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RESEARCH

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In vitro bioactivities and gastrointestinal simulation validate ethnomedicinal efficacy of five fermented *kodo*-based Himalayan traditional drinks and bioaccessibility of bioactive components

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Abstract

Tongba, *chhyang*, *nigar* and *raksi* are popular traditional drinks served in Nepal, northern and north eastern part of India and Tibetan plateau which are claimed as medicinal and considered as elements of high altitude ethnopharmacology. In this research, *kodo* (finger millet) based traditional beverages such as *tongba*, *chhyang*, *nigar* and *raksi* samples, collected from Singalila ridge of the Himalayas, were analysed through various qualitative and quantitative biochemical parameters. Total phenolic content (gallic acid equivalent) was estimated in a varied range from 936.26 ± 6.87 µg/mL (*nigar*) to 96.36 ± 1.22 µg/mL (*chimphing raksi*) while highest free fatty acid was measured in *khokim raksi* (2.11%). Antioxidant activity was evaluated using in vitro assays (DPPH assay, iodometric assay and in vitro anti-lipid peroxidation assay); antibacterial assay was conducted using well diffusion method. *Tongba* was found to be the most potential sample in antioxidant assays with a DPPH scavenging value of 85.31 ± 1.54% while *nigar* was the sample with most promising antibacterial activity. Furthermore, special emphasis was given on GC-MS based metabolite profiling and metabolomic elucidation. Responsible candidates detected by GC-MS analysis were discussed to correlate results of preliminary biochemical screenings and in vitro bioactivities. In vitro gastrointestinal digestion was carried out to figure out bioaccessibility of the bioactive groups of compounds in simulated human GI tract where DPPH assay, total phenol content and lipid composition (acid value) were considered as parameters. Most of the bioavailable bioactive antioxidants and phenolics were estimated to be decreased in the gastric phase reflecting the highest rate of digestion in the stomach while breakdown of lipid composition was dominated by the intestinal phase. Overall results suggest that antioxidative phenolics and fatty acids from these beverages possess bioactivities in their bioavailable form which may be associated to their ethnomedicinal properties.

Keywords Traditional beverages, Antioxidant, Antibacterial, Gastrointestinal simulation, GC-MS analysis

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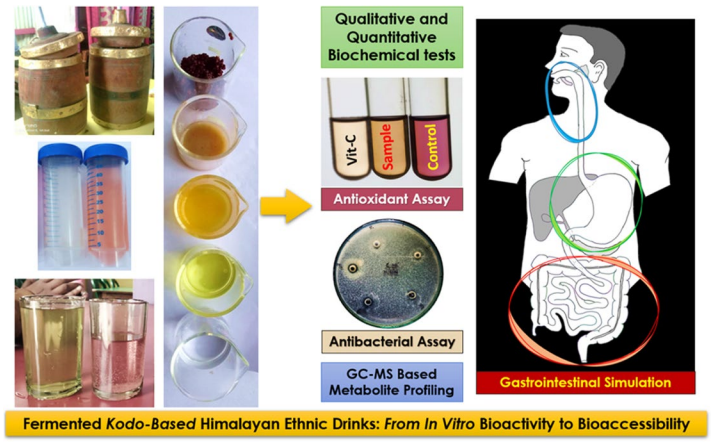
Graphical Abstract



Introduction

Food production and availability differ depending on altitude, soil, weather condition and other ecological aspects. Therefore, food habits and food requirements of the people living at high-altitude are different from the rest of the world (Majumder et al. 2021a). Picôn-Reâtegui (1978), pioneer scientist in this field, demonstrated how high-altitude effects human nutrition, metabolism and other physiological activities. Himalaya's ethnoecology stands upon lifestyle of people belonging to different ethnicities (Nepalese and Tibetan ethnic community). Several ethnic foods and beverages and ethnomedicines enrichen with natural products found in the Himalayas are useful to inhabitants and tourists which help to endure the altitudinal stresses, recover from various high-altitude sicknesses and get acclimatized with the environmental temperature that remains frigid throughout the year (Majumder et al. 2021a).

Perched atop the elevation of 2400–3600 m, the Singalila ridge of the Eastern Himalaya, is situated in the Indo-Nepal region including parts of Darjeeling and West Sikkim districts of India and Mechi zone of Nepal. Besides being a habitat of endangered animals, birds and plants, Singalila National Park is also considered as a land to conserve people belonging to various ethnic groups such as Sherpas, Tibetans, Rais, Tamangs, Lepchas, Limbus, Gurungs etc. (India Biodiversity Portal, 2016). Indigenous traditional knowledge of these people is associated to the ethnopharmacology of Singalila where use of various medicinal plants and preparation of traditional foods and fermented beverages play important role. Panda (2018) reported an extensive field survey on ninety-two threatened ethnomedicinal plants of Singalila National Park including their ethnic drug dosimetry. According to the report, more than fifty diseases and disorders, including sciatica, arthritis, asthma, blood dysentery, diarrhoea, snake bite, insect bites, throat pain, high blood pressure and other cardiovascular illnesses, inflammation etc. can be treated by using distinct parts of those ethnomedicinal plants (Panda, 2018).

Fermented foods are used in many countries because of their ability to promote health and prevent diseases. Most of the traditional fermented foods contain probiotics defined as beneficial bacteria. But the nature of the products and basic ingredients (substrates and fermentation starters) vary around the world (Saeed et al. 2022). The ethno-foodology of Singalila is also very distinctive and diverse, enriched with a plenty of traditional foods which include cereal-based fermented beverages like, *tongba* or *tumba*, *chhyang* or *chhaang*, *nigar*, *raksi* etc. (Majumder et al. 2021a). Traditional knowledge on food preservation and fermentation is an immense part of food management in the Himalayas where arrangement of important raw food materials throughout year is exceedingly difficult. So, preservation is carried out to increase the shelf-life of food materials (Majumder et al. 2021a) where traditional fermentation technologies are used. *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, *tongba*, *chhyang*, *nigar*, *raksi* etc. are some of the fermented ethnic foods consumed in the high altitudes of Darjeeling and Kalimpong districts of West Bengal; Sikkim; and Nepal (Majumder et al. 2021a).

Tongba, a finger millet-based fermented drink, is an important token of the *Limbu* community's tradition. *Tongba* is drunk regularly by many people belonging to Tibetan, Nepali and other ethnic groups who live high altitude places of Nepal, northern and north eastern part of India and Tibetan plateau. *Tongba* has many medicinal properties mainly anti-inflammatory or pain-relieving activity as demonstrated by Dangal et al. (2021) and Majumder et al. (2022a). Dangal et al. (2021) reported the traditional preparation of this drink. Traditionally, the culture is aged even up to six months where the fermentation culture matures exhibiting intense flavours (Harmayani et al. 2019). Another ethnic beverage of this region is *chhyang* or *kodo ko jaanr* (finger-millet-based) which is mild-alcoholic, sweet-flavoured and one of the most nutritious drinks with high calories and vitamins (Ray et al. 2016). Cereal grains such as rice or millet are mixed with dried yeast starter called *marcha* (about 1–2% by the substrate weight) and put in an earthen pot or jar (Ray et al. 2016) for solid state fermentation. Fermentation period of this beverage varies with seasons; as three days for summer and ten days for winter (Ray et al. 2016). *Raksi* or *rakshi* is the distilled version of the beverage *chhyang* which was reported to contain volatiles having medicinal properties to prevent every disorder or disease referred to as high altitude sickness (Majumder et al. 2021a). Fusion of different additives or ethnomedicinal natural products such as, flowers of *chimphing* [*Tetrataenium nepalense* (D. Don) Manden] or root of *khokim* [*Rheum emodi* Wall. ex Meissner] is considered as a regular practice in the preparation of *raksi* in Singalila (Majumder et al. 2021a). Beside finger-millet (*Eleusine coracana* Gaertn.) or *ragi* (Hindi) or *kodo* (Nepalese), whole grains of wheat, rice and sorghum or a blend of diverse types of grains are also used as substrates for the preparation of *chhyang*, *nigar* and *raksi*. Traditionally cultured dried yeast, i.e., *marcha* or *khesung* (source of molds, bacteria and yeast) is the only starter used in fermentation of these beverages. Starter culture *marcha* is prepared by pounding glutinous rice soaked in water overnight (Yamamoto and Matsumoto, 2011) and that is the only source of fermenting microbes in these beverages. So, inclusion of rice grown amylolytic filamentous yeasts (*Aspergillus oryzae*, *Rhizopus oryzae* and *Saccharomycopsis fibuligera*) and probiotic lactic acid bacteria in the microbiome of a rice-based-starter (*marcha*) is natural. This microflora is reported to produce various bioactive metabolites (Yamamoto and Matsumoto, 2011; Olee et al. 2022).

Traditional foods are an integral part of the diet of people living in the high-altitude regions of the Himalayas and often consumed by visitors as well. So, investigation of such underexplored foods needs to be carried out to elucidate their role in the beneficial effects on human health. Traditional foods are generally considered healthy; therefore, the foodomics study on traditional foods is essential to validate acclaimed properties. In this research, five *kodo* (finger millet) based fermented beverages- *tongba*, *chhyang*, *nigar* and *raksi* (both *khokim raksi* and *chimphing raksi*) were collected from Singalila ridge of the Himalayas which were subjected to several qualitative and quantitative biochemical analysis to characterize those in a comparative way and evaluate the ethnomedicinal claims. As part of our ongoing effort to explore biological activities of different Himalayan ethnic beverages, our present study has been designed to explore in vitro antioxidant and antibacterial properties. Induction potentials of these beverages on the lipid peroxidation of goat liver homogenate were also tested to ascertain their hepatoprotective activity. GC-MS based volatile profiling has been done to discuss their biochemical profiles comprehensively through comparative analysis. Bioactivity and bioaccessibility cumulatively describe bioavailability of a food or drug (Fernández-García et al. 2009). So, another important objective of this research was to conduct in vitro gastrointestinal digestion using a static simulation model where in vitro assays (DPPH assay and determination of total phenol content and lipid composition) were considered parameters to evaluate the catabolism nature of these beverages and to figure out bioaccessibility of the bioactive compounds of these beverages in simulated human digestive system.

Materials and methods

Sample collection

The sample collection procedure was conducted on the trekking route of Singalila National Park (Fig. 1) located in the Darjeeling district of India and Indo-Nepal border. Samples were collected while travelling across some old human settlements or villages situated at the buffer zone of the national park as following; Dhotrey, Tonglu, Tumling, Gairibans, Kalipokhri, Bikheybhanjyang, Sandakphu, Gurdum, Srikhola and Rimbik.

