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In vitro bioactivities and simulated gastrointestinal digestion studies on *Guras*-based traditional fermented drinks collected from Singalila ridge of the Himalayas

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Abstract

Guras or *Rhododendron* wine and its distilled form (*Raksi*) are popular traditional and therapeutic drinks served in the Rhododendron growing regions of the Himalayas, mainly in the northern and northeastern part of India, Nepal and Indo-Nepal Singalila ridge. In this research, *lali Guras* (*Rhododendron arboreum* Sm.) based traditional beverages such as unfermented decoction (as control), fermented *Guras* wine and distilled spirit *Guras ko Raksi* were collected from Singalila ridge of the Himalayas for analysis. *In vitro* experiments such as physicochemical and qualitative and quantitative biochemical tests; antioxidant assays (DPPH and iodometric assay); antibacterial assay; and simulated gastrointestinal digestion were carried out. Phytochemically rich unfermented decoction exhibited the best results in total phenolic content assay (82.18±1.13 mg/100 mL gallic acid equivalent) and fatty acid quantification (1.86±0.08%) test while the wine was found to be the most potential sample in all bioactivity tests. The wine was found to contain high amounts of glycoside and fermented distilled liquor *Guras Raksi* showed high presence of glycerol. Both the fermented samples exhibited high rate of digestion in simulated human digestion system compared to the phenolic rich unfermented decoction. This research validated the therapeutic potential of these *Guras*-based beverages in high-altitude conditions like Singalila by focusing on their biochemistry.

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1. Introduction

Fermentation contributes to a longstanding food sustainability by assisting in the production, preservation, and safety of food components. In high-altitude human settlements such as the Himalayan region, traditional fermentation techniques play a crucial role in food and beverage management by extending their shelf life where sourcing essential raw food materials year-round poses significant challenges (1). The ethno-foodology of Singalila is distinctive, enriched with culturally diverse traditional foods. *Kinema* and *Masauyra* (fermented legume products); *Chhurpi*, *Chhu/Sheden*, *Philu*, *Somar* (dairy products); *Gundruk*, *Sinki*, *Khalpi*, *Mesu* (fermented vegetables); *Sel-roti*; meat and fish pickles; and alcoholic beverages like *Guras*, *Tongba*, *Chhyang*, *Nigar*, *Raksi* etc. are some of the important ethnic fermented foods consumed in the high altitudes of Darjeeling and Kalimpong districts of West Bengal; Sikkim; and Nepal (1).

Guras or *Buransh* (*Rhododendron arboreum* Sm.) is one of the important species of flowers that have several health benefits and are used as a viable substrate for the production of wine (2) in high-altitude settlements of the Himalayas such as Darjeeling, Uttarakhand,

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Himanchal Pradesh, Arunachal Pradesh, Nagaland, Jammu and Kashmir, etc. Rhododendron is an enormous genus of more than a thousand species, and it belongs to the Ericaceae family. The Singalila ridge of the Himalayas has been a hub of diverse flora including Rhododendrons (3). At an altitude of 2400-3600 meters, the Singalila ridge, is situated in the Indo-Nepal region including parts of Darjeeling and West Sikkim districts of India and Mechi zone of Nepal which also separates mountain ranges of West Bengal from other Himalayan ranges to its west. The famous naturalist Sir Joseph Hooker discovered twenty-five new species of Rhododendrons in the Himalayas; and Singalila National Park has been a source of eighteen of them to be called as the land of Rhododendrons (4). Among all species, *lali Guras* (*Rhododendron arboreum* Sm.) is generally used to prepare fermented beverages in this region. Rhododendron flowers are a rich source of carbohydrates, chlorogenic acids, quinic acid, coumaric acid and other hydroxycinnamic acids, polyphenols, flavones, ursolic acid etc. (2,5–7). *Guras* flowers have antioxidant, anti-inflammatory and cholinergic activity which is also used as ethnomedicine to treat diarrhoea and blood dysentery (8). Several ethnic beverages i.e., *Raksi*, *Chhyang*, *Tongba*, *Guras*/Rhododendron flower juice or wine, last but not least tea are served in many places of Singalila ridge and neighbouring hills as a remedy for high-altitude sicknesses including muscle pain (1). Both juice and fermented wine from *Guras* exhibit ethnomedicinal properties such as, anti-inflammatory (painkilling ability), antidiabetic, cardioprotective, hepatoprotective, antiallergic, antioxidant and anticancer activities (2,5–7). Yadav et al. (2) prepared wine from Rhododendron flowers (from Uttarakhand) in laboratory and determined the pH, and total soluble solid, antioxidant activity, total phenolic content, and total flavonoid content of it.

Not long ago, our research team have conducted similar research works on Japanese camellia (*Camellia japonica*) and tea (*Camellia sinensis*) flower wines (16,24) where development of fermented wines from those flower petals, *in vitro* biochemical characterization and fermentation metabolomics along with the wine metabolite profiling were carried out successfully (16,24). In this research, unfermented flower decoction, fermented *Guras* wine and distilled *Guras Raksi* were collected from Singalila. A range of *in vitro* techniques- preliminary experiments like physicochemical analysis, qualitative biochemical tests, quantification of total phenol content and free fatty acid, *in vitro* antioxidant assays and antibacterial activity were carried out to characterize the *Guras* samples. Furthermore, another important objective of this research was conduction of *in vitro* gastrointestinal digestion using the static simulation model (9) to evaluate the digestion process of *Guras* beverages in human digestive system and to figure out bioaccessibility of the bioactive compounds (phenolics, lipid composition and antioxidants) present in these ethnic beverages.

2. Materials and Methods

2.1. Collection of samples

Samples of *Guras* or fermented Rhododendron-based beverages were collected from an old and renowned tavern in Gairibas (27°02'52"N, 88°01'47"E; Elevation: 2581 m; Figure 1). Gairibas is one of the oldest human settlements in Singalila National Park (1), located at Indo-Nepal region of the Himalayas.

