 20 min. Supernatant containing hemoglobin (Hb) was diluted with PBS (pH: 7.4) to final concentration 5 mg/dL. 0.5 mL of Hb was incubated in presence of 20 μL juice for 10 min and then added with 0.5 mL glucose solution (2%) containing 200 mg/mL gentamycin for 72 h. Glycated hemoglobin (HbA₁c) was calculated by measuring absorbance at 443 nm spectrophotometrically 19. Percentage of inhibition of HbA₁c formation by juices was calculated as follows: % inhibition of HbA₁c = [(Ac - Aj) / Ac] x100 where Ac becomes absorbance of control and Aj absorbance in presence of juice.

**Free radicals induced DNA damage**

Method described by Chang et al. 20 was adopted with suitable modifications to assess genomic DNA damage. Reaction was carried out in tris-buffer (pH 7.4) at 37°C. FeCl₃, H₂O₂ were used to generate hydroxyl radicals (OH). In an Eppendorf tube, for control test, genomic DNA (2 μL) was incubated with
5 µL of tris-buffer. In another set, genomic DNA (2 µL) along with 5 µL of tris-buffer was enacted with FeCl₃ (5 µL) and 10 µL of 30% H₂O₂. The next set was prepared with genomic DNA (2 µL), 5 µL of tris-buffer and 5µl of juices and incubated for 10 min at room temperature. FeCl₃ (5 µL) and 10 µL of 30% H₂O₂ were added to induce free radical reaction. Volume of reaction mixture was equated with addition of tris-buffer. Tubes were incubated at 37°C for 15 min. Thereafter, 3 µL of 6X gel loading dye was added. Electrophoresis was performed on 0.8% agarose gel containing 3 µL ethidium bromide (10 mg/mL), at 85 V for 35 minutes. Gel was viewed under transilluminating UV light and photographed (Bio-Rad Chemi Doc™ XRS+ with Image Lab™ Software).

**Intestinal α-glucosidase activity assay**

One gram of rat intestinal acetone powder was suspended in 10 mL of normal saline and sonicated for 3-4 min using probe sonicator. Centrifugation was carried out at 7500 rpm for 30 min at 4°C and supernatant was treated as crude intestinal α-glucosidase enzyme. In a 96 well plate, 20 μL test sample was constituted with 100 μL phosphate buffer (100 mM, pH 6.8) and incubated with 50 μL of crude intestinal α-glucosidase enzyme for 10 min. Microplate was placed on spectrophotometer platform and 50 μL p-nitrophenyl-α-D-glucopyranoside (5 mM) substrate was added. Kinetics of reaction was recorded for 5 min at interval of one min at 405 nm as function of enzyme activity. Individual blank for each test sample was prepared to nullify background absorbance. Enzyme activity over time was plotted with obtained absorbance values.

**Statistical analysis**

One-way ANOVA followed by Tukey’s multiple comparison tests was applied to compare differences within the groups. Unpaired t-test (two tailed) with Welch’s correction was applied to compare differences between the groups. Criterion for statistical significance was p<0.05. Statistical analyses were performed using GraphPad PRISM® Version 5.01 (GraphPad Software. Inc., CA, USA).

**Results and Discussion**

Steady increase in sugar sweetened beverages consumption even as little as two serving per week, has been linked with increased risk for onset of weight gain, development of Type 2 diabetes mellitus, hypertension, cardiovascular diseases and other chronic illnesses among all age groups. Progression of these diseases is closely linked with development of insulin resistance, pancreatic β-cell dysfunction, visceral adiposity, dysglycemia, dyslipidemia and inflammation. Furthermore, large spikes in blood glucose levels and insulin response have been recorded following consumption of sucrose-sweetened beverage within first hour. Similar picture emerged in our study when rats were orally administered high sucrose solution in fasting state (Fig. 1a). There was sharp rise in plasma glucose level following oral administration of sucrose and glucose level remained high in these rats even at 120 min. However, when rats were primed with leaf or the root’s juice of *R. sativus* before sucrose...
feeding, the postprandial glycemic rise was dramatically assuaged. Decrease in sucrose induced glycemic increase was more potent in leaf juice primed rats than that in the root juice primed rats (Fig. 1a). Attenuation of glycemic spike at 30th min after sucrose feeding was significant (p<0.01) in leaf juice primed rats than in root juice when compared with the rise in control group rats at this time point. Later, 60th min (p<0.01 in case of root juice priming and, p<0.001 in case of leaf juice) and onwards up to 120 min both leaf and root juice priming significantly (p<0.001) decreased sucrose induced postprandial glycemic rise in rats (Fig. 1a).