Six-month aged traditionally brewed culture of *tongba* (TNB) (before adding hot water as the drink is served) was collected from the village Kalipokhri (27°04'45"N, 88°01'03"E; Fig. 1) situated at an altitude of 3040 m (one the highest points of Singalila ridge). *Chhyang* (CHN) and *nigar* (NGR) were collected from a household located in Gurdum, a picturesque hamlet in the Himalayas at an altitude of 2643 m (27°07'16"N, 88°03'26"E; Fig. 1). Both CHN and NGR were collected from a single batch of fermented culture. *Nigar* was a slightly transparent yellowish liquid which was acquired by separating the yellowish supernatant produced during fermentation of cereal (*kodo*) after three days. This liquid is a popular alcoholic beverage consumed in the high altitudes of

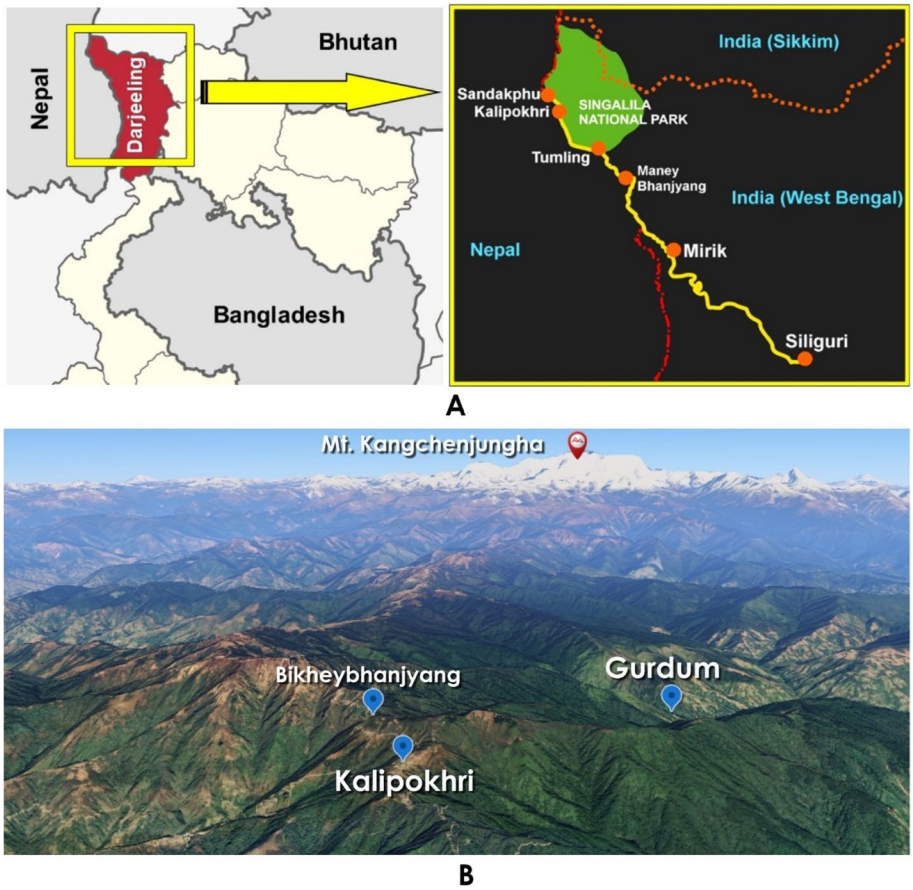


Fig. 1 **A** Location of Singalila National Park situated in Darjeeling district and adjoining Indo-Nepal region. **B** 3D view of Singalila ridge of the Himalayas emphasizing sample collection sites i.e., Kalipokhri (*Tongba*), Gurdum (*Chhyang* and *nigar*) and Bikheybhanjyang (*raksi*) obtained from Google Earth (https://earth.google.com/web/)

Darjeeling, Kalimpong, Sikkim and Nepal. After separating *nigar*, the residue or fermented cereal grains (*kodo*) were left for further fermentation which was later collected (after ten days of fermentation period), thoroughly smashed by mixing water and was filtered through a cloth strainer to obtain the cloudy-white liquor called *chhyang*. Some brewers often mix both the supernatant and grains together to prepare a single beverage. The same culture, without smashing the grains (as done for *chhyang*), is incubated for aging to prepare *tongba* which is offered after adding hot water as mentioned above. Finger-millet *raksi* (*kodo ko raksi*), both *khokim* added *kodo ko raksi* (KKR) and *chimphing* added *kodo ko raksi* (CKR) were collected from a very old and locally famous tavern at Bikheybhanjyang (27°05'32"N, 88°00'47"E; elevation: 3142 m) located between Kalipokhri village and Sandakphu, the highest point of the ridge (Fig. 1).

Finger-millet or *kodo* was found to be the most available substrate for brewing in the study area. So, in this context, only *kodo* based beverages (Fig. 2) were collected. Samples (50 mL of each) were collected in sterilized plastic tubes (Tarsons, India) to avoid contamination and kept inside an ice box.

Qualitative tests

Following the protocols of Kancherla et al. (2019); Das et al. (2020); Majumder et al. (2021b), presence of various bioactive constituents such as terpenoid, glycoside, phenol, flavonoid, coumarin, tannin, protein, steroid and alkaloid were determined in the samples (TNB, CHN, NGR, KKR and CKR) through respective qualitative biochemical tests (Table 1). Glycerol identification test was done following Dunstan's test. Qualitative test for fatty acid was done following Cox and Pearson's phenolphthalein test developed by Hiremath et al. 2007. Table 1 delivers the protocols followed in qualitative tests. A heatmap of the results was prepared in Microsoft Excel (v2303) for a comparative approach.

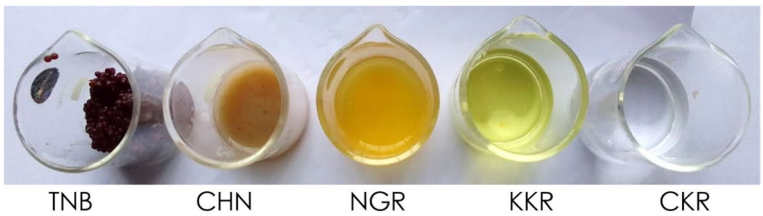


Fig. 2 Five fermented *kodo*-based Himalayan ethnic beverages; *Tongba* (TNB), *chhyang* (CHN), *nigar* (NGR), *khokim raksi* (KKR) and *chimphing raksi* (CKR)

Table 1 Methods followed in qualitative biochemical tests

Group of molecules	Qualitative detection method
Glycoside	0.5 mL of sample were evaporated and dissolved in 1 mL glacial acetic acid. 1 drop of 10% $FeCl_3$ solution followed by 1 mL of Conc. H_2SO_4 was added by the side of test tube. Appearance of brown colour rings at the interface would indicate the presence of glycosides.
Glycerol	Dunstan's test was used for identification of glycerol. Phenolphthalein drops in borax solution give pink colour. If addition of a sample to this solution leads to the disappearance of pink colour in cold which however reappears on heating and again disappears on cooling would indicate the presence of glycerol.
Phenol	In 2 mL of sample, 200 µL of Folin–Ciocalteu reagent and 2 mL 20% (w/v) Na_2CO_3 were added. Appearance of blue colour would indicate the presence of phenol.
Flavonoid	Alkaline reagent test was done here. Two drops of NaOH solution were added to 2 mL of sample. Initially, a deep yellow colour appeared but it gradually became colourless by adding few drops of dilute HCl, indicating that flavonoids were present.
Coumarin	Few drops of NaOH solution was added to 1 mL of sample. Yellow coloration would indicate the presence of coumarin
Tannin	To 0.5 mL sample and few drops of HNO_3 was added. The reddish to yellow colour of the solution would indicate the presence of tannins.
Fatty acid	Few drops of phenolphthalein solution was added into a test tube followed by two drops of diluted NaOH solution. A pink colour will appear. Few drops of sample was then added and shaken. A disappearance of pink colour would indicate the presence of fatty acids in the sample.
Protein	Biuret test was followed here. Two drops of 3% $CuSO_4$ and few drops of 10% NaOH were added to 1 mL of sample, violet or red colour formation would indicating the presence of proteins.
Terpenoid	Horizon test was followed here. Two millilitres of trichloroacetic acid was added to 1 mL of sample. The presence of terpenoids was confirmed by the formation of a red precipitate.
Steroid	For test of 0.5 mL samples were evaporated and dissolved in 2 mL chloroform. 2 mL of concentrated H_2SO_4 was introduced carefully by the sidewall of the test tube. Formation of red colour ring would confirm the presence of steroid.
Alkaloid	Dragendorff 's test was done. By adding 1 mL of Dragendorff"s reagent to 2 mL of sample, an orange red precipitation would indicate the presence of alkaloids.

Quantification of TPC (total phenol content)

Total phenolic content in samples was quantified by the Folin–Ciocalteu method developed by Blainski et al. (2013). 200 µL of sample was taken in 2800 µL of distilled water which was mixed with 500 µL of Folin–Ciocalteu reagent (SRL, India) and 2 mL of 20% (w/v) sodium carbonate (SRL, India) thoroughly (Majumder et al. 2022b). The mixture was left in the 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 (µg GAE/mL). The data were expressed as means of three replicates ± Standard Deviation (SD).

Quantification of FFA (free fatty acid)

Following the protocol of Agee (1938), 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 (Agee, 1938) 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 0.1 N KOH until pale pink colour persisted for 10 s. The end point of the titration was considered as acid value. The FFA (free fatty acid content %) was calculated by diving

the acid value with 2. This assay has also been established as one of the chemical techniques used to determine the lipid composition (https://people.umass.edu/~mcclemen/581Lipids.html). The data were expressed as means of three replicates ± Standard Deviation (SD).

In vitro antioxidant activity

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

Following the protocol of Majumder et al. (2022b), 200 µL of each sample was taken in a test tube containing 2800 µL of 100 mM 2,2-diphenyl-1-picrylhydrazyl or DPPH (HiMedia, India) solution and was left 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:

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

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

Besides this conventional experiment i.e., "DPPH inhibition kinetics" was also included in this study (Majumder et al. 2022b). A reaction kinetics for 30 min was programmed in "Scanning Kinetics" application installed in the Cary-60 UV-vis spectrophotometer (Agilent) for this assay. TI_{50} (time needed to reach the steady state) was also calculated graphically from the plot of absorbance of DPPH solution vs. time. DPPH inhibition kinetics was an observational analysis suitable for this comparative studies.

Iodometric assay

Antioxidant activity was also determined using this titrimetric assay developed for alcoholic beverages (Majumder et al. 2022b). 1 mL of 1% starch indicator was added to 20 mL of sample 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).

In vitro anti-lipid peroxidation assay

Protocol of Rahman et al. (2015) was followed to assess the in vitro lipid peroxidation inhibition activity. Results of this assay were calculated from the standard curve of vitamin E (tocopheryl acetate) ($R^2 = 0.9973$; $y=0.0077x+0.0321$). Results were expressed as mg TAE/ mL (TAE: tocopheryl acetate equivalent). The study has been performed using goat liver homogenate as the lipid source. The data were expressed as means of three replicates ± Standard Deviation (SD).

In vitro antibacterial activity

Well diffusion method (Ghosh et al. 2020) was performed to assess the antibacterial activity of crude samples. Overnight grown cultures of two Gram-negative bacteria i.e., *Escherichia coli* and *Klebsiella pneumoniae* and two Gram-positive bacteria i.e., *Staphylococcus aureus* and *Bacillus subtilis* were used for this experiment. Diameters (mm) of the inhibition zones formed surrounding the well containing samples were recorded as results which were corresponding to quantification of antibacterial activity.