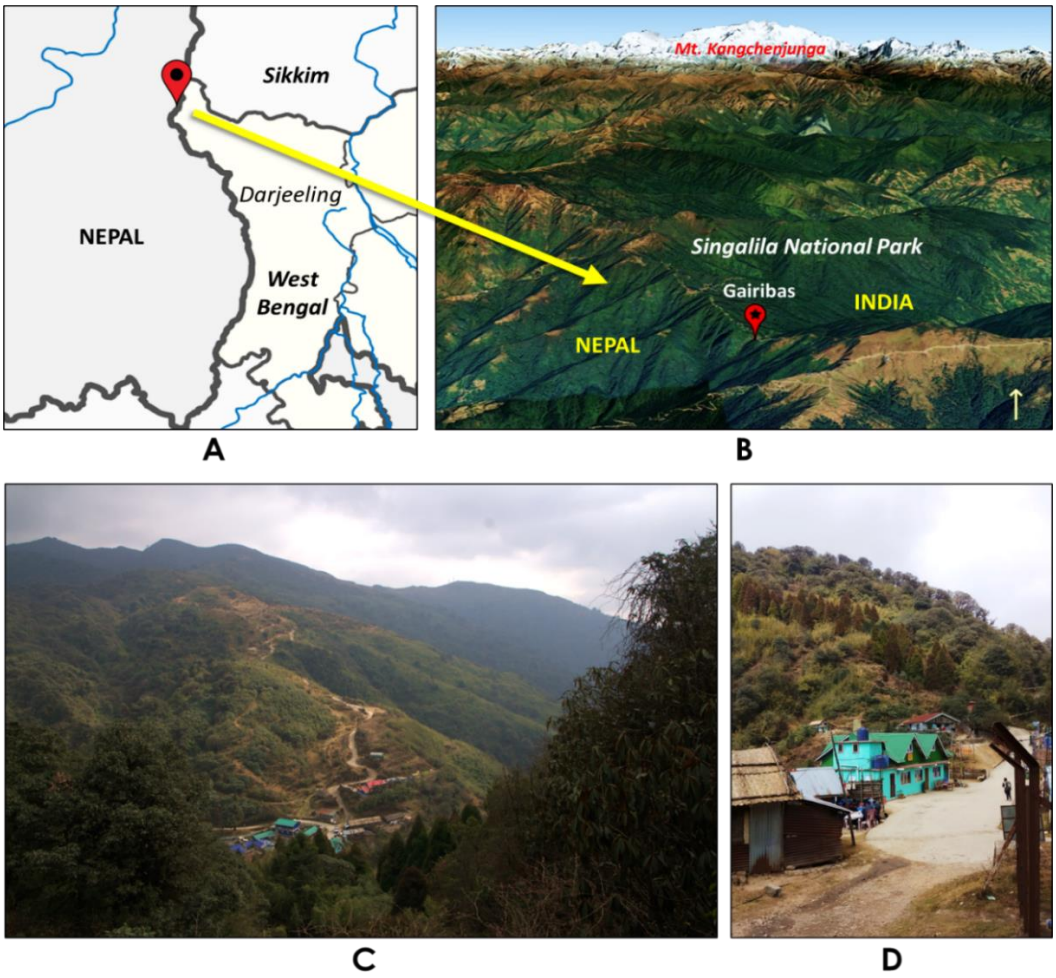

Figure 1. A: The map of Indo-Nepal regions of Darjeeling Himalayas pointing Gairibas at the Singalila ridge; B: The Google Earth Pro- image showing Gairibas in the Singalila Forest (Coordinates: 27°02'52"N, 88°01'47"E; Elevation: 2581 m); C: Bird's-eye view of Gairibas [Location: Singalila National Park, Darjeeling (India); Ilam, Mechi Zone, Eastern Region (Nepal)]; D: *Guras* sample collection site in the village- Gairibas

Depending on brewing stages, three different samples from a same batch were collected such as, unfermented Rhododendron flower decoction (**RFD**) (considered as control); fermented sample- *Guras* wine (**GW**); and the *Raksi* or distilled/spirit version of the fermented *Guras,* locally called *Guras ko Raksi* (**GR**). Different brewing stages were chosen to ensure a comparative biochemical analysis. Brewers prepared the decoction of fully bloomed *lali Guras* or red Rhododendron (*Rhododendron arboreum* Sm.) flowers (Figure 2-A; Figure 2-B) harvested from Rhododendron forest of Gairibas; carried out the fermentation process using starter culture called *marcha* (three weeks of fermentation period was given) (Figure 2-D); and completed the distillation process (Figure 2-C); following their secret age-old traditional knowledge of brewing. From decoction, 50 mL were kept aside as control sample (**RFD**) which was immediately transferred in laboratory for analysis. Then, after three weeks of fermentation, **GW** and **GR** were collected. Three replicas (50 mL of each sample) were collected in sterilized plastic tubes (Tarsons, India) (Figure 2-E) to avoid contamination and kept inside a portable refrigerator. All the biochemical tests were carried out in triplicates and results were expressed as mean ± SD (n=3). A schematic illustration of the traditional brewing method of

Guras wine (GW) and *Guras Raksi* (GR) from *lali Guras* (Rhododendron flowers) including decoction, fermentation by *marcha* and distillation has been given in Figure 3.

