

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 spikes 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 conditions^{6,7}. 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,

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monitoring of postprandial glycemia and glycemic excursion has become an important step to manage glycemic control^{7,9}.

The dietetics has been referred to as the primary strategy for treatment of postprandial hyperglycemia⁷. Consumption of green vegetables has been shown to be a promising dietary milieu in maintaining glucose homeostasis both in experimental animals¹⁰ and clinical studies^{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 urticaria¹³. Recently, the multifactorial antidiabetic properties in *R. sativus* have been discussed¹⁴. 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 earlier¹⁵. 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_{0-120 min.} mg/dL/hr) was calculated following trapezoidal rules¹⁶. 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 (AlCl₃.6H₂O), bovine serum albumin (BSA), crude rat intestinal

acetone powder, gallic acid, gentamycin, β -nicotinamide adenine dinucleotide hydrate (NADH), nitro-blue tetrazolium (NBT), p-nitrophenyl 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 μ L juices, 2.5 mL distill water, 250 μ L of 1N Folin-Ciocalteu reagent and 250 μ L Na_2CO_3 (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 μ L of juices with equal volume of $\text{AlCl}_3 \cdot 6\text{H}_2\text{O}$ (2% w/v) prepared in methanol as described earlier¹⁵. 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 ($\text{O}_2^{\cdot-}$) scavenging

Protection of NBT reduction by $\text{O}_2^{\cdot-}$ 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 μ L NBT (50 μ M), 100 μ L NADH (78 μ M) and 70 μ L test sample into Eppendorf tubes. Reaction was started by addition 100 μ L of PMS (1M) to the mixture and incubated at 25°C for 5 min. Absorbance was measured spectrophotometrically at 560 nm. Percentage of $\text{O}_2^{\cdot-}$ radical scavenging activity by juices was calculated as follows: $[(A_c - A_j)/A_c] \times 100$ where, A_c represents

absorbance of control samples without juice and A_j , absorbance in presence of juice.

Advanced glycation end-products (AGEs)

200 μ L BSA (10 mg/mL in 67 mM sodium phosphate buffer containing 3 mM sodium azide, pH 7.4) was incubated with 200 μ L glucose solution (50 mg/mL in sodium phosphate buffer, pH 7.4) in presence of 200 μ L 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 μ L 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 μ L of 10% TCA. Washed pellet was dissolved in 572 μ L of alkaline phosphate buffer (pH 10.6).

Fluorescence of vesperlysine-like (λ_{exc} 370 nm; λ_{em} 440 nm) and pentosidine-like (λ_{exc} 335 nm; λ_{em} 385 nm) AGEs was measured spectrophotofluorometrically¹⁸. Percentage inhibition of AGEs formation in presence of juices was calculated as follows: $[(f_c - f_j)/f_c] \times 100$ where f_c represented fluorescence of control samples and f_j 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 μ L juice for 10 min and then added with 0.5 mL glucose solution (2%) containing 200 mg/mL gentamycin for 72 h. Glycated hemoglobin (HbA_{1c}) was calculated by measuring absorbance at 443 nm spectrophotometrically¹⁹. Percentage of inhibition of HbA_{1c} formation by juices was calculated as follows: % inhibition of HbA_{1c} = $[(A_c - A_j) / A_c] \times 100$ where A_c becomes absorbance of control and A_j , 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. FeCl_3 , H_2O_2 were used to generate hydroxyl radicals ($\cdot\text{OH}$). In an Eppendorf tube, for control test, genomic DNA (2 μ L) 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 FeCl_3 (5 μL) and 10 μL of 30% H_2O_2 . The next set was prepared with genomic DNA (2 μL), 5 μL of tris-buffer and 5 μL of juices and incubated for 10 min at room temperature. FeCl_3 (5 μL) and 10 μL of 30% H_2O_2 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 DocTM XRS+ with Image LabTM 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 activity²¹. 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 inflammation²². Furthermore, large spikes in blood glucose levels and insulin response have been recorded following consumption of sucrose-sweetened beverage within first hour²³. 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

