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Full Length Research Article

In vitro, *In silico* and Chemical Profiling of Timbur *Raksi*, A Lesser-Known Ethnic Fermented Beverage from Darjeeling Himalaya

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Abstract

Fermentation is one of the ancient techniques for food preservation. Timbur *raksi*, is a distilled fermented alcoholic beverage from the high-altitude region of Darjeeling Himalaya. Here within we have done the antioxidant, GC-MS and molecular docking. Our present study aims to claim this brew as an effective stress-releasing fermented drink by reducing reactive oxygen species (ROS). At the higher concentration (200µg/ml), the methanolic extract of the sample showed 60% of inhibition. GC-MS analysis of the sample showed 43 bioactive compounds. These bioactive compounds can act as scavengers for reactive oxygen species, anti-inflammatory and anticancerous. The molecular docking study was done with antioxidant transcription factor to validate our *invitro* analysis. This investigation on *raksi* opened a new field in ethnobiology, However, further study should be done on its load of microflora and its nutrition aspect.

***Key words*:** Ethnobiology, *Zanthoxylum*, *Raksi*, Antioxidant, GC-MS, Molecular docking

India is a place of diverse communities and widely distributed throughout the area. Every culture has a different ethnic identity. This ethnic uniqueness may vary depending on the region of the inhabitants and other environmental conditions [1]. One of the influencing factors for changing the food habit and behavior of every community is altitude. A huge population in India belongs to high altitude, and fermented food and beverages are an integral part of this high-altitude region. These home-based fermented foods and beverages are regarded as ethnoecological constituents of this region for being stated as therapeutically valuable to indigenous people and capable of fighting against a lot of high-altitude sicknesses [2]. Fermentation is a traditional ethnic practice to reduce the spoilage of any food product and to increase nutrient availability. *Raksi* or *rakshi* is a distilled alcoholic beverage, produced from locally fermented conventional food, which has been used as a remedy for various diseases regularly by the native inhabitants living in high altitudes of the Himalayas. This distilled beverage has a distinguishing aroma made from fermented starchy materials where fermented loads of finger millet, maize, wheat, canna, cassava roots and *Rhododendron*, fruits of *Zanthoxylum* were also distilled to get *raksi* [3]. *Zanthoxylum acanthopodium* DC. (Family: Rutaceae) or locally known as Timbur in Darjeeling Himalayas, is a locally grown shrub up to 6 cm in height. *Zanthoxylum acanthopodium* DC.is a medicinal plant, mainly used in various diseases such as cholera, gastric diseases, and diabetes and [4]. Timbur raksi (TR) is also one of the important ethnic brews in Darjeeling Himalaya used as an anti-inflammatory stress-releasing high-altitude drink. Different types of *raksi* are well-recognized for their various health claim in the field of ethnobiology but no scientific analysis work has been done on it. The ethnomedicinal claim as a stress-releasing and anti-inflammatory drink should be studied properly in a scientific way. In this scenario, our study on TR will open a new area in the field of ethnobiology. Therefore, the main of our study is to evaluate the health-beneficial properties of TR. Here within we have done the antioxidant, GC-MS analysis of the TR. Moreover, *in silico* molecular docking is also done to validate our claim TR is a stress-releasing high-altitude drink.

MATERIALS AND METHODS

Sample collection and preparation

Freshly prepared Timbur *raksi* was collected from the Darjeeling market (Latitude: 27°2' 9.6252" N and longitude 88°15'45.6192"E) from an elderly person. The sample was collected in sterilized glass bottles to avoid microbial contamination. Collected samples were filtrated using Whatman No.1 filter paper. Filtrated samples were lyophilized using a rotary evaporator. The dried powder was kept in the refrigerator for further analysis.

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In vitro antioxidant assay
DPPH assay

The free radical DPPH antioxidant assay was performed following the method of Bhattacharjee *et al*. [5] with slight modifications. Various concentrations (50–200 µg/ml) of the TR sample were prepared and mixed appropriately with freshly produced 1 mM DPPH solution and left in dark for 45 minutes. The absorbance was taken at 517 nm using Bio-Rad micro plate. Ascorbic acid is used as standard. The percent of inhibition was calculated using the following formula:

Percent of scavenging: $(A_0\text{-}A_1) / A_0 * 100$
[Where A0: Absorbance of the control, A1: absorbance of samples].

Estimation of phenolic and flavonoid content

The total phenol content was estimated by the Folin-Ciocalteu method and the total flavonoid content was determined using the aluminum chloride ($AlCl_3$) method. Both phenol and flavonoid content was estimated by the method of Lala *et al*. [6].

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Statistical analysis

The entire qualitative data have been reported as the mean ± SD of three extents. ANOVA was used for statistical analysis and representation, followed by Dunnett's multiple comparison tests where $\alpha < 0.001$ was statistically significant.

GC-MS analysis

The biologically active compounds of TR sample were identified by Gas chromatography and Mass spectroscopy (GC-MS) analysis. The standard methodology was already discussed in one of the previous papers [7].