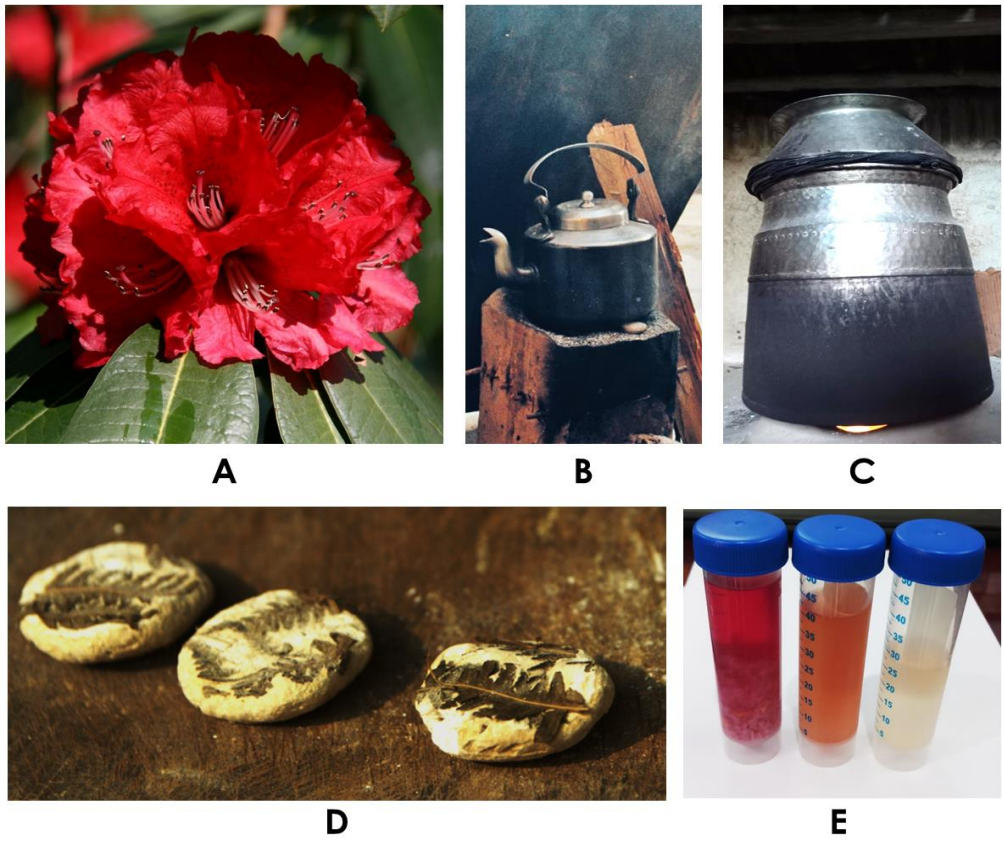

Figure 2. A: Fully bloomed *lali Guras* or Rhododendron flower; B: Preparation of decoction in an aluminum kettle; C: A glimpse at traditional distillation method; D: Ethnic rice-based starter *marcha*; E: Collected samples- RFD or unfermented Rhododendron decoction, GW or fermented *Guras* wine and GR or distilled version- *Guras ko Raksi* (from left to right)

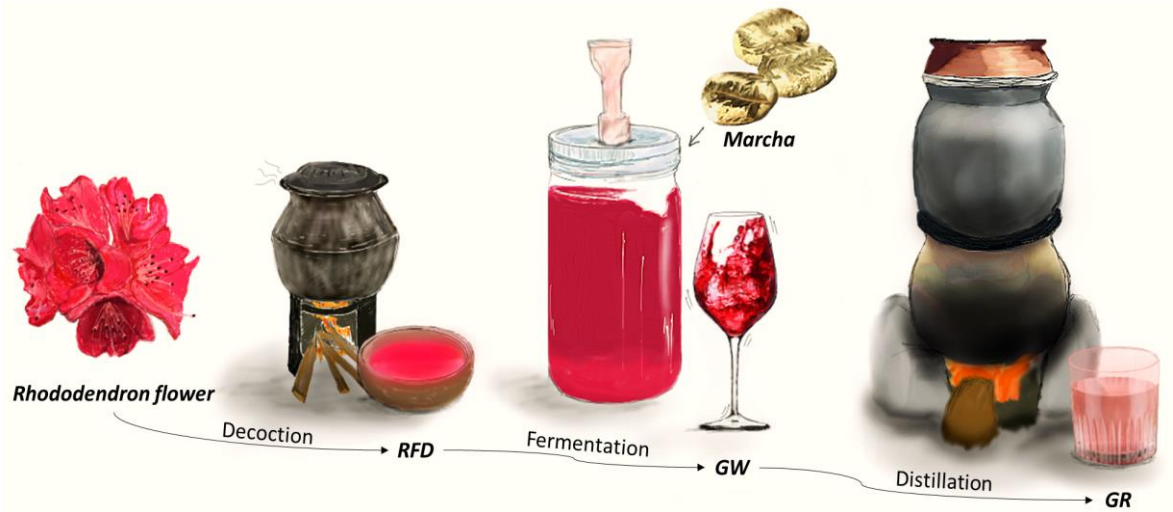

Figure 3. Schematic illustration showing traditional brewing methods of *Guras* wine (GW) and *Guras ko Raksi* (GR) from *lali Guras* (Rhododendron flowers) including decoction, fermentation by *marcha* and distillation.

2.2. Physicochemical analysis

Physicochemical parameters such as pH (for acidity), specific gravity (Sg) and alcohol by volume (%ABV) were determined following the protocol of Majumder et al. (10). pH was measured by using LMPH10 pH meter (Labman Co.) to investigate the changes in acidity of the decoction due to fermentation. Specific gravity (Sg) of the fermented samples were determined mathematically by calculating the ratio of the specific weight of sample to the specific weight of water at 4°C. Alcohol percentage or %ABV in fermented samples (**GW** and **GR**) were calculated by entering OG (original gravity or specific gravity of **RFD**) and FG (final gravity or specific of fermented beverages) values into an online ABV calculator (11,16,24). Experiments were carried out in triplicates and results were expressed as mean ± SD (n=3).

2.3. Qualitative biochemical tests

Presence of various bioactive constituents such as glycoside, glycerol, reducing sugar, saponin, phenol, flavonoid, coumarin, tannin, fatty acid, terpenoid, steroid and alkaloid were determined in the samples using the protocols of qualitative biochemical tests demonstrated in various literatures (9,12–14).

2.4. Quantification of TPC (total phenol content)

Folin–Ciocalteu method (15) was followed to quantify the total phenolic content in samples. 200 µL of each sample was taken and its final volume was made upto 3 mL with distilled water. To which 500 µL of Folin–Ciocalteu reagent (SRL, India), and 2 mL of 20% (w/v) sodium carbonate (SRL, India). The mixture was left in dark for sixty minutes, and absorbance was measured at 650 nm in a UV-vis spectrophotometer (Cary-60, Agilent). TPC was measured against gallic acid standard curve ($R^2 = 0.9975$; $y = 0.0043x - 0.1672$) and results were expressed as gallic acid equivalent (mg GAE/100 mL) (16). The data were expressed as means of three replicates ± Standard Deviation (SD).