GC-MS based metabolite profiling

Sample preparation for GC-MS analysis was done following the method standardized for alcoholic beverage analysis (Majumder et al. 2021c). One mL of each sample was evaporated and dissolved in 1 mL methanol (Merck) as the polarity of methanol as an organic solvent also shows proximity with water or ethanol, solvents merely of edible beverages. GCMS-QP2010 Plus (Shimadzu Co., Japan) having a DB-5 fused-silica capillary column (30 m x 0.25 mm x 0.25 µm), equipped with a quadrupole mass spectrometer, was used. Split injection (with a ratio of 20:1) technique was performed where injection volume was 1 µL. Column Oven Temperature was 80 °C. Helium gas was used as carrier agent at 1.21 mL/ min. Injection temperature, interface temperature and ion source temperature were adjusted to 260 °C, 270 °C and 230 °C, respectively. Mass spectra were recorded at 5 scan/sec with a scanning rate of 40–650 m/z. The chromatogram (TIC or Total Ion Chromatogram) was based on the intensity of fragments produced by the ionization. Data Acquisition and control of the GCMS-QP2010 Plus is carried out by GCMSsolution software. Utilizing a probability-based matching method, the spectra were compared to the databases (Wiley and NIST) for compound identification (Majumder et al. 2020). The area percentages provided do not represent necessarily the quantitative measure of the contents. However, according to the guidelines by Shimadzu Co., the peak area corresponds to the amount of compound present in a sample (https://www.shimadzu.eu.com/sites/shimadzu.seg/files/SEG/GCMSBASIC.pdf). So, based on this interpretation and other literature (Acharyya et al. 2021; Chakraborty et al. 2023), peak area percentage (%) was considered for quantification of the amount of each compound detected and further quantitative analysis was carried out.

Chemical classification of each compound was based on ChEBI (Chemical Entities of Biological Interest) ontology (https://www.ebi.ac.uk/chebi/) and PubChem database (https://pubchem.ncbi.nlm.nih.gov/).

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 (Sánchez-Velázquez et al. 2021). Protocol reported by Brodkorb et al. (2019), as "INFOGEST static in vitro simulation of gastrointestinal food digestion", 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 as suggested by Shen et al. (2023).

The digestion procedure was divided into three successive phases oral (OP), gastric (GP) and intestinal (IP). The simulated salivary fluid (SSF), simulated gastric fluid (SGF), and simulated intestinal fluid (SIF) were prepared using appropriate amounts of enzymes, electrolyte stock solutions, and calcium dichloride ($CaCl_2$) following the protocol given by Shen et al. (2023) with slight modifications. The oral phase involved dilution of each sample 1:1 (vol/vol) with simulated salivary fluid (SSF) where 1500 U/mL alpha-amylase (SRL, India) and 100 µL 0.3 M $CaCl_2$ were added. Incubation was done for 2 min maintaining the pH at 7.0. The oral digestion mixture (final OP solution) was then diluted 1:1 (vol/vol) with simulated gastric fluid (SGF) containing 2500 U/mL gastric enzyme- pepsin (SRL, India) and 17.5 µL $CaCl_2$ (0.3 M) and incubated at pH 3.0 for 2 h. HCl was used to lower the pH. The gastric digestion mixture (final GP solution) was then diluted in 1:1 (vol/vol) ratio with simulated intestinal fluid (SIF) containing 10 mM bile salt (SRL, India), 2000 U/mL pancreatic enzyme- pancreatin (SRL, India) and 100 µL $CaCl_2$ (0.3 M). The pH of the mixture was adjusted to 6.0. The resultant mixture was cultured at 37 °C for 2 h. The experimental conditions for the digestion procedure, such as pH, time of digestion and enzyme activity, were previously standardized by Minekus et al. (2014). Incubation temperature for each phase was done at 37 °C. A schematic representation of the experimental procedure are presented in Fig. 3, providing information of the process and conditions as maintained. During the gastrointestinal simulation, samples i.e., OP, GP and IP were collected after corresponding phases i.e., oral digestion, stomach or gastric digestion and intestinal digestion for subsequent analysis.

Antioxidant activity (DPPH assay), total phenolic content (TPC) and lipid composition (acid value) 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). A line illustration of human GI tract (Fig. 3) was digitally drawn in Sketchbook (android) software (v5.3.1) to use for pictorial representation of this data. GI tract in each of the illustrations were coloured by Gradient Fill Tool at ArcSoft PhotoStudio v4.1.3 C using the colour codes (as RGB colour mode) which were acquired from the heatmaps

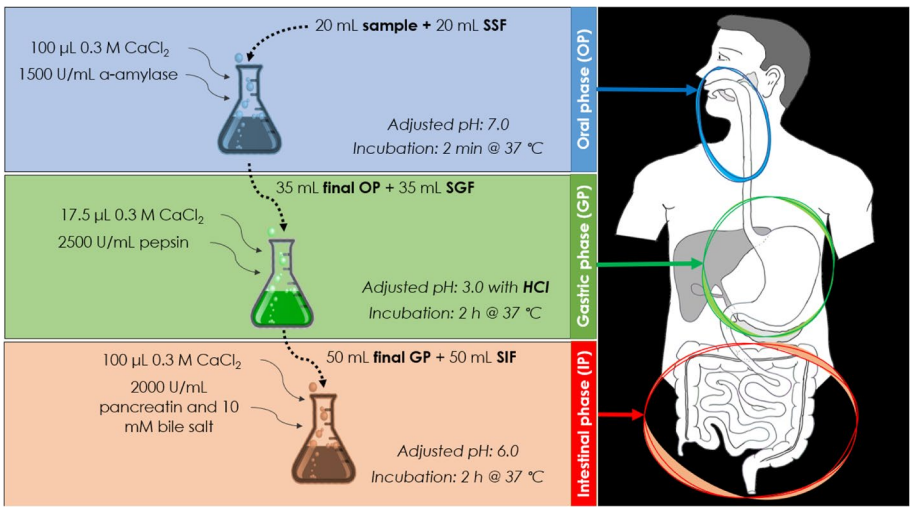


Fig. 3 Schematic diagram showing flowchart of the procedure of gastrointestinal simulation (left) and the line illustration of human GI tract used for pictorial demonstration of the results (SSF: simulated buffer for salivary fluid; OP: oral phase; SGF: simulated buffer for gastric fluid; GP: gastric phase; SIF: simulated buffer for intestinal fluid)

produced with the numerical results in Microsoft Excel v2303. Three distinct colour scales were used to prepare heatmaps of three different parameters. Colour scales were chosen as following, Yellow (RGB: 255, 255, 0) – Dark Purple (125, 0, 146) for antioxidant assay results, Dark Blue (RGB: 18, 3, 55) – White (RGB: 255, 255, 255) for total phenol content results and Red (RGB: 255, 0, 0) – White (RGB: 255, 255, 255) for lipid composition to demonstrate the rate of digestion.

The effect of in vitro GID on the content of DPPH, TPC and FFA was evaluated with the bioaccessibility index (*BI*) calculated with the following equation (Sollano-Mendieta et al. 2021):

$$BI(\%) = \frac{A}{B} \times 100$$

where *A* is the amount of each compound (TPC and FFA) or antioxidant capacity (DPPH) quantified in each digestion phase (OP, GP and IP), *B* is the amount of each compound (TPC and %FFA) or antioxidant capacity (DPPH) quantified in the undigested food matrix (crude sample).

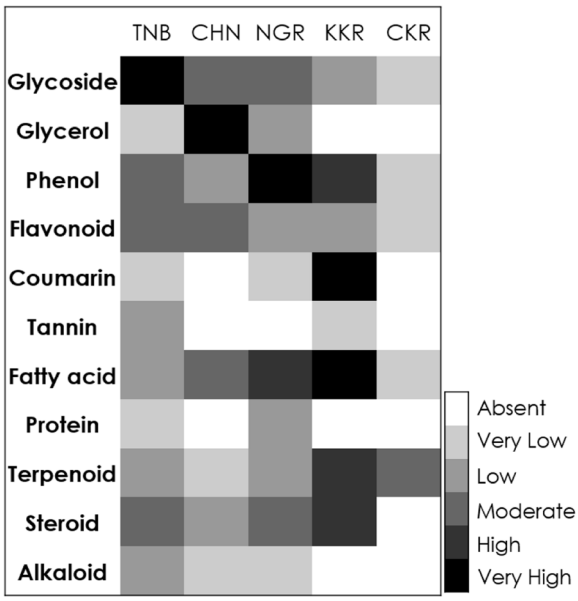


Fig. 4 Monochromatic heatmap showing results of qualitative tests assessed to detect the presence of different bioactive groups of molecules in ethnic beverage samples- *Tongba* (TNB), *chhyang* (CHN), *nigar* (NGR), *khokim raksi* (KKR) and *chimphing raksi* (CKR)

Statistical analysis

Data obtained from the 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). "Data Analysis Tools" of Microsoft Excel was used to get the correlation coefficient. The test for statistical difference was performed using one-way ANOVA (analysis of variance). To calculate the similarity matrix and cluster analysis, the data were transferred to the NTSYS-pc (v2.10e) software. The cluster analysis was performed using UPGMA method based on Jaccard similarity coefficient. The Pearson correlation matrix in Excel was also built using the Correlation tool from the Analysis ToolPak add-in. The dendrogram produced from the results of similarity matrix and cluster analysis by NTSYS-pc (v2.10e) was based on binary system of presence and absence while Pearson correlation relies on numerical data (peak area%) rather than a binary system.

Results

Qualitative tests

Comparative analysis based on qualitative biochemical tests revealed presence of various bioactive molecules in all collected beverage samples. A monochromatic heatmap (Fig. 4) has been generated using the results of these tests. Glycoside was noticed very high in TNB followed by CHN and NGR and detected least in *raksis* (distilled liquors). Glycerol was detected highest in CHN by Dunstan's test. NGR and KKR were detected as samples with high phenolics through the indication test. Flavonoid was found in higher quantity in TNB and CHN compared to others. Fatty acids, terpenoids and steroids were detected maximum in KKR. Alkaloids were detected only in undistilled samples, TNB, CHN and NGR. *Raksi* sample- CKR did not respond well in qualitative tests except for terpenoid. However, quantitative biochemical tests and GC-MS analysis were carried out to find out further explanations as elucidated below.

Quantification of TPC (total phenol content)

TPC was found substantially high in both NGR and KKR compared to rest of the samples. Quantified phenolic value for the sample NGR was recorded as highest with a TPC of 936.26 ± 6.87 µg GAE/mL, for KKR it was 886.96 ± 5.76 µg GAE/mL. Result for CKR was minimum (96.36 ± 1.22 µg GAE/mL) among all. Results of TPC have been graphically represented in Fig. 5A.

Quantification of FFA (free fatty acids)

The highest value of FFA was measured in KKR (2.11%) followed by NGR (1.97%) and least was determined in CKR. The trend of FFA was similar to the results of comparative fatty acid indication test (Fig. 4). The result of this experiment has been graphically represented in Fig. 5B.