Total glycemic load calculated as area under the curve (AUC) shows that priming rats either with leaf juice or root juice of *R. sativus* significantly (p<0.001) reduced sucrose induced glycemic load (Fig. 1b). Interestingly, leaf juice was more potent (p<0.01) in assuaging sucrose induced glycemic load than root juice (Fig. 1b) These findings highlight importance of *R. sativus* leaves which is mostly discarded by consumers for preference of root. The reason *R. sativus* leaves juice displayed more potent antihyperglycemic activity may be due to the presence of high content of antidiabetic compounds sulforaphanes14, 24.

The first hour high spike in plasma glucose levels also depend on digestibility of carbohydrates. Faster is the digestion and absorption, rapid will be the glycemic spikes. To slow down digestion of carbohydrates, inhibition of pancreatic α-amylase and intestinal α-glucosidase activities have been an important target. Aqueous extract of radish leaves has been shown to possess potent pancreatic α-amylase and intestinal α-glucosidase inhibitory activity25. Data presented in Fig. 2 shows that leaf juice of *R. sativus* inhibited intestinal α-glucosidase activity better than the root juice. However, this activity could not reach level of statistical significance in our study. Therefore, the role of intestinal α-glucosidase activity alone as observed in our study cannot fully be ascribed to the observed antihyperglycemic activity in rats. *R. sativus* has been observed to regulate hormones related with glucose metabolism, maintaining the balance between glucose absorption and uptake, increasing insulin sensitivity by enhancing synthesis of adiponectin and decreasing insulin resistance by inhibiting protein-tyrosine phosphatase 1β activity24, 26, 27.

Flavonoids have been reported as active phytochemicals modulating number of enzymes activities28. Leaf juice presented twelve time higher flavonoids content than that present in root juice (Table 1). Therefore, higher flavonoids content in leaf juice of *R. sativus* may be ascribed to better intestinal α-glucosidase inhibitory activity as observed in our study.

Postprandial glycemic excursion disturbs metabolic homeostasis in mild to moderate hyperglycemic patients6. Although, the absolute codification of time to measure postprandial glycemic excursion is not yet decided, the peak time of up to 72 min post meal has been considered suitable7. Furthermore, postprandial spikes (delta-glucose) of 30-50 mg/dL in blood glucose have been considered as normal at these time points7. Fig. 3 presents postprandial glycemic excursion in rats
following sucrose administration. It is evident from Fig. 3 that there was more than 58 mg/dL glycemic spike at 30th minute in juice-unprimed rats. However, when rats were primed with leaf or root juice before sucrose feeding, decrease (29%, p<0.05) in postprandial glycemic excursion was observed (Fig. 3). Similarly, feeding rats' juice of *R. sativus* leaf or root significantly (p<0.001) attenuated postprandial glycemic spikes between 60 and 90 min post-sucrose administration (Fig. 3). The superiority of leaf juice over the root's juice in mitigating postprandial glycemic excursion (at 60th and 90th min) is clearly distinguished although the differences could not reach level of statistical significance (Fig. 3).

Acute postprandial hyperglycemia accelerates overproduction of reactive oxygen species and increases formation of non-enzymatically glycated proteins (AGEs)⁸. Similarly, increased glycated hemoglobin (HbA1c) levels are considered reflection of postprandial glycemic excursion and total postprandial glycemic load⁷. Therefore, scavenging of overtly generated reactive oxygen species and prevention of AGEs formation has been considered important aspects to reduce risk of macro- and micro vascular complications development. In a recent study, levels of plasma fluorescent AGEs pentosidine-like AGEs in particular, were observed associated with development of major adverse cardiovascular events in Type 2 diabetes cases²⁹. Analysis presented in Table 1 shows that juice of *R. sativus* leaf as well as root scavenged O$_2^-$ radical potently. Root juice was more potent (p<0.004) than leaf juice (Table 1). The reason might be due to the presence of more polyphenol content (p<0.0001) in root juice than present in leaf juice (Table 1). Similarly, protection against hydroxyl radical (•OH) induced damage to genomic DNA was also observed by juices in our study (Fig. 4). Protection against formation of pentosidine-like AGEs (p<0.0002) and vesperlysine-like AGEs (p<0.017) was also observed more in root juice than leaf juice (Table 1). The protection offered by leaf juice against glycation of hemoglobin (HbA1c) was however, marginally better (p<0.0545) than that offered by root juice.

In conclusion, our research demonstrated that *R. sativus* possess potentials that may reduce sucrose or high glycemic-index diets induced postprandial hyperglycemia and check the abrupt postprandial hyperglycemic spikes. Simultaneously, presence of potent antioxidant activities and principles offering protection against build up of AGEs and HbA1c formation may offer additional benefits in preventing/delaying development of hyperglycemia induced complications. Consumption of *R. sativus* leaves and roots either as salads, smoothies or as juice before main meal therefore, may become an easy and convenient dietary therapeutic milieu to counter diet induced hyperglycemia and harmful effects of postprandial hyperglycemia. Further research on diabetic animal models and clinical conditions are required to substantiate these observations.

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**Conflict of interest**
All the authors declare that they have no conflict of interest financial or otherwise.
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