2.5. Quantification of FFA (free fatty acid)

Following the protocol of Patterson (17), fatty acids in these ethnic beverages were quantified for a comparative analysis. The same assessment was also carried out further during *in vitro* GID (gastrointestinal digestion) analysis to evaluate the fate of lipid compositions in simulated GI condition. Following the titrimetric isopropyl alcohol method (18) this test was carried out with slight modifications. In 1 mL of each sample 10 mL of isopropyl alcohol (Merck) and 100 µL of phenolphthalein indicator (SRL, India) were added and shaken. This mixture was titrated against 0.1N KOH until appearance of pale pink colour lasting for 10 sec. The end point of the titration was considered as acid value. The FFA (free fatty acid content %) was calculated by dividing the acid value with 2. This assay has also been established as one of the chemical techniques used to determine the lipid composition (9). The data were expressed as means of three replicates ± Standard Deviation (SD).

2.6. In vitro antioxidant activity

2.6.1. DPPH (2,2-diphenyl-1-picrylhydrazyl) assay

Protocol of Majumder et al. (10,16) was followed to conduct this assay. 200 µL of each sample was taken in a test tube containing 2800 µL of 2,2-diphenyl-1-picrylhydrazyl or DPPH (HiMedia, India) solution which was incubated for 20 min in dark condition. Decreasing absorbance was recorded at 517 nm for quantification of the antioxidant activity. Result of this

assay was expressed as mean ± SD (n = 3) of three replicates. DPPH scavenging percentage was calculated using the following equation:

$$Free\ radical \text{ scavenging percentage } (\%) = \frac{C_{Abs} - S_{Abs}}{C_{Abs}} \times 100 \quad (1)$$

Where C_{Abs} is absorbance recorded for DPPH solution (control) and S_{Abs} is absorbance recorded in DPPH solution injected with sample.

2.6.2. Iodometric assay

Antioxidant activity was also determined using this titrimetric assay developed by Majumder et al. (9,16) for analysis of alcoholic beverages. 1 mL of 1% starch indicator was added to 20 mL of sample and dissolved in 150 mL of distilled water. Titration was done with 0.005 mol/L iodine solution. The redox titration endpoint was decided by the first iodine excess that is complexed with starch, giving a deep bluish violet colour. Ascorbic acid was used as reference (R^2 = 0.9999; y = 3.9022x - 0.206) to quantify the activity. Results have been expressed as µg AAE/ mL (AAE: ascorbic acid equivalent). The data were expressed as means of three replicates ± Standard Deviation (SD).

2.7. In vitro antibacterial activity

Well diffusion method (19) was performed to assess the antibacterial activity of crude samples. Overnight grown cultures of two Gram-positive bacteria i.e., *Staphylococcus aureus* and *Bacillus subtilis* and two Gram-negative bacteria i.e., *Escherichia coli* and *Klebsiella pneumoniae* were used for this experiment. Culture plates were made using the media (Mueller Hinton Agar) inoculated with 100 µL inoculum. For the well diffusion method, three wells were made in the dish with a cork-borer and 100 µL of each sample were pipetted into the wells. The petri-dishes were then incubated overnight at 37°C. Diameter of the inhibition zone around each sample containing well was corresponding to the antibacterial activity.

2.8. In vitro GID (gastrointestinal digestion)

This procedure comprised consecutive steps simulating different conditions along the gastrointestinal tract. In this research, *in vitro* GID was used to determine the bioaccessibility of compounds (9) present in *Guras* samples. "INFOGEST static *in vitro* simulation of gastrointestinal food digestion" protocol (20) was followed to design this experiment. Using this method, beverage samples were subjected to oral, gastric and intestinal digestion where parameters such as electrolytes, enzymes, bile, dilution, pH and time of digestion were maintained exactly as reported by Shen et al. (21). Total phenolic content (TPC), lipid composition (acid value) and antioxidant activity (DPPH assay) were measured to investigate the digestibility and release of food constituents under simulated gastrointestinal conditions. All analyses were performed in triplicate. The data were expressed as means of three replicates ± Standard Deviation (SD).

2.9. Statistical analysis

Data obtained from results of various experiments during this research were analysed statistically using Microsoft Excel (v2303). The data were expressed as means of three replicates ± Standard Deviation (SD). The test for statistical difference was performed using one-way ANOVA (analysis of variance). The level of significance was set at $P < 0.05$.

2.10. Preparation of diagrams

Graphical representations of the experimental data i.e., line graphs and bar graphs were prepared in Microsoft Excel (v2303). Illustrations of the schematic diagrams and graphical abstract were digitally drawn in Autodesk SketchBook (v8.7.1).

3. Results and Discussion

3.1. Physicochemical analysis

The pH of the fermentation substrate/ decoction- **RFD** was recorded to be 5.73±0.22. It was suitable for brewing since acidic pH is reported as fermentation friendly. According to Majumder et al. (16), maintaining an acidic condition (by lowering pH near 5) during brewing of a beverage may stimulate the rate of fermentation process. The pH was seen to decrease upon completion of fermentation as a lower pH of 3.84±0.08 was determined in the wine sample- **GW**. However, pH of its spirit liquor or *Raksi* (**GR**) was significantly high (P<0.05) like 4.66±0.19 (Figure 4-A). Specific gravity was also recorded to be decreased successively upon subsequent stages (Figure 4-B). Specific gravity of the tested wine (0.994±0.005) was significantly higher (P<0.05) than that of its distilled version or *Raksi* (0.944±0.005) which was inversely proportional to the values of alcohol percentage or %ABV. Around 13.5% alcohol was measured in **GW** while alcohol percentage for the sample **GR** was recorded about 20% (Figure 4-C). Results of physicochemical analysis have been graphically represented in Figure 4.