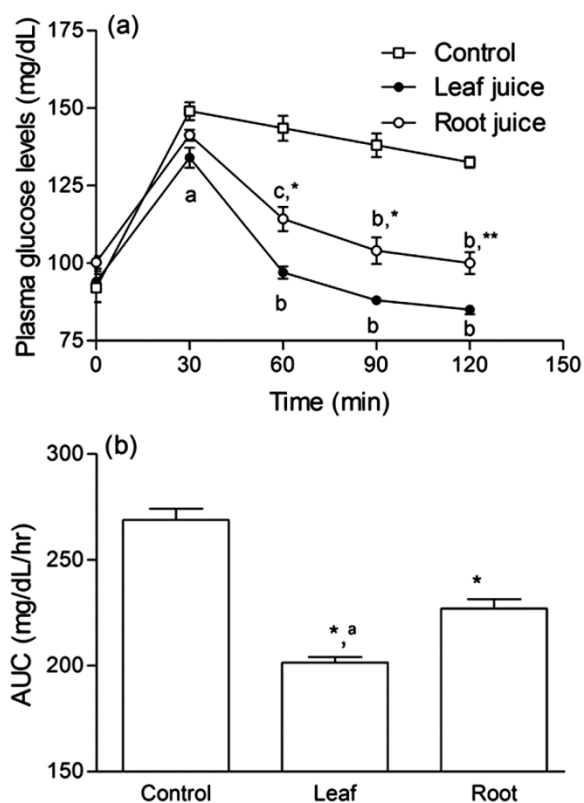


Fig. 1 — Sucrose induced postprandial glycemia under influence of leaf and root juice of *R. sativus* in rats. (a) Glycemic curve over time following oral sucrose feeding to rats juice-unprimed (control) as well as juice-primed with *R. sativus* leaf and root juice respectively. Values represent mean \pm SD, n=4. Tukey's multiple comparison test followed by ANOVA was applied to compare differences within the groups. ^a $p < 0.01$ (control vs leaf juice), ^b $p < 0.001$ when compared with control group, ^c $p < 0.01$ when compared with control, * $p < 0.05$ and ** $p < 0.05$ when compared with leaf juice group. (b) AUC (area under the curve, glucose load over time). Tukey's multiple comparison test followed by ANOVA was applied to find differences within the groups. * $p < 0.001$ when compared with control and ^a $p < 0.01$ when compared with root juice consumed group.

feeding, the postprandial glycaemic rise was dramatically assuaged. Decrease in sucrose induced glycaemic increase was more potent in leaf juice primed rats than that in the root juice primed rats (Fig. 1a). Attenuation of glycaemic 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 glycaemic rise in rats (Fig. 1a).

Total glycaemic 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 glycaemic load (Fig. 1b). Interestingly, leaf juice was more potent ($p < 0.01$) in assuaging sucrose induced glycaemic 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 antihyperglycaemic activity may be due to the presence of high content of antidiabetic compounds sulforaphanes^{14,24}.

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 glycaemic 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 antihyperglycaemic 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 1 β activity^{14,26,27}.

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 glycaemic excursion disturbs metabolic homeostasis in mild to moderate hyperglycaemic patients⁶. Although, the absolute codification of time to measure postprandial glycaemic 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 glycaemic excursion in rats

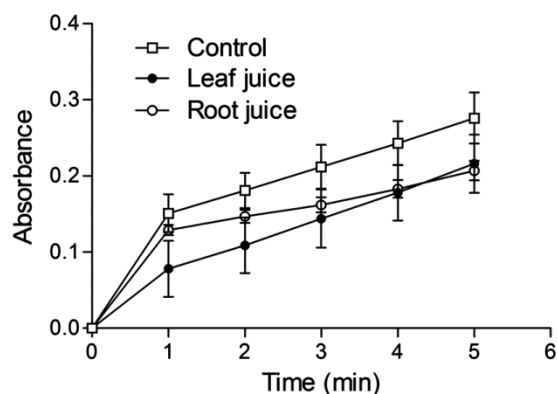


Fig. 2 — Kinetic assay of rat intestinal α -glucosidase activity under influence of *R. sativus* leaf and root juice. It appears from the figure that under influence of leaf and root juice of *R. sativus*, the enzyme activity is slowed down; differences between control and treatments at respective time points however, could not reach statistical significance.