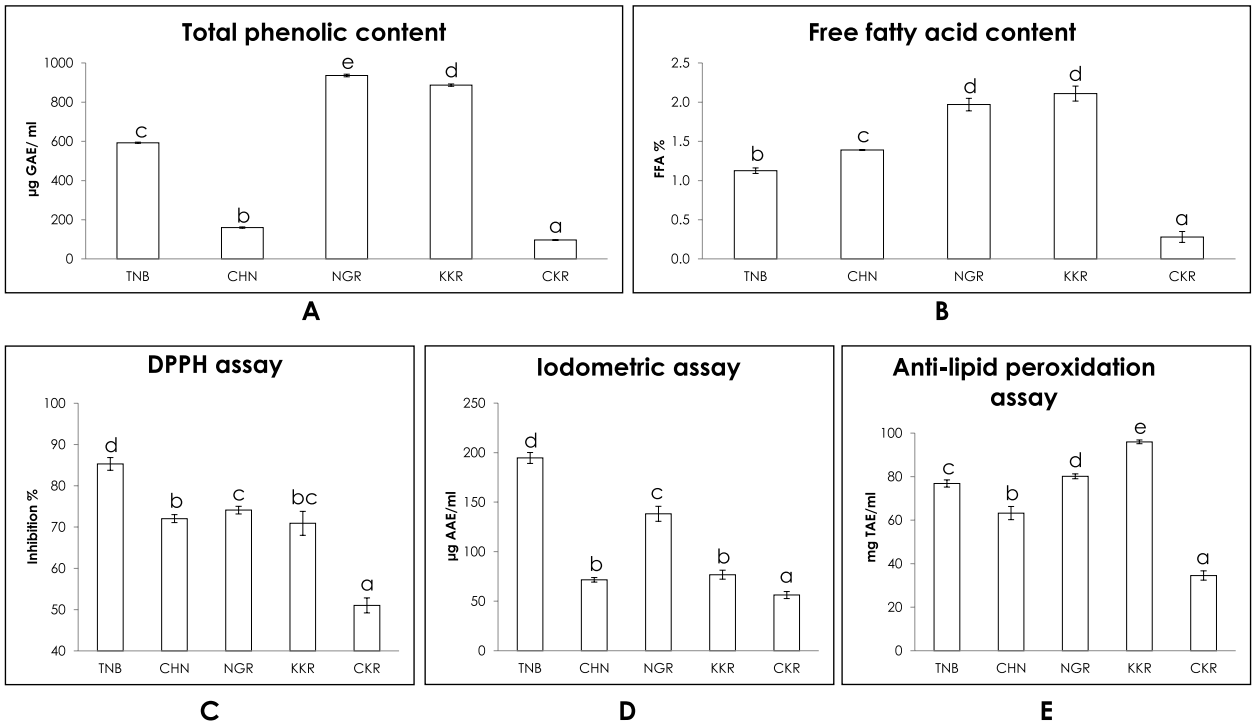


Fig. 5 Results of **A** total phenolic content or TPC; **B** free fatty acid content (FFA %); **C** DPPH assay and **D** iodometric assay for in vitro antioxidant activity; and **E** anti-lipid peroxidation or hepatoprotective assay as assessed in *Tongba* (TNB), *chhyang* (CHN), *nigar* (NGR), *khokim raksi* (KKR) and *chimphing raksi* (CKR). Values are means ± standard deviation, means with different superscripts in each figure are significantly different ($P < .05$). [GAE: gallic acid equivalent; FFA: free fatty acid; AAE: ascorbic acid equivalent; TAE: tocopheryl acetate equivalent]

In vitro antioxidant activity

DPPH assay

This assay revealed TNB, NGR and CHN as outstanding free-radical scavengers for scavenging 85.31 ± 1.54%, 74.11 ± 0.92% and 72.05 ± 0.96% DPPH solution, respectively. Regarding the distilled (*raksi*) samples, KKR was found to possess promising antioxidant activity for scavenging 70.91 ± 2.9% DPPH (Fig. 5C).

Antiradical property of these ethnic beverages was monitored as a function of time to observe the nature of inhibition and to differentiate samples on the basis of this nature. Sharper the decline of absorbance graph faster was the rate of inhibition. Figure 6 has provided the graphs of DPPH inhibition kinetics. Among all, CHN and KKR exhibited faster and sharper declining graphs during the first 10 min (depicting faster release of antioxidants) and further became stable and parallel to the x-axis or reached the steady state (Fig. 6B and D). Consecutively, TNB and NGR exhibited prominent and declined graphs (Fig. 6A and C) where the declining rate was found least for the sample CKR due to its low antioxidant property (Fig. 6E).

Iodometric assay

Results of iodometric assay was similar to the comparative trend found in DPPH assay as TNB showed highest antioxidant property with a recorded value of 194.6 ± 5.58 µg AAE/ mL followed by NGR (138.22 ± 7.56 µg AAE/ mL) while CKR was again at the bottom of the list. The result of this experiment has been provided in the following graph (Fig. 5D).

Lipid peroxidation inhibition assay

Anti-lipid peroxidation activity was considered as another parameter to judge the antioxidant property with an intention to interpret the hepatoprotective activity at the same time. Among all samples, KKR showed the maximum level of lipid peroxidation inhibition with an estimated value of 95.96 ± 0.86 mg TAE/mL followed by NGR (80.12 ± 0.64 mg TAE/mL). Figure 5E depicts the anti-lipid peroxidation efficacy of samples calculated from the tocopheryl acetate standard curve. Specific antioxidant volatiles that could probably protect liver cells from oxidative damage have also been identified by GC-MS as elucidated in the "Discussion" section.

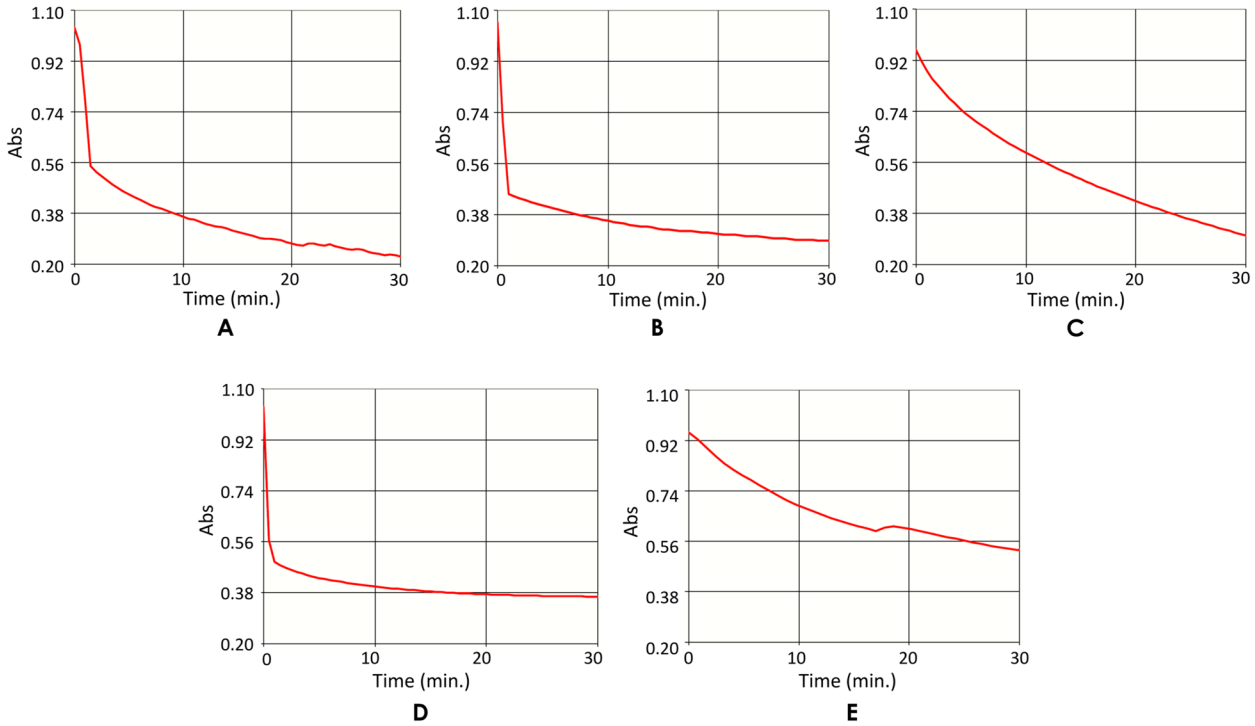


Fig. 6 Results of spectrophotometric DPPH inhibition kinetics study presenting comparative absorbance kinetics graphs produced by different fermented ethnic beverages i.e., TNB or *tongba* (**A**), CHN or *chhyang* (**B**), NGR or *nigar* (**C**), KKR or *khokim raksi* (**D**) and CKR or *chimphing raksi* (**E**) [Abs: absorbance at 517 nm]

In vitro antibacterial activity

The diameter (mm) of the inhibition zone around each sample containing well against each bacterium was recorded as given in Table 2 and photographs of antibacterial plates have been provided in Fig. 7. NGR was found to be the most potential antibacterial sample for showing inhibition zones against all the tested bacteria including maximum inhibition zone against *Escherichia coli* (9 mm). TNB and CKR revealed selective antibacterial activity against *Staphylococcus aureus* and KKR inhibited *Escherichia coli*'s growth only. Furthermore GC-MS analysis focused on this finding by showing

Table 2 Results of antibacterial assay or inhibition zones (diameter in mm) produced by samples- *Tongba* (TNB), *chhyang* (CHN), *nigar* (NGR), *khokim raksi* (KKR) and *chimphing raksi* (CKR)

	Escherichia coli	*Klebsiella pneumonia*	*Staphylococcus aureus*	*Bacillus subtilis*
TNB	-	-	7 mm	-
CHN	-	-	-	-
NGR	9 mm	6 mm	6 mm[a]	8 mm[a]
KKR	7 mm	-	-	-
CKR	-	-	6 mm	-

[a] Partial inhibition

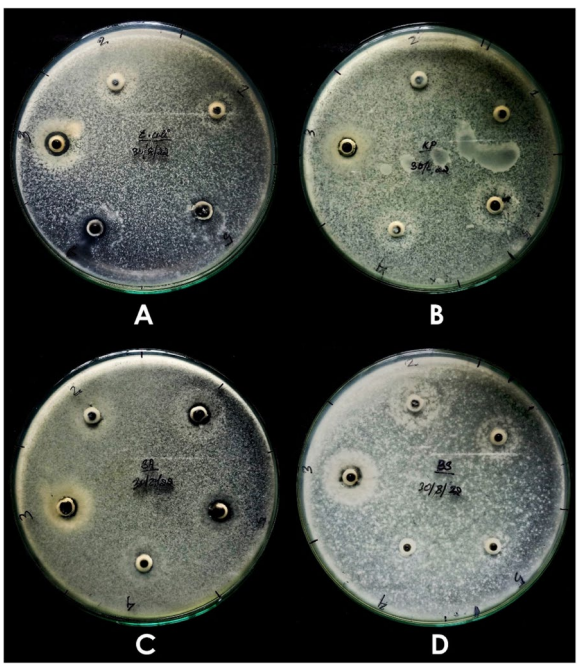


Fig. 7 Pictures of bacterial pour plates (**A** *Escherichia coli*; **B** *Klebsiella pneumoniae*; **C** *Staphylococcus aureus* and **D** *Bacillus subtilis*) showing inhibition zones produced by samples i.e., *Tongba* (TNB), *chhyang* (CHN), *nigar* (NGR), *khokim raksi* (KKR) and *chimphing raksi* (CKR) (coded as 1–5 accordingly)

antibacterial components present in these samples. CHN failed to show any antibacterial property.

Volatile profiles of ethnic beverages

GC-MS analysis revealed- a total of twenty-five metabolites in TNB including ethyl α-D-glucopyranoside (α-EG) as the major compound with 53.95% peak area; twenty-two compounds in glycerol (76.88% peak area) rich sample CHN; thirty-one compounds in NGR which was rich in sugar alcohol (glycerol) and other fermented carbohydrate derivatives followed by fatty acid- oleic acid. Regarding *raksi* samples, linoleate derivatives (23.68% total peak area) were detected as major volatile composition in KKR along with coumarins such as meranzin and auraptene, whereas CKR was abundant in amino acid derived metabolites including major compound cyclo-Gly-Pro (a cyclic dipeptide) occupying 26.47% peak area. The list of volatile metabolites has been provided in Table 3.

The hierarchical cluster analysis generated two major clusters (Fig. 8). First cluster was divided into a subgroup (TNB and CHN) and sample NGR. Only two positive correlations were found in the Pearson correlation matrix (Table 4) among different individuals. Strong correlation was found between CHN and NGR (53% similarity) and a small correlation was found between TNB and NGR (2%).