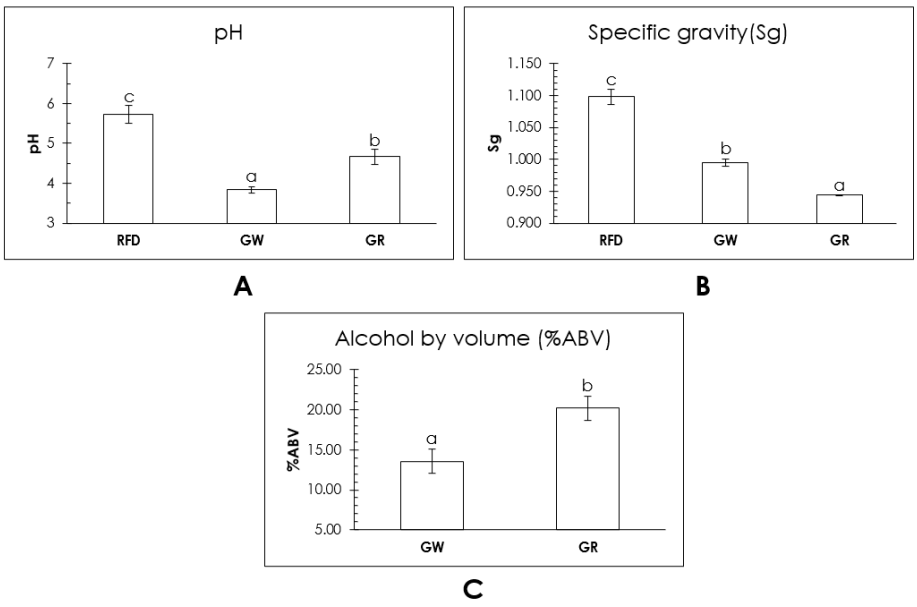

Figure 4. Results of physicochemical analysis: pH (A), specific gravity or Sg (B) and alcohol by volume or %ABV (C). Error bars represent ±SD or standard deviation (n=3). In each chart/graph, different letters above the error bars signify significant difference among samples ($P < .05$).

3.2. Qualitative biochemical tests

Qualitative biochemical tests revealed presence of various bioactive molecules in *Guras* samples. Table 1 has been prepared with results of these tests in a comparative way.

Table 1. Results of qualitative biochemical tests [**+++**(High), **++**(Moderate), **+**(Low), **-** (Absent)]

Name of molecules	RFD	GW	GR
Glycoside	++	+++	+
Glycerol	+	++	+++
Reducing sugar	+++	++	+
Saponin	+	++	+
Phenol	+++	++	+
Flavonoid	+++	++	+
Coumarin	+++	++	+
Tannin	+	+	-
Fatty acid	+++	++	+
Terpenoid	++	+	+
Steroid	++	++	+
Alkaloid	+	-	-

Reducing sugar, glycoside and glycerol are markers of different stages of fermentation. Reducing sugar is corresponding to the unfermented carbohydrates (sugars) present in the unfermented decoction (**RFD**) while glycoside and sugar alcohol (glycerol) are typical fermented metabolites. So, by considering these three results we have successfully evaluated the progress in fermentation process taking their alterations into account. Reducing sugar was found high in the unfermented **RFD** while fermentation and further distillation led in reduction of sugar content and increase of fermented metabolites such as glycoside (in **GW**) and glycerol (in **GR**). Experiments revealed remarkable presence of bioactive phytochemical groups in **RFD** followed by **GR**. Comparatively, distilled sample **GR** resulted low in most of the tests except glycerol. Alkaloids were absent in fermented samples.

3.3. Quantification of TPC (total phenol content)

Total phenolic content (TPC) for the control or unfermented sample **RFD** was recorded significantly higher (P<0.05) with a value of 82.18±1.13 mg GAE/ 100 mL while, TPC of fermented samples were determined to be 59.54±1.99 mg GAE/ 100 mL and 46.04±3.99 mg GAE/ 100 mL for **GW** and **GR** respectively (Figure 5-A). Result of this assay was corresponding to the qualitative test for phenol.

3.4. Quantification of FFA (free fatty acid)

Free fatty acid percentage was recorded significantly high (P<0.05) in the control or **RFD** compared to others which was equivalent to the qualitative result (Table 1). The estimated fatty acid of **RFD** (2.1±0.09%) was determined to be decreased to 1.86±0.08% after fermentation and further to 0.91±0.05% after distillation (Figure 5-B).

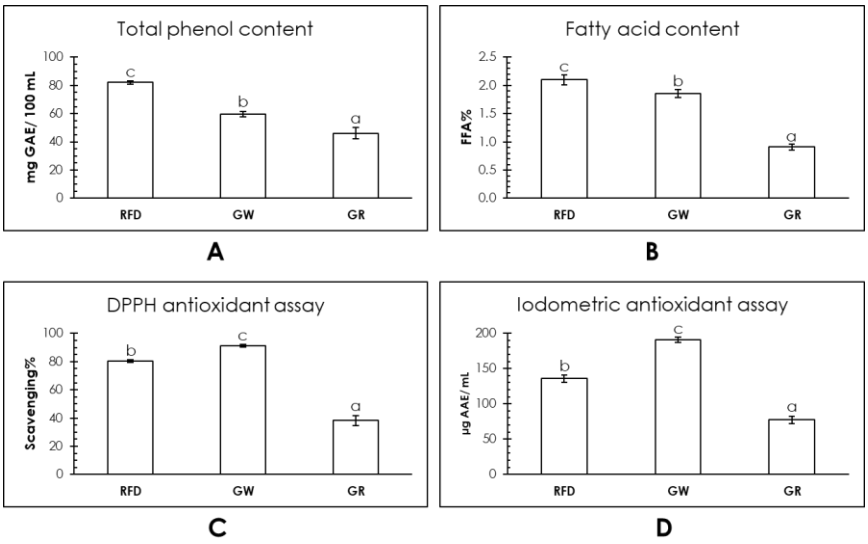

Figure 5. Results of various quantitative biochemical tests. A: Total phenol content or TPC analysis; B: Fatty acid content (free fatty acid %); C: *In vitro* antioxidant activity- DPPH assay; D: *In vitro* antioxidant activity- iodometric assay. Error bars represent ±SD or standard deviation (n=3). In each chart/graph, different letters above the error bars signify significant difference among samples ($P < .05$). [GAE: gallic acid equivalent; FFA: free fatty acid; AAE: ascorbic acid equivalent]

3.5. In vitro antioxidant activity

Antioxidant activity is one of the major bioactivities (associated to various therapeutic properties including anticancer activity) reported in *Guras*-based beverages (both in the unfermented decoction and fermented wine). Therefore, DPPH assay and iodometric assay were considered to judge the antioxidant activity of the collected samples in this *in vitro* study. Fermented *Guras* wine (**GW**) exhibited significant high (P<0.05) antioxidant activities followed by unfermented **RFD** in both the assays performed (Figure 5). The DPPH free radical scavenging property of **GW** was recorded to be 91.34±0.95% (Figure 5-C) and its iodine reducing potential was determined to be an equivalent of 190.93±3.49 µg ascorbic acid (Figure 5-D). Abundant quantity of phenols in Rhododendron flower (7) might be the reason behind high antioxidant activity of **RFD** (80.53±0.91% DPPH inhibition). The distilled *Guras Raksi* did not present any potentiality as antioxidant which was comparable with its biochemical quality. Results of TPC, %FFA, DPPH assay and iodometric assay have been graphically represented in Figure 5.