Table 1 — Phytochemicals analysis and *in vitro* biological activities in leaf and root juice of *R. sativus*

	pH	Total polyphenol ($\mu\text{g/mL}$, GAE)	Total flavonoids ($\mu\text{g/mL}$, RE)	%Super oxide radical scavenging	% AGEs inhibition		% HbA1c inhibition
					p-like	v-like	
Leaf juice	5.3 \pm 0.04	123 \pm 2	185 \pm 8 ^b	81.4 \pm 2.0	51.72 \pm 2.9	21.88 \pm 1.1	91.12 \pm 0.3 ^f
Root juice	5.4 \pm 0.15	573 \pm 9 ^a	15.7 \pm 0.5	92.5 \pm 1.4 ^c	94.88 \pm 1.9 ^d	26.96 \pm 0.4 ^e	79.71 \pm 4.8

Values represent mean \pm SD, $n \geq 3$. GAE: Gallic acid equivalent, RE: Rutin equivalent, AGEs: advanced glycation end-products. Two types of AGEs were measured in this analysis 1) pentosidine-like (p-like) AGEs and vesperlysine like (v-like) AGEs. Unpaired t-test (two tailed) with Welch's correction was applied to compare differences between parameters of leaf and root juice. ^a $p < 0.0001$, ^b $p < 0.0007$, ^c $p < 0.004$, ^d $p < 0.0002$, ^e $p < 0.017$, ^f $p < 0.0545$ when compared with their respective counterparts.

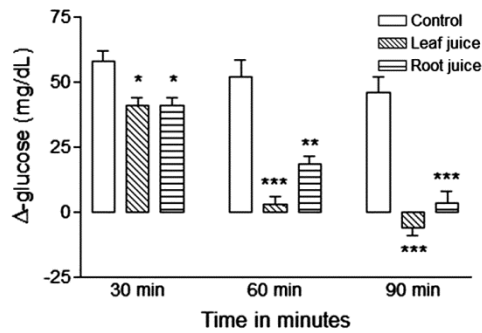


Fig. 3 — Sucrose induced postprandial glycaemic excursion under influence of leaf and root juice of *R. sativus* in rats at different peak times. Values represent mean \pm SD, n=4. Tukey's multiple comparison test followed by ANOVA was applied to compare differences within the groups. *p<0.05, **p<0.01, and ***p<0.001 when compared with their respective control groups.

following sucrose administration. It is evident from Fig. 3 that there was more than 58 mg/dL glycaemic 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 glycaemic excursion was observed (Fig. 3). Similarly, feeding rats' juice of *R. sativus* leaf or root significantly (p<0.001) attenuated postprandial glycaemic spikes between 60 and 90 min post-sucrose administration (Fig. 3). The superiority of leaf juice over the root's juice in mitigating postprandial glycaemic 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 (HbA_{1c}) levels are considered reflection of postprandial glycaemic excursion and total postprandial glycaemic 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 O₂⁻ 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

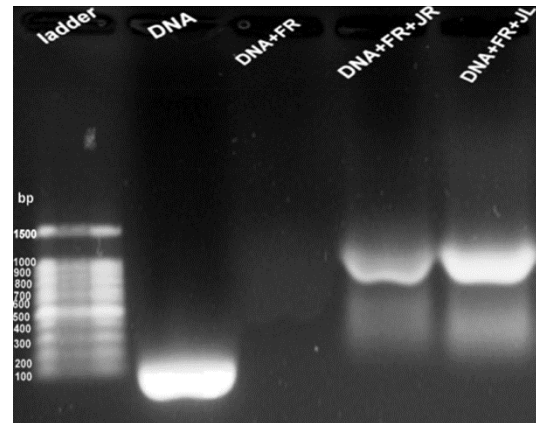


Fig. 4 — Influence of *R. sativus* leaf (JL) and root (JR) juices on free radicals induced damage to genomic DNA. FR; Fenton reagent generated free radicals, bp; base pairs.

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 (HbA_{1c}) 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 glycaemic-index diets induced postprandial hyperglycemia and check the abrupt postprandial hyperglycaemic spikes. Simultaneously, presence of potent antioxidant activities and principles offering protection against build up of AGEs and HbA_{1c} 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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