Moreover, this research highlights bioactivities of entire groups of molecules present in the sampled beverages rather than induvial effects of each metabolite. Hence, these ethnic beverages can be utilized as a form of complementary or alternative therapy to tap into their therapeutic potential. Secondary metabolites such as glycosides, phenolics, fatty acid and amino acid derivatives, terpenoids, alkaloids etc. and candidates responsible for antioxidant and antimicrobial properties were revealed by GC-MS based metabolite profiling which have been emphasized in the "Discussion" section. The graphs in Fig. 9 were produced using the total peak area shared by diverse groups of components (fatty acid derivatives; amino acid derivatives; phenolics; terpenoids and steroids; alkaloids; glycosides; and fermented sugar catabolites i.e., alcohols and organic acids) to study the distribution of volatiles in analysed beverages.

In vitro GID (gastrointestinal digestion)

The outcome of in vitro digestion experiment revealed the measures of components' breakdown and the bioaccessibility of nutritionally important components. Selected parameters (antioxidant activity, total phenol and lipid composition) were found to decrease after every step of digestion (Table 5). For crude samples, results of antioxidant activity (DPPH scavenging %) were determined to be 85.31 ± 1.55%, 72.05 ± 0.96%, 74.11 ± 0.92%, 70.91 ± 2.91% and 51.02 ± 1.81% for TNB, CHN, NGR, KKR and CKR, respectively but after completion of in vitro GID, antioxidant values were recorded insignificant with values that ranged between 3 and 5% only. Similarly, after completion of intestinal digestion, TPC values (μg GAE/mL) for TNB, CHN, NGR, KKR and CKR were seen to be decreased from 592.96 ± 2.68, 159.76 ± 3.42, 936.26 ± 6.87, 886.96 ± 5.76 and 96.36 ± 1.22 to 21.97 ± 2.74, 12.21 ± 3.11, 29.93 ± 4.23, 24.21 ± 1.02 and 9.25 ± 2, respectively. Acid values (%FFA) for the estimation of lipid compositions were quantified to be 1.126 ± 0.035%, 1.39 ± 0.004%, 1.97 ± 0.08%, 2.11 ± 0.095% and 0.28 ± 0.07% in TNB, CHN, NGR, KKR and CKR, which were estimated to be decreased to 0.02 ± 0.01%, 0.04 ± 0.01%, 0.12 ± 0.04%, 0.27 ± 0.08% and 0.02 ± 0.01%, respectively.

Pictorial representation of the results as recorded in Table 5 has been given in Fig. 10 where illustrations of human GI tract were self-explanatory. A total of fifteen pictures demonstrating results of three different parameters performed with five different samples have been collaged here (Fig. 10). In the antioxidant activity assay, the yellow colour gradually changing into dark purple down the GI tract is depicting the digestion of antioxidant components. Similarly, illustrations corresponding to other two assays have colour gradients (from darker to lighter shade down the GI tract) which were used to describe the bioaccessibility and digestion or decrease of targeted bioactive components in human GI tract.

Discussion

In several regions of Japan and China, finger millet grains are malted and fermented to obtain different traditional fermented drinks such as finger millet's *sake* and *huanhjing* (Majumder et al. 2022a). Previously, our research team evaluated the metabolomic similarities between Japanese *sake* and Himalayan *tongba* (Majumder et al. 2022a) by demonstrating influences of both substrate (*kodo* or finger-millet) and rice-based starters (*koji* mold and *khesung* or *marcha*) in their metabolite profiles. In this research, all the sampled Himalayan ethnic beverages were *kodo*-based and fermented by the common starter called *marcha*. So, there was certainly a common factor i.e., influence of raw materials used in the fermentation process which influenced metabolite profiles of all the fermented beverages. However, preparation techniques were different as described in the methodology. Therefore, individual fermentation period, serving procedure, separation technique, distillation process and effect of additives were reasons

Table 3 The lists of metabolites (derived from GC-MS analysis) arranged according to their chemical class or type and their chromatogram peak area% [*Tongba* (TNB), *chhyang* (CHN), *nigar* (NGR), *khokim raksi* (KKR) and *chimphing raksi* (CKR)]

Name of compound	Chromatogram peak area %				
Fatty acid derivatives	*TNB*	*CHN*	*NGR*	*KKR*	*CKR*
Ethyl 4-hydroxybutyrate	-	-	2.56	-	-
Caprylic triglyceride	-	-	-	0.44	-
Ethyl pentadecanoate	0.29	-	-	-	-
Methyl 14-methylpentadecanoate	0.36	-	-	-	-
Palmitic acid	-	5.51	5.62	-	-
Ethyl palmitate	1.01	-	-	-	-
Methyl palmitate	-	-	-	2.18	-
cis-9-Hexadecenal	-	0.49	1.29	-	-
Ethyl linoleate	1.47	-	-	2.58	-
Methyl linoleate	-	-	-	20.84	-
Linoelaidic acid	-	-	0.08	-	-
Ethyl linolenate	2.64	-	-	-	-
Oleic acid	-	1.7	13.33	-	-
Methyl oleate	0.67	0.12	0.16	-	-
Oleic acid, monoester with propane-1,2-diol	-	-	0.7	-	-
Stearic acid	-	-	1.51	-	-
Methyl stearate	-	-	0.09	2.94	-
Methyl petroselinate	-	-	-	11.96	-
cis-Vaccenic acid	-	10.04	-	-	-
Linoleyl alcohol	-	0.08	-	0.26	-
2-Octadecenal	-	-	0.21	-	-
Oxirane, hexadecyl-	-	-	0.08	-	-
Methyl arachidate	-	0.11	0.12	-	-
Arachidyl alcohol	1.96	-	-	-	-
3-Cyclopentylpropionic acid, 2-dimethylaminoethyl ester	0.46	-	-	-	-
2,5-Di(trifluoromethyl)benzoic acid, 3-hexadecyl ester	1.05	-	-	-	-
1-(2,3-Dimethoxypropoxy)-2-methoxyhexadecane	5.79	-	-	-	-
4-(2-Methoxyhexadecoxymethyl)-2,2-dimethyl-1,3-dioxolane	1.48	-	-	-	-
Methyl 2-methoxypropenoate	-	-	2.12	-	-
Octyl methacrylate	-	-	-	-	4.59
5-Chlorovaleric acid, 2,5-dichlorophenyl ester	-	-	-	-	1.14
(7R)-cis-anti-cis-Tricyclo[7.3.0.0(2,6)]dodecan-7-ol	-	-	-	-	2.05
Amino acid derivatives	*TNB*	*CHN*	*NGR*	*KKR*	*CKR*
cyclo (L-Leu-L-Pro)	16.96	0.16	-	-	-
cyclo-Gly-Pro	-	-	-	-	26.47
pGlu-phe-pro	-	-	-	-	5.67
5-Pyrrolidino-2-pyrrolidone	-	-	-	-	16.77
Pyroglutamic acid	0.32	-	-	-	-
Actinomycin C2	1.43	-	-	-	-
Bis(2-(Dimethylamino)ethyl) ether	-	0.36	-	-	-
N,N,N′-Triethylethylenediamine	-	-	-	-	8.2
Cyclopropanecarboxamide, N-cycloheptyl	-	-	-	-	17.11
Lidocaine	-	-	-	8.03	-
Lidocaine benzylbenzoate	-	-	-	1.56	2.51
Phenolics	*TNB*	*CHN*	*NGR*	*KKR*	*CKR*
Tyrosol	2.72	-	1.85	-	-
5-Methylcyclohexane-1,3-diol	0.37	-	-	-	-

Table 3 (continued)

Name of compound	Chromatogram peak area %				
Phenylacetaldehyde	-	-	2.07	-	-
Phenethyl alcohol	-	-	5.91	-	-
Hydroquinone	-	-	0.99	-	-
Meranzin	-	-	-	7.26	-
Auraptene	-	-	-	14.44	-
Phenyl acetate	-	-	-	-	6.84
Terpenoids and steroids	*TNB*	*CHN*	*NGR*	*KKR*	*CKR*
Neophytadiene	0.42	0.31	0.25	-	-
Phytol	0.26	0.08	-	-	-
Farnesol	0.37	-	-	-	-
beta-Sitosterol	0.85	0.29	0.52	-	-
β-Sitosterol-methyl ether	0.85	-	-	-	-
Cholesta-3,5-diene	-	0.2	0.21	-	-
Clionasterol	-	0.26	0.78	-	-
24-Norursa-3,12-diene	-	-	0.83	-	-
Sandacanol	-	-	-	0.93	-
Nootkatone	-	-	-	0.92	-
Copalol	-	-	-	2.1	-
Squalene	-	-	-	2.56	-
Geranylgeraniol formate	-	-	-	1.46	-
Etiocholanolone glucuronide	-	-	-	1.17	-
Driman-8,11-diol	-	-	-	2.13	-
Geranyllinalool	-	-	-	-	4.62
Calamendiol	-	-	-	-	2.53
Alkaloids	*TNB*	*CHN*	*NGR*	*KKR*	*CKR*
2-[1,2-Dihydroxyethyl]-9-[.beta.-d-ribofuranosyl]purine (Nebularine derivative)	-	0.63	-	-	-
Dihydroergotamine	2.7	-	-	-	-
Glycosides	*TNB*	*CHN*	*NGR*	*KKR*	*CKR*
Ethyl α-D-glucopyranoside	53.95	0.83	3.18	-	-
Methyl α-D-galactopyranoside	-	-	1.1	-	-
Fermented sugar alcohols and other catabolites	*TNB*	*CHN*	*NGR*	*KKR*	*CKR*
1,3-Methylene-d-arabitol	1.25	-	-	-	-
Glycerol	-	76.88	17.44	-	-
4 H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-	-	-	19.4	-	-
Succinic acid monoethyl ester	-	-	6.12	-	-
Ethanol, 2-[2-[(tetrahydro-2 H-pyran-2-yl)oxy]ethoxy]-	-	-	0.94	-	-
5-Hydroxymethyl-2-furfural	-	-	8.19	-	-
Levoglucosan	-	-	2.01	-	-
Other compounds	*TNB*	*CHN*	*NGR*	*KKR*	*CKR*
Phthalic acid	0.37	0.17	-	16.24	-
Bis(tridecyl) phthalate	-	-	0.35	-	-
Isobutyl tridec-2-yn-1-yl phthalate	-	-	-	-	1.51
12-Crown-4	-	1.61	-	-	-
Musk Ambrette	-	0.13	-	-	-
Diltiazem	-	0.05	-	-	-