3.6. In vitro antibacterial activity

The antibacterial efficacy of *Guras* samples was evaluated through agar diffusion assay on bacterial cultures. No complete halo of inhibition zones were observed around the wells containing fermented sample **GW** and **GR**, the control **RFD**. However, fermented sample **GW** was found to produce partial but prominent inhibition zones against Gram-positive *Bacillus subtilis* and Gram-negative *Klebsiella pneumoniae*. Quantifying outcomes was sidestepped, with visuals of culture plates included instead (Figure 6).

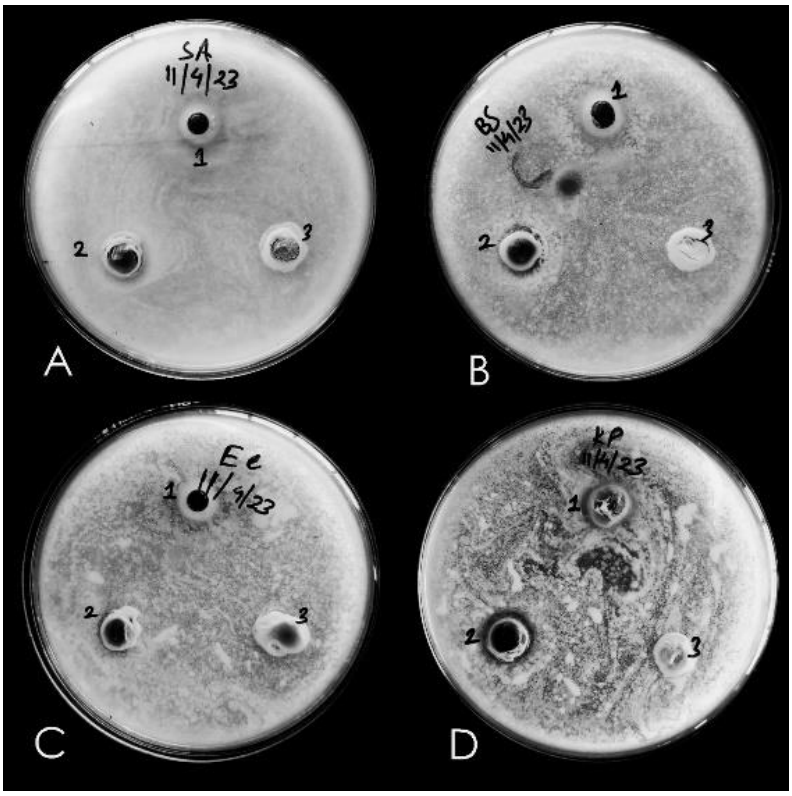

Figure 6. Resulting culture plates showing antibacterial activity of samples RFD or Rhododendron flower decoction (1); GW or *Guras* wine (2) and GR or *Guras ko Raksi* (3) against selected Gram-positive bacteria i.e., *Staphylococcus aureus* (A) and *Bacillus subtilis* (B) and Gram-negative bacteria i.e., *Escherichia coli* (C) and *Klebsiella pneumoniae* (C). GW or *Guras* wine (2) exhibited partial inhibition zones against Gram-positive *Bacillus subtilis* (B) and Gram-negative *Klebsiella pneumoniae* (D).

3.7. In vitro GID (gastrointestinal digestion)

Bioactivity alone cannot define the nutritional value of a food or beverage. So, to understand the nutritional efficiency, bioaccessibility or digestion of that food in human digestive system has also to be taken into consideration which assessed in this research as well. The *in vitro* digestion procedure is advantageous being fast, low cost, safe, less laborious experiment that needs no ethical consents as applied to *in vivo* models. Furthermore, *in vitro* digestion models are more reproducible as these allow for higher control of the experimental variables compared to *in vivo* models (31). *In vitro* digestion models or GI simulations have advanced the area of digestion studies in a simple and more cost-effective way. Investigation of structural modifications, digestibility, and release of food constituents can be done under simulated conditions which has been incorporated in this research.

This *in vitro* experiment showed breakdown and bioaccessibility of nutritionally important components present in *Guras* samples under simulated human gastrointestinal condition. Results of this experiment have been given in Table 2. Rate of digestion in each phase was calculated (in percentage) from the total digestion recorded for each parameter. In the stomach/gastric phase, the digestion rate of total phenol content was highest with calculated values of 77%, 69% and 68% for **RFD**, **GW** and **GR** respectively whereas more than 85% digestion of lipid composition was observed in the last phase of intestine.

Table 2. Results of *in vitro* gastrointestinal digestion of antioxidants, total phenolic content and lipid composition tested in RFD (Rhododendron flower decoction), GW (*Guras* wine) and GR (*Guras ko Raksi*)

Parameters	GID phases	RFD	GW	GR
Total phenol content or TPC (mg GAE/ 100 mL)	Crude sample	82.18±1.13^{b}	59.54±1.99^{d}	46.04±3.99^{c}
	Oral phase	80.63±2.18^{b}	52.92±2.22^{c}	44.44±2.03^{c}
	Gastric phase	67.41±1.77^{a}	21.09±0.97^{b}	18.31±3.51^{b}
	Intestinal phase	65.1±2.81^{a}	13.4±1.87^{a}	7.69±3.09^{a}
Lipid composition (acid value or %FFA)	Crude sample	2.1±0.09^{c}	1.86±0.08^{c}	0.91±0.05^{c}
	Oral phase	2.1±0.05^{c}	1.81±0.02^{c}	0.9±0.06^{c}
	Gastric phase	1.88±0.06^{b}	1.7±0.03^{b}	0.82±0.06^{b}
	Intestinal phase	0.39±0.04^{a}	0.29±0.09^{a}	0.3±0.09^{a}
Antioxidant activity (DPPH scavenging %)	Crude sample	80.53±0.91^{c}	91.34±0.95^{d}	38.2±3.67^{c}
	Oral phase	79.55±1.27^{c}	82.8±0.99^{c}	35.35±4.11^{c}
	Gastric phase	69.4±2.27^{b}	33.31±1.2^{b}	4.5±1.6^{b}
	Intestinal phase	52.01±2.3^{a}	7.81±2.46^{a}	1.93±0.77^{a}

Note: In each parameter, different superscript letters (column-wise) represent statistically ($P < .05$) different groups. [TPC: total phenol content; GAE: gallic acid equivalent; FFA: free fatty acid; DPPH: 2,2-diphenyl-1-picrylhydrazyl]

Figure 7 is the pictorial representation of the results. The figures clearly indicated that, total phenol content and other antioxidant components of samples were mainly digested during the gastric phase which was corresponding to the significant curvature observed in that phase for those two parameters. Whereas a reduction in lipid composition was observed in the intestine. Unlike fermented samples, unfermented **RFD** exhibited a stable, hard-to-digest nature as changes in both total phenol content and antioxidant activity parameters were recorded least in **RFD**.

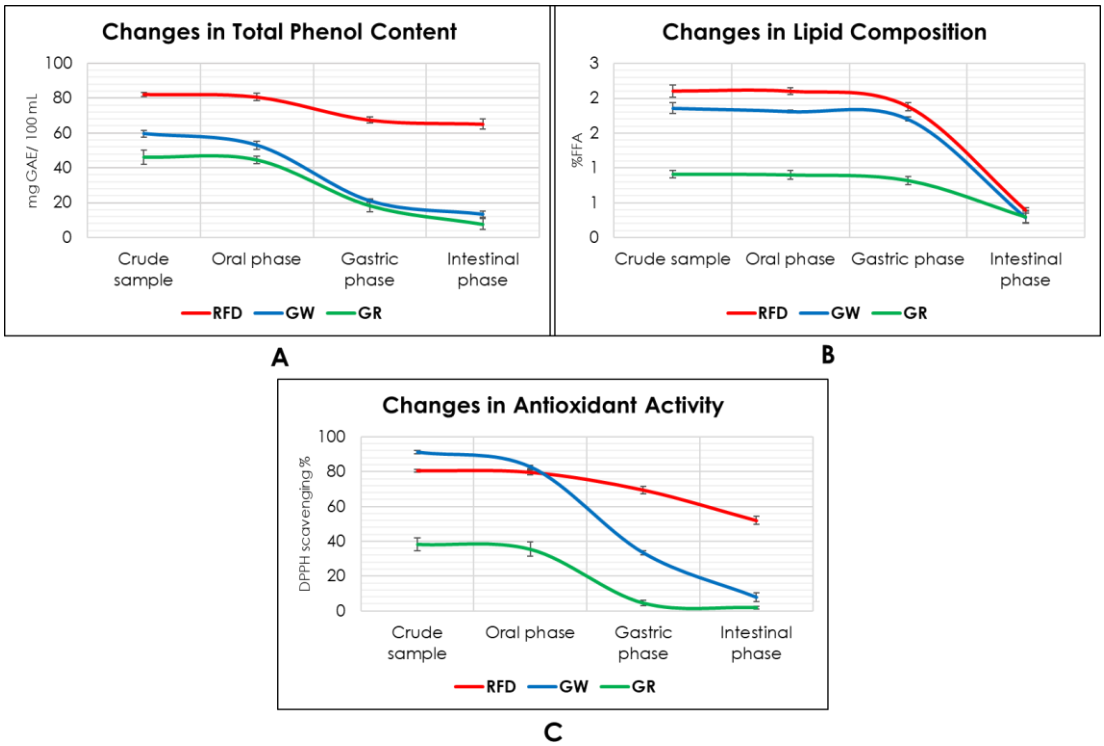

Figure 7. Graphical representations of the results of *in vitro* gastrointestinal digestion (data given in Table 5). A: Total phenol content or TPC assay; B: Lipid composition (acid value or %FFA); C: Antioxidant activity (DPPH scavenging %)

3.8. Discussion

Physicochemical properties like pH, specific gravity and %ABV are important parameters to measure the fermentation rate and physicochemical acceptability of fermented beverages. Physicochemical alterations also indicate biological and chemical changes during fermentation such as microbial growth; utilization of nutrients; microbial breakdown or oxidation of substrate metabolites; production of alcohol, organic acids, and other secondary metabolites; changes in viscosity etc. (10). During fermentation of foods and beverages, fermenting yeasts and bacteria cause acidification by releasing organic acids as secondary metabolites which makes the broth acidic and accelerate the fermentation rate as well (10,16). As a result, fermented foods or drinks possess a tangy flavour. Eminent chemists from the brewing field Liebmann and Rosenblatt (22) and Guymon et al. (23) described importance of pH in judging the quality of distilled alcoholic beverages. Estimated pH values of typical wines ranges between 3 to 4 (24) while pH of edible commercial spirits including distilled wine (brandy) ranges from 4 to 5 (22,23). Therefore, pH values of both wine (3.84±0.08) and spirit or *Raksi* (4.66±0.19) samples analysed in this research was satisfactory. Moreover, the distillation process can be held responsible behind the gain in pH for the sample **GR**. Kanwar et al. (25) also demonstrated effect of distillation processes on rising pH levels while studying with some cereal based ethnic drinks of Himachal Pradesh. Elevated pH levels generally result in increased glycerol production (26) which is also corresponding to the results of qualitative glycerol test (Table 1). Specific gravity helps to determine the desired level of viscidness and alcohol percentage thus indicates the endpoint after the desired level of fermentation. After fermentation, specific gravity of **RFD** was found to be decreased (Figure 4-B) indicating production of alcohol. Generally, distilled spirits/ liquors around the world (whiskey, rum, brandy, vodka etc.) contain high proportion of alcohol compared to undistilled fermented

drinks like beer and wine. It was corresponding to our results because the estimated %ABV was significantly higher (P<0.05) in **GR** compared to **GW**. Utilization of reducing sugar by fermenting microbes results in production of ethanol, organic acids, CO_2, glycerol, glycoside etc. These chemical changes are typical of beverage fermentation and were corresponding to the results i.e., decrease in reducing sugar content and specific gravity; and increase in acidity, alcohol percentage (%ABV), glycerol and glycoside contents etc. as shown in Figure 4 and Table 1.