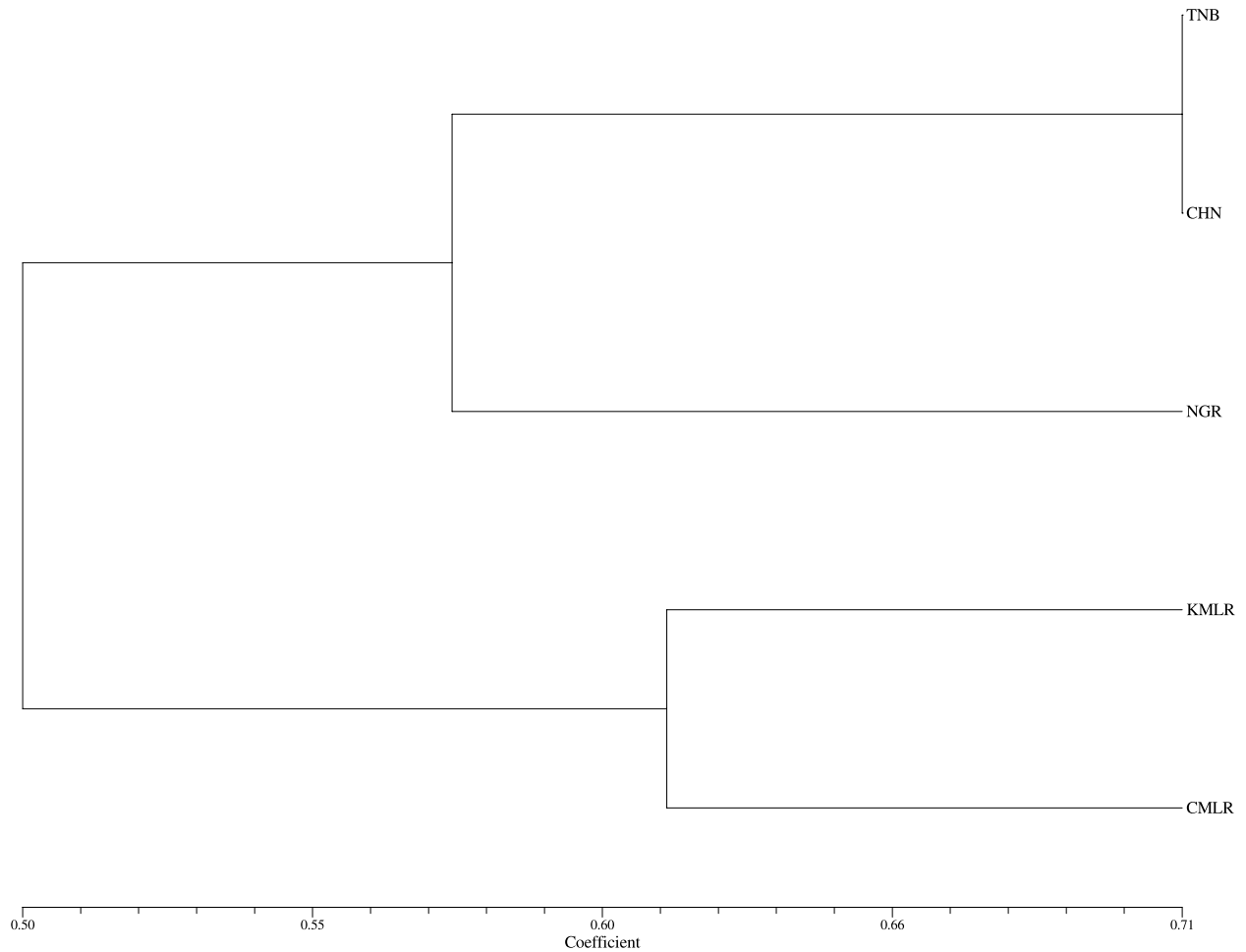


Fig. 8 NTSYS-pc (v2.10e) derived similarity matrix and cluster analysis result of five ethnic beverage samples (based on binary system of presence and absence of metabolites). [*Tongba* (TNB), *chhyang* (CHN), *nigar* (NGR), *khokim raksi* (KKR) and *chimphing raksi* (CKR)]

Table 4 The Pearson correlation matrix of five ethnic beverages (based on GC-MS peak area% of metabolites) [*Tongba* (TNB), *chhyang* (CHN), *nigar* (NGR), *khokim raksi* (KKR) and *chimphing raksi* (CKR)]

	TNB	CHN	NGR	KKR	CKR
TNB	1				
CHN	-0.02	1			
NGR	0.02	0.53	1		
KKR	-0.05	-0.05	-0.11	1	
CKR	-0.07	-0.05	-0.12	-0.09	1

behind variations found in results which have been discussed during interpreting results.

The results of qualitative tests as presented in Fig. 4 revealed presence of bioactive groups of molecules in the selected ethnic beverages. Coumarins were detected too high in *khokim raksi* or KKR to validate its high phenol content (Fig. 4). Interestingly, the additive agent- *khokim* (*Rheum emodi* Wall. ex Meissner) was reported as an abundant source of antioxidative polyphenols, namely signature anthraquinone metabolites (such as emodin and rhein) and coumarins (Zargar et al. 2011; Park and Lee, 2021). However, NGR exhibited the best result in phenol indication test but subsequent tests for phenolic constituents revealed neither coumarin nor flavonoid was responsible for NGR's high phenolics (Fig. 4). Distilled *raksi* samples (KKR and CKR) were devoid of alkaloids and proteins indicating distillation process as a reason behind this separation. Perhaps terpenoids (mainly monoterpenoids) being volatile in nature, were carried into the distilled portion along with condensed alcohol which was the reason behind detection of high terpenoids in both *raksi* samples (Fig. 4).

A total of 41.2% peak area of KKR included derivatives and esters of various fatty acids (Fig. 9) including major compound Methyl linoleate (20.84%). Fatty acid

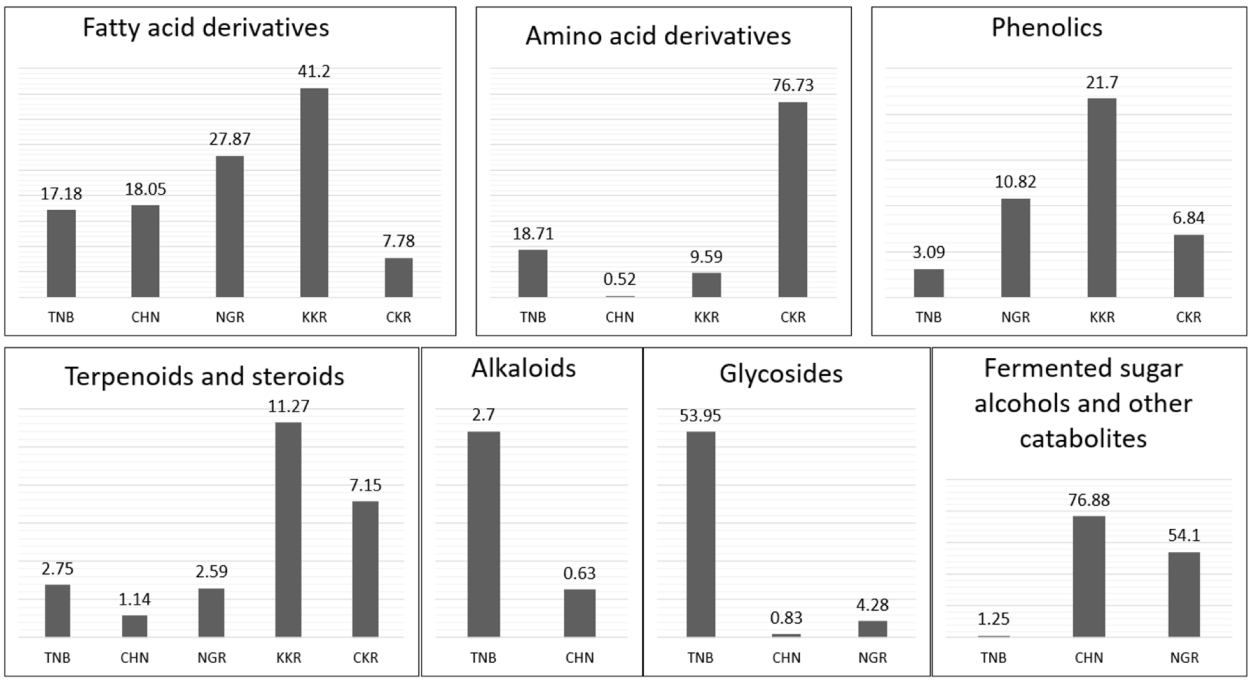


Fig. 9 Distribution of different types of metabolites based on GC-MS derived peak area% [*Tongba* (TNB), *chhyang* (CHN), *nigar* (NGR), *khokim raksi* (KKR) and *chimphing raksi* (CKR)]

Table 5 Results of in vitro gastrointestinal digestion of antioxidants, total phenolic content and lipid composition tested in *Tongba* (TNB), *chhyang* (CHN), *nigar* (NGR), *khokim raksi* (KKR) and *chimphing raksi* (CKR)

Parameters	GID phases	TNB	CHN	NGR	KKR	CKR
Antioxidant activity (DPPH scavenging %)	*Crude sample*	85.31 ± 1.55^{d}	72.05 ± 0.96^{b}	74.11 ± 0.92^{c}	70.91 ± 2.91^{bc}	51.02 ± 1.81^{a}
	Oral phase	79.17 ± 1.34^{d}	68.09 ± 0.35^{c}	68.88 ± 1.1^{c}	62.22 ± 1.88^{b}	42.47 ± 0.67^{a}
	BI (%)	92.81	94.51	92.94	87.74	83.24
	Gastric phase	9.36 ± 0.63^{b}	9.93 ± 1.03^{b}	7.25 ± 0.78^{a}	10.73 ± 0.61^{c}	7.65 ± 0.37^{a}
	BI (%)	10.97	13.78	9.78	15.13	14.99
	Intestinal phase	4.7 ± 1.06^{a}	4.28 ± 0.93^{a}	3.66 ± 1.43^{a}	3.55 ± 1.3^{a}	3.04 ± 1.08^{a}
	BI (%)	5.51	5.94	4.94	5.01	5.96
Total phenol or **TPC** (μg GAE/ mL)	*Crude sample*	592.96 ± 2.68^{c}	159.76 ± 3.42^{b}	936.26 ± 6.87^{e}	886.96 ± 5.76^{d}	96.36 ± 1.22^{a}
	Oral phase	587.85 ± 2.46^{c}	151.94 ± 2.86^{b}	900.24 ± 3.97^{e}	878.09 ± 4.26^{d}	81.03 ± 1.73^{a}
	BI (%)	99.14	95.1	96.15	99	84.09
	Gastric phase	34.70 ± 3.94^{c}	23.98 ± 4.27^{b}	87.02 ± 1.47^{e}	74.30 ± 3.99^{d}	18.66 ± 1.42^{a}
	BI (%)	5.85	15.01	9.29	8.38	19.37
	Intestinal phase	21.97 ± 2.74^{c}	12.21 ± 3.11^{b}	29.93 ± 4.23^{d}	24.21 ± 1.02^{cd}	9.25 ± 2^{a}
	BI (%)	3.71	7.64	3.2	1.6	9.6
Lipid composition (acid value or %FFA)	*Crude sample*	1.126 ± 0.035^{b}	1.39 ± 0.004^{c}	1.97 ± 0.08^{d}	2.11 ± 0.095^{d}	0.28 ± 0.07^{a}
	Oral phase	1.12 ± 0.04^{b}	1.34 ± 0.01^{c}	1.92 ± 0.05^{d}	2.01 ± 0.07^{d}	0.21 ± 0.09^{a}
	BI (%)	99.47	96.4	97.46	95.26	75
	Gastric phase	1.09 ± 0.05^{b}	1.12 ± 0.04^{b}	1.27 ± 0.06^{c}	1.98 ± 0.09^{d}	0.21 ± 0.07^{a}
	BI (%)	96.8	80.58	64.47	93.84	75
	Intestinal phase	0.02 ± 0.01^{a}	0.04 ± 0.01^{a}	0.12 ± 0.04^{a}	0.27 ± 0.08^{a}	0.02 ± 0.01^{a}
	BI (%)	1.78	2.88	6.09	12.8	7.14