Typical phytochemicals such as phenol, flavonoid, coumarin, tannin, fatty acid, terpenoid, steroid and alkaloid were detected high in unfermented **RFD** (Table 1) compared others because, during the initial phase, a significant number of molecules could originate exclusively from freshly harvested Rhododendron flowers, devoid of any involvement from external organisms.

Reports describing the Rhododendron flower as a source of chlorogenic acids and various natural phenol components (7) certainly validated the high phenol content determined in **RFD** during biochemical tests (Figure 5-A). However, possible reasons behind further reduction in phenolic compounds, fatty acids and other phytochemicals during fermentation might be the microbial degradation of phenols during fermentation, oxidation of phenols during storage and the generation of diverse secondary metabolites from fermentation that made the proportion of phenols low in the final broths which can be evaluated by detailed metabolomic study.

The plant cuticle is composed of cutin (a polymer of cross-esterified hydroxyfatty acids) and a mixture of long-chain hydrocarbons mainly lipid derivatives (long chain fatty acids, fatty alcohols, alkanes etc.), known collectively as wax (27). Flower petal is one of the most substantial sources of such wax metabolites which might be the reason behind **RFD**'s high lipid content. Moreover, Flower petal, specifically of Rhododendron, was earlier reported to contain a large amount of fatty acids (28,29).

Fermentation led increase in antioxidant activity was detected in *Guras* wine which exhibited better results in DPPH and iodometric assays compared to its unfermented version. Fermented sample **GW**'s antibacterial activity against *Bacillus subtilis* and *Klebsiella pneumoniae* also validated efficacy of this high-altitude beverage to treat high-altitude's gastrointestinal and respiratory infections. Presence of bioactive molecules like phenol, flavonoids, coumarin, cardiac glycosides, terpenoids and steroids could be the reason behind tested *in vitro* bioactivities of Guras beverages which may also possess other medicinal properties (cardioprotective, neuroprotective, gastroprotective, respiratory-protective, antiinflammatory) to treat other high altitude sicknesses as suggested by Majumder et al. (9). Further insight into metabolomics on *Guras* would be affirmative.

The breakdown rate of unfermented **RFD** was lowest during gastro-intestinal simulation as revealed by total phenol content estimation and antioxidant activity (Table 2; Figure 7). Further, Rhododendron's chlorogenic acids and quinic acid compounds could likely account for the persistent and hard-to-digest nature of **RFD**. Previously this phenolic acid has been observed to remain unchanged within the gut during the process of microbe-independent metabolism (30). *In vitro* colonic biotransformation experiment by Naranjo Pinta et al. (30) revealed that in human digestive system, quinic acid before being absorbed can be transformed into simpler compounds by the gut microbiota. According to that study, gut microbiota play role in the absorption and metabolism of food bioactives like flavonoids other phenolic acids that remain intact while reaching the colon. In summary, the findings indicate that while **RFD** might not be readily digestible within the human gastrointestinal tract, it can undergo biotransformation into more easily digestible forms such as **GW** and **GR**, facilitated by

traditional fermentation technology. *In vitro* GID results can be further described after detailed study with metabolites of these beverages.

Recently, Majumder et al. (9) carried out gastro-intestinal simulation experiment with some fermented *kodo*-based ethnic beverages. Their research revealed stomach/gastric phase as the leading phase for digestion of antioxidants such as phenolic components and intestine to lead in the breakdown of most of the lipids, which is corresponding to the results of our research. Earlier, Sollano-Mendieta et al. (32) also reported high rate of reduction in antioxidant activity (DPPH assay) and total phenol content (TPC) during *in vitro* gastric or stomach phase. In human GI tract, involvement of bile acids to facilitate lipid digestion in the small intestine has been well established (33) which is corresponding to the quantified high lipid digestion during intestinal phase in this experiment.

4. Conclusions

The ethno-foodology of Singalila and adjoining high-altitude places of Darjeeling, Sikkim, and Nepal talks about various fermented and probiotic foods that are yet to be explored. The current study was designed as a novel approach combining bioactivity and bioaccessibility of Himalayan ethnic beverage- fermented *Guras* (Rhododendron) wine and distilled *Guras Raksi* through *in vitro* assessments. Unfermented decoction was also considered among the samples because the decoction is also often consumed to avail its ethnomedicinal properties. Biochemical properties, *in vitro* bioactivities and gastrointestinal simulation study have evaluated ethnomedicinal potential of these traditional beverages. Medicinal activities of these beverages are associated to consumption. However, consumption of alcohol containing beverages is a matter of concern, therefore intaking of a limited quantity of these traditional beverages in high altitudinal areas can be an effective regimen towards coping against various high-altitude induced ailments owing to cumulative effects of the constituent metabolites. Due to the remoteness of villages of Singalila from large-scale markets, the indigenous populations rely heavily on locally produced and packaged beverages to tolerate the trials of a colder climate. Tourists also often indulge in these beverages to quench their thirst and benefit from their medicinal properties, which can help them to alleviate high-altitude sicknesses including headache and muscle pain. This *in vitro* study unveiled the therapeutic potentials of fermented *Guras* from Singalila, discovering it as “more than a sip”. For further insight work may be planned on metabolite profiling, pharmacology of ethnic beverages, *in silico* and *in vivo* experiments to investigate the medicinal properties more in depth.

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Author Contributions

S.M. conceived the idea. S.M. and M.B. designed the protocols. S.M. did the sample collection and performed all the biochemical experiments. A.G. and S.M. performed antibacterial assay. S.M. and M.B. did the statistical analysis. S.M. analysed and compiled the

data and wrote this paper. M.B. supervised the whole research. All the authors read and approved the manuscript.

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The data supporting the findings of the article is available within the article.

Conflicts of Interest

All authors declare no conflict of interest, financial or otherwise.

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