The superscript letters in the same column represent statistically ($P < .05$) different groups. [*BI* Bioaccessibility Index, *DPPH* 2,2-diphenyl-1-picrylhydrazyl, *TPC* Total phenol content, *GAE* Gallic acid equivalent, *FFA* Free fatty acid]

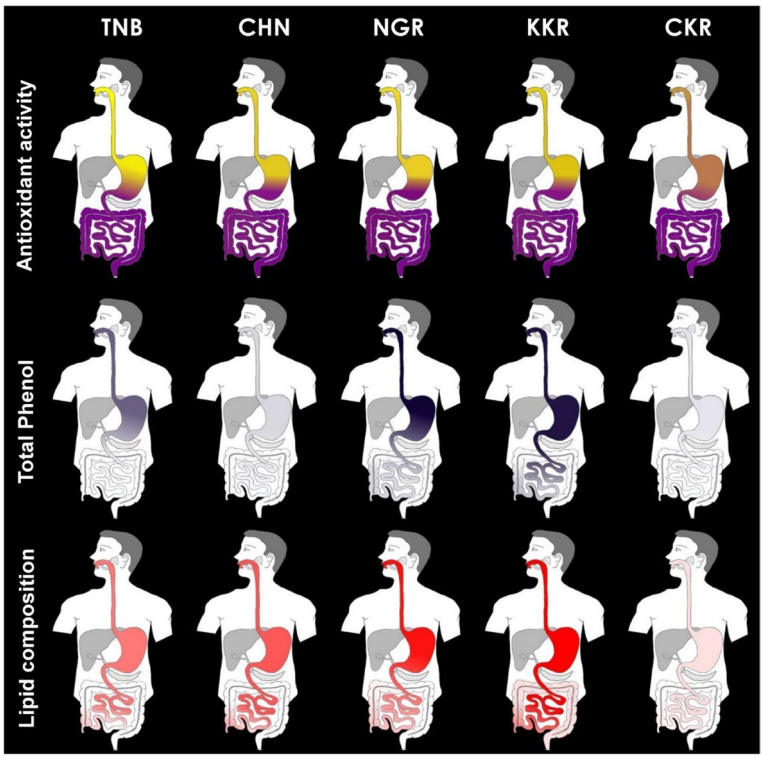


Fig. 10 Overall result of gastrointestinal simulation study which was based on three different parameters performed with five ethnic beverage samples [*Tongba* (TNB), *chhyang* (CHN), *nigar* (NGR), *khokim raksi* (KKR) and *chimphing raksi* (CKR)], the pictorial version of Table 5. The change in the shade of colour down the GI tract of each illustration depicts the digestion and bioaccessibility of each composition. [Colour scales were chosen as following, Yellow (RGB: 255, 255, 0) – Dark Purple (125, 0, 146) for antioxidant assay results, Dark Blue (RGB: 18, 3, 55) – White (RGB: 255, 255, 255) for total phenol content results and Red (RGB: 255, 0, 0) – White (RGB: 255, 255, 255) for lipid composition]

abundancy (27.87%) was also found in NGR led by oleic acid derivatives. Comparative standings based on fatty acid metabolome follow this order i.e., KKR, NGR, CHN, TNB and CKR (Fig. 9) which was also reflected in qualitative and quantitative test results (Figs. 4 and 5B). Total phenol content comprises one (phenolic acids) or more (polyphenols) aromatic rings with attached hydroxyl groups in their structures (Minatel et al. 2017). TPC was quantified substantially high in both NGR and KKR compared to other samples (Fig. 5A). Phenolics detected in NGR were of single aromatic rings i.e., tyrosol, phenylacetaldehyde, phenethyl alcohol and hydroquinone (Table 3). Polyphenol coumarins such as meranzin and aueaptene were detected as major volatile components in KKR responsible for high TPC (Fig. 5A) and coumarin content (Fig. 4). Higher phenolic content is responsible for bioactivities; therefore, NGR and KKR were expected to exhibit good antioxidant and antibacterial activities which were further supported by respective assays as mentioned in the results. Abundance of terpenoid biosynthesis pathway derived products were found in distilled beverages as a total of seven different terpenoids and steroids such as sandacanol, nootkatone, copalol, squalene, geranylgeraniol formate, etiocholanolone glucuronide and driman-8,11-diol were detected in KKR while CKR was found to contain terpenoids with notable peaks i.e., geranyllinalool (4.62%) and calamendiol (2.53%). Previously, qualitative test showed presence of alkaloid in collected undistilled ethnic beverages. Further, GC-MS revealed alkaloids, such as dihydroergotamine (2.7%) and 2-[1,2-dihydroxyethyl]-9-[.beta.-d-ribofuranosyl]purine (Nebularine derivative) (0.63%) as detected in TNB and CHN, respectively. Fermented carbohydrate catabolite products such as sugar alcohols (mainly glycerol), Maillard reaction products and glycosides were detected in undistilled beverages. In qualitative tests cardiac-glycosides were detected high in TNB followed by NGR and CHN which was further validated by GC-MS analysis revealing corresponding peaks of major compound ethyl α-D-glucopyranoside in TNB (53.95%) followed by NGR (3.18%) and CHN (0.83%). The presence of glycerol was found inversely proportional to glycosides in undistilled beverages (Fig. 4). 76.88% peak area in the chromatogram of CHN was occupied with glycerol. NGR had a noteworthy peak of glycerol (17.44%) among its carbohydrate derived fermented products. Karki

(2013), reported abundance of phenolic content and fatty acids in a similar beverage- referred as traditional millet beer from Nepal. Guo et al. (2018) reported high total polyphenol content determined by Folin–Ciocalteu colorimetric method in traditionally prepared and commercially available millet wine samples from China. The antioxidant capacity was also evaluated which revealed promising DPPH radical-scavenging activity (Guo et al. 2018). Guo et al. (2018) also analysed the amino acid profile that revealed abundance amount of glutamic acid (Glu), leucin (Leu), glycine (Gly) and proline (Pro) in various millet wine samples and their result was found corresponding to our results because peptides detected in our samples (mainly in CKR) were composed of those amino acids (cyclo-Leu-Pro, cyclo-Gly-Pro, pGlu-Phe-Pro, pyroglutamic acid etc.).

Hierarchical cluster analysis was based on binary system of presence and absence of individual metabolites which revealed similarities between TNB and CHN. CHN is the filtered version of TNB prepared by smashing the same *kodo* grains which are served as TNB after pouring hot water. The same glycoside (α-EG), dipeptide (cyclo-Leu-Pro), terpenoids (phytol, neophytadiene and sitosterol) were detected in both TNB and CHN (Table 3) resulting comparatively highest level of metabolomic similarity with a similarity coefficient of 0.71 in the similarity matrix (Fig. 8). GC-MS analysis suggests that NGR joined the group (first cluster) due to the presence of glycerol, α-EG, oleic and palmitic acid, neophytadiene, sitosterol and clionasterol as detected in TNB and/or CHN. Second cluster was divided into KKR and CKR. Both the *raksi* samples (KKR and CKR) were also close to each other showing a similarity coefficient of 0.62. Interestingly, this clustering completely outgrouped distilled *raksi* samples from the group of undistilled beverages TNB, CHN and NGR.

Based on chromatogram derived peak areas, the high degree of correlation (revealed by Pearson correlation matrix) between CHN and NGR (Table 4) was due to obtaining notable peak areas of glycerol (major compound for both) and other common components having equivalent peak area% in the chromatograms such as fatty acids (Derivatives of palmitic acid and oleic acid) terpenoids (Neophytadiene; β-sitosterol; cholesta-3,5-diene; and Clionasterol) and glycoside α-EG.

Antioxidant activities of ethnic beverages have been analyzed through DPPH assay, iodometric assay and anti-lipid peroxidation assay where excellent activities were noticed in TNB, NGR, CHN and KKR. According to GC-MS based metabolite profiling, nine components out of twenty-five metabolites of TNB were reported as antioxidative, these were major compound- cyclo (L-Leu-L-Pro) (Deepak et al. 2021), oleic acid (Wei et al. 2016), palmitic acid, β-sitosterol, phytol, neophytadiene, farnesol, tyrosol and actinomycin C2 (Majumder et al. 2022a). Following TNB, NGR was the sample with maximum antioxidant activity which was detected with large peaks of numerous potential antioxidant components. The list includes major compound- oleic acid, palmitic acid, β-sitosterol, neophytadiene, tyrosol, hydroquinone (Elwakil et al. 2000), phenylacetaldehyde (Choi et al. 2020), DDMP (4 H-pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-), 5-hydroxymethyl-2-furfural (Majumder et al. 2021c), and succinic acid monoethyl ester (Saravanan and Pari, 2006). Some of these antioxidant candidates, as mentioned in both TNB and NGR, were also spotted in the metabolome of glycerin rich sample- CHN which were cyclo(L-Leu-L-Pro), phytol, neophytadiene, palmitic acid, oleic acid and β-sitosterol. Regarding *raksi* samples, nootkatone, palmitic acid, squalene, geranylgeraniol, auraptene (Majumder et al. 2021a), linoleic acid (Fagali and Catalá, 2008), meranzin (Naseri et al. 2013) etc. were some of the major antioxidant components of KKR. Crude sample of CKR did not result well in antioxidant assays, though cyclo-Gly-Pro and pGlu-Phe-Pro, two of the peptides detected in CKR were reported for antioxidant properties (Kiran et al. 2018; Tran et al. 2022).

The aspect of the DPPH kinetic study was completely different from the aspect of conventionally studied total antioxidant capacity. Knowledge of kinetic parameters such as free radical scavenging efficiency and time required to reach steady state provides additional information about antioxidant behavior. According to Sánchez-Moreno et al. (1998), this analysis is more informative than calculation of scavenging percentage determinations at fixed endpoint. The application of this kinetic study will definitely help in the effective use of any antioxidant food products. Absorbance kinetics were also used to determine the time required for achieving 50% of the total antioxidant activity (TI_{50}) shown by each sample. KKR took the least time, of about 0.6 min, to reach its TEC_{50} followed by CHN (0.8 min), TNB (1.5 min), CKR (5.09 min) and NGR (9.1 min). Perhaps the total antioxidant capacity exhibited by NGR was higher than most of the samples including KKR but here, KKR was found to be active as the fastest (not highest) antioxidant releasing sample among all while NGR was the slowest one. To be precise, the level of antioxidant release by these samples in human GI tract has also been analyzed in this research by simulating a static in vitro GI model.

The antioxidant activity by above-mentioned antioxidant metabolites, might be responsible for significant anti-lipid peroxidation because lipid peroxidation is a chain of oxidative degradations occurred in lipid molecules. Peroxidative breakdown of membrane lipids is

thought to play a key role in numerous disease pathologies directly associated with hepatic injury including alcoholic liver disease or ALD (Ali et al. 2019). In this experiment, liver cells (goat liver homogenate) were used to evaluate anti-lipid peroxidation activity by ethnic beverage samples. So, in the meantime, hepatoprotective efficacy, that too by some fermented alcoholic beverages, were interpreted from this research. Not only, a huge number of antioxidative fermented metabolites have been found but also volatiles were specifically reported as hepatoprotective. For example, four metabolites from KKR (highest activity recorded) i.e., nootkatone (Kurdi et al. 2018), squalene (Sumi et al. 2020), major coumarin compounds auraptene (Gao et al. 2018) and meranzin (Tian et al. 2019) were reported to confer strong hepatoprotective actions. Sujila et al. (2014) and Gupta et al. (2019) demonstrated hepatoprotective efficacy of β-sitosterol (detected in TNB, CHN and NGR) and phytol (detected in TNB and CHN) particularly in alcohol-induced liver toxicity. Sarna et al. (2016) reported tyrosol as preventative of high fat diet-induced hepatic oxidative stress which was detected in TNB and NGR. Moreover, NGR's major compound 5-hydroxymethyl-2-furfural (Li et al. 2015) and farnesol (Abukhalil et al. 2020) of TNB were also reported to possess hepato-protectivity. Validating the metabolomics, no such compounds were detected in sample CKR which was found with least in vitro anti-lipid peroxidation activity.

In antibacterial assay, all the beverages except CHN exhibited inhibition zones, but in different patterns which were further investigated through the GC-MS derived results. Ericsson et al. (2001) classified "infections commonly encountered at high altitude" into six distinct categories where they have mentioned *Escherichia coli* as a major enteropathogenic bacteria responsible for gastrointestinal infection at high altitude. Pneumonia is also considered as an infamous bacterial infection linked to respiratory illness with high mortality rate encountered in the mountains and surrounding lowlands (Ericsson et al. 2001). Cold and dry weather conditions are also associated with pneumonia mortality (Davis et al. 2016). NGR's efficacy against *Klebsiella pneumoniae* and *Bacillus subtilis* (responsible for fatal pneumonia) and activity of both NGR and KKR against enteropathogenic *Escherichia coli* validated potentiality of these Himalayan beverages to prevent high-altitude infections. GC-MS analysis revealed such compounds in every single sample which were responsible for selective antibacterial properties as observed in the plates (Fig. 7; Table 2). For example, tyrosol (2.72% in TNB and 1.85% in NGR) was reported to have selective and substantial antibacterial property against *Staphylococcus aureus* (Abdel-Rhman and Rizk, 2016). In this research, three samples (TNB, NGR and CKR) were found to inhibit *Staphylococcus aureus* (Table 2) and among which, TNB and NGR were detected with tyrosol (Table 3). CKR's phenyl acetate and NGR's phenylacetaldehyde also have antibacterial effects against *Staphylococcus aureus*, as reported by Kim et al. (2004). A recent study *in silico* research has shown bioactivity of cyclopropanecarboxamide, one of the major compounds of CKR (detected as cyclopropanecarboxamide, N-cycloheptyl), against dihydropteroate synthase of *Staphylococcus aureus* (Edet et al. 2023). In contrast, TNB produced a better halo (7 mm) against this bacterium which was due to presence of other selective anti-staphylococcal compounds actinomycin C2 and farnesol. Actinomycin type of antibiotics have antibacterial activity against a variety of bacteria, most of which are Gram-positive, such as *Staphylococcus aureus* (Shah et al. 2017). Farnesol was also reported to inhibit biofilm formation (Jabra-Rizk et al. 2006) and HMG-CoA reductase activity to hamper the mevalonate pathway of *Staphylococcus aureus* (Kaneko et al. 2011). Recently, hydroquinone has also been reported as useful for inhibiting biofilm formation and virulence of *Staphylococcus aureus* (Kim et al. 2022), so, detection of this phenol in NGR was also relevant. NGR's major compound phenethyl alcohol (5.91%) was noted as selectively antibacterial towards Gram-negative bacteria which was reflected in the antibacterial assay as well. Previously, Lucchini et al. (1993) reported bactericidal activity of phenethyl alcohol on *Escherichia coli*. In further research, growth of *Klebsiella pneumoniae* was found inhibited by phenylethyl alcohol and later this compound was specified as bacteriostatic for all Gram-negative bacteria (The American Society for Microbiology, 2011). Overall, this analysis affirms NGR as the only sample to inhibit growth of all the four bacteria assessed. Surprisingly, most of the antibacterial components were also detected in NGR as discussed above which might also produce a synergistic effect beyond selective effects for antibacterial activities. KKR's bioactive coumarin metabolite and major compound auraptene (14.44% peak area) was also reported to inhibit the growth of human pathogenic strains of *Escherichia coli* (Charmforoshan et al. 2022; Curini et al. 2012). Furthermore, lidocaine (a total of 8.59% peak area along with its benzylbenzoate derivative), another major compound of KKR, was reported to possess a bacteriostatic activity over *Escherichia coli* (Sakuragi et al. 1999). So, the consequence of the presence of both auraptene and lidocaine in KKR was formation of a prominent halo (7 mm) by the sample against *Escherichia coli* as shown in Fig. 7. At last, but not least, CHN did not respond well in the antibacterial assay, and it was also acceptable because about 95% of the area of its chromatogram was occupied

mostly by glycerol (76.88%) followed by some fatty acid derivatives which may not possess relevant antibacterial properties.

Metabolites of these beverages not only possess antioxidant and antibacterial properties but are also reported for other bioactivities. Literature study revealed that some metabolites were previously reported to prevent and treat high-altitude illnesses including inflammation and pain, respiratory illnesses (COPD, lung injury, bronchitis, laryngitis, tracheitis, cough and cold and bacterial infections), cardiovascular diseases (hypertension, high LDL cholesterol and coronary artery disease), neurological stresses (nerve weakness, headache, dizziness, fatigue, shortness of breath, loss of appetite, sleep problems, vertigo etc.), altitudinal gastroenterological problems (mainly indigestion) and other diseases and disorders which can be collectively termed as altitude sickness. Metabolites such as auraptene and cyclo-Gly-Pro were previously reported to attenuate edema, a major high-altitude sickness (Keshavarzi et al. 2021; Ferro et al. 2015). Inflammation (body muscle inflammation, joint pains, peripheral edema etc.) and high altitude are two sides of a coin. Interestingly, in Singalila and neighbouring mountains, drinks like *tongba*, *chhyang*, *nigar*, *guras* (rhododendron wine) and *raksi*s are served as anti-inflammatory healing beverages (Majumder et al. 2021a). Beverages studied in this research may exhibit pain-relieving property possibly because of containing anti-inflammatory compounds detected by GC-MS i.e., auraptene (Bibak et al. 2019) and meranzin (Patel, 2022) in KKR; cyclo-Gly-Pro (Ferro et al. 2015) in CKR, and cis-vaccenic acid (Jacome-Sosa et al. 2016) in CHN. Monoterpene-coumarin auraptene is a potential vasodilator and prevents hypertension to exhibit cardio-protectivity; while meranzin improves atherosclerosis; and cyclo-Gly-Pro prevents cardio-metabolic disorders by normalizing systolic blood pressure as reported (Arabi et al. 2021; Li et al. 2019, 2020). Auraptene can attenuate chronic gastritis and also possesses anti-gastric-cancer activity (Sekiguchi et al. 2012). *Raksi* compounds auraptene and cyclo-Gly-Pro are reported as potential neuroprotective components (Bibak et al. 2019; Majumder et al. 2021a). Specifically, auraptene exhibits a variety of bioactivities linked to high altitude neurological and psychiatric illness like anxiety, depression, dizziness, and Alzheimer's disease (Arabi et al. 2021; Majumder et al. 2021a) and meranzin also plays role in preventing depression (Liu et al. 2021). Ethyl α-D-glucopyranoside, the major glycoside metabolite of *tongba* is reported to produce collagen, and scientifically, it makes *tongba* beneficial for retention of body's moisture in high-altitude condition unlike other commercial and marketed fermented beverages (Majumder et al. 2022a).

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 (Minekus et al. 2014). 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 (Oomen et al. 2002) which has been incorporated in this research. Through both DPPH assay and TPC test, digestion rate of the targeted compounds (total antioxidants and phenolics) was found highest in gastric/ stomach phase. Previously, Sollano-Mendieta et al. (2021) reported high reduction or breakdown rate of antioxidant activity (DPPH assay) and total phenol content (TPC) during in vitro gastric or stomach phase. The breakdown rate for lipid composition was found high in intestinal condition. In human GI tract, involvement of bile acids to facilitate lipid digestion in the small intestine has been well established (Staels and Fonseca, 2009) which is corresponding to the quantified high lipid digestion during intestinal phase in this experiment. The result was further evaluated incorporating the bioaccessibility index (BI). The rate of digestion or degradation was calculated comparing mean BI (%) of successive digestion phases. For example, digestion of antioxidant components and TPC of all samples were high during gastric digestion (mimicking stomach phase) with 77.3% and 83.1% respectively, while digestion process of lipid composition was dominated by intestinal phase with around 76% decrease (Table 5).

In this research, discussed bioactivities such as antioxidant, hepatoprotective and antibacterial activities; and bioaccessibility as analysed through selected in vitro techniques cumulatively validated nutritional efficiency and bioavailability of these ethnic beverages. Results of in vitro GID study showed that these beverages are entirely digestible and bioactive components (antioxidants, phenolics and lipid composition) are easily absorbable or bioaccessible within the human GI tract.

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 properties of Himalayan ethnic beverages through in vitro assessments. Bioactivities, volatile profiling and gastrointestinal simulation study have conclusively validated ethnomedicinal potential of these traditional beverages. This article describes the ethnomedicinal values of *tongba*, *chhyang*, *nigar* and *raksi* with reference to bioaccessibility. Secondary metabolites of these beverages exhibit moisture retention activity by collagen production (α-EG); antioxidant activities, antibacterial properties against various high-altitude infections; hepatoprotective activity and other high-altitude sickness preventive activities that could construct research opportunities in the field of food science and pharmacology. 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 ailments owing to cumulative effects of the constituent metabolites. Fermented foods and beverages hold deep cultural significance within the diverse ethnic groups of the country. The studied traditional alcoholic beverages not only offer a refreshing experience but also play a vital role in the social and cultural fabric of the tribal communities residing in Darjeeling, Sikkim, Nepal, and other high-altitude regions. Due to the remoteness of these locations 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 sickness. However, it is noteworthy that the region lacks a large-scale production unit or industry dedicated to local alcoholic beverages. By embracing advanced food-biotechnology or fermentation technology, the region can unlock the potential for large-scale production of these culturally significant beverages. This initiative aims to satisfy the needs of both local residents and tourists while simultaneously fostering economic growth. A significant concern of this venture may be the exploitation of and encroachment on long standing traditional customs. Since these beverages are strongly intertwined with the origin, habitat, religion, and overall way of life of tribes, they tend to consider these ethnic drinks as an integral part of their cultural heritage. Consequently, they strive to safeguard and preserve their traditional knowledge, shielding it from external influences and exposure. The current results may advise future in-depth studies. Further work may be planned on pharmacology of ethnic beverages, *in silico* and in vivo experiments to investigate the medicinal properties more in depth.

Abbreviations

GC-MS	Gas chromatography-mass spectrometry
TNG	*Tongba*
CHN	*Chhyang*
NGR	*Nigar*
KKR	*Khokim* added *kodo ko raksi*
CKR	*Chimphing* added *kodo ko raksi*
DPPH	2,2-Diphenyl-1-picrylhydrazyl
DDMP	4 H-pyran-4-one, 2,3-Dihydro-3,5-dihydroxy-6-methyl-
α-EG	ethyl α-D-glucopyranoside

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Authors' contributions

SM conceived the idea. SM and MB designed the protocols. SM and PS did the sample collection procedure. SM, AG, SC and SN performed all the biochemical experiments. SS and SA performed antibacterial tests. SM, SDS and MB did the statistical analysis. MB supervised the whole research. SM compiled data and wrote the draft of manuscript. SM, SA and MB revised the manuscript. All the authors read and approved the manuscript.

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All data analysed during this study are included in this article.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

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Competing interests

The authors declare that they have no competing interests.

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