In silico molecular docking

Different bioactive compounds were identified in the GC-MS analysis of TR sample. These compounds were used for *in silico* molecular docking. Antioxidant transcription factor FOXO3 protein (forkhead-box class O3; PDB ID: 2UZK) was taken for molecular docking study. The protein structure was downloaded from PDB database (https://www.rcsb.org/). PDB structures were modified into pdbqt in the Autodock Vina tool. The obtained compounds from GC-MS were used as a ligand for the docking study. NCBI PubChem database was used for ligand molecules download. Further SMILES server is used to convert the sdf to pdb format. Finally using the Auto-dock vina tool (http://vina.scripps.edu/) molecular docking was done.

RESULTS AND DISCUSSION

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

Nowadays oxidative stress is one of the major reasons for to formation of Reactive oxygen and nitrogen species for to accumulation of various diseases. Several fruits are used to neutralize this ROS/RNS and are known as antioxidants [8]. DPPH is one of the significant assays to study free radical scavenging capacity. TR sample showed 56±.43% at its highest concentration which is at 200 µg/ml (Fig 1A). To provide stability DPPH can accept hydrogen and electron, and act as a source of natural antioxidants [9]. With the increase in the concentration of the TR sample gradual decrease in coloration was observed. DPPH assay illustrated the sufficient amount of antioxidants in the sample (Fig 1A).

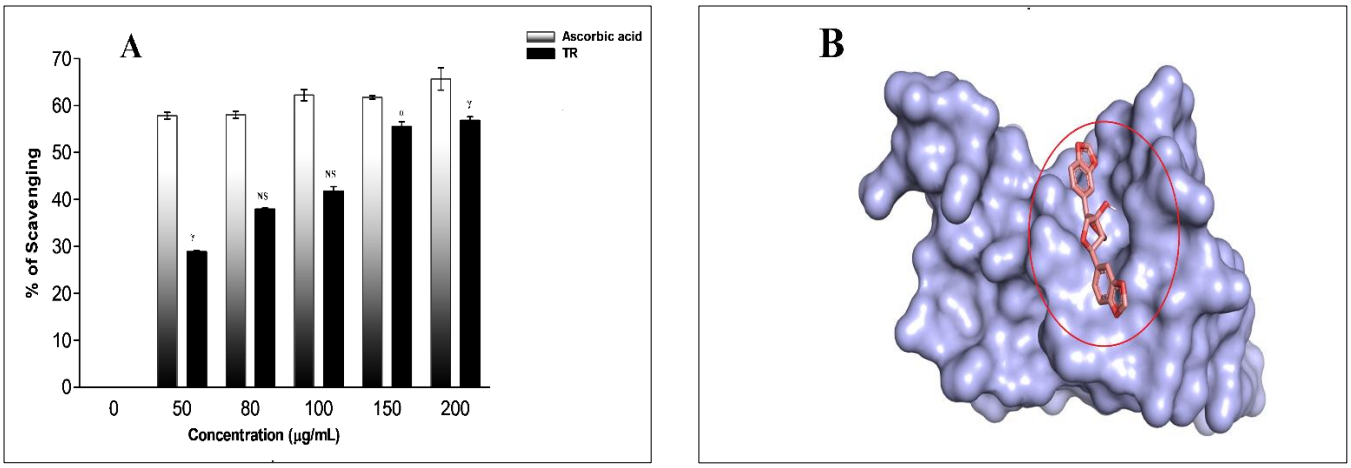

Fig 1 (A) DPPH scavenging assay of TR sample and standard ascorbic acid; (B) Molecular docking with FOXO protein and the STIGMAST-5 compound

Estimation of phenolic and flavonoid content

Aromatic rings and redox potential are the main features of phenolic compounds. Phenol can reduce free radicals. Moreover, Flavonoids are also ROS/RNS scavengers [10]. The phenolic compound content of the TR sample is 56±5 gallic acid equivalent per 100 mg of the sample. The flavonoid content of the sample is 45±6%.

GC-MS analysis and in silico molecular docking

A total of 43 compounds were detected in GC-MS analysis (Table 1). Most of these compounds are long-chain fatty acid moiety and their derivatives such as hexadecanoic acid, Palmitoleic acid, and 11-Octadecenoic acid. Palmitoleic acid act as an antibacterial agent against gram-positive bacteria [11]. STIGMAST-5-EN-3-OL, (3.BETA.)- or Betasitosterol plays a crucial role in reducing stress [12]. 9-Hexadecenoic acid plays important role as an anti-inflammatory and antioxidative agent [13].

In silico molecular docking is also done with the compounds from TR and one oxidative stress-related protein that is FOXO (forkhead-box class O3). Foxo is a DNA-binding protein and a transcription factor. Several reports are there that FOXO plays a crucial role in oxidative stress. Our *in-silico* study results in the binding sufficient affinity with FOXO and

several compounds [14]. Out of which STIGMAST-5-EN-3-OL, (3.BETA.)- or Betasitosterol shows the binding affinity of -5.8 Kcal/mol (Fig 1B). This result shows the compounds of TR can modulate the proteins responsible for stress generation.

Table 1 GC-MS analysis of TR sample

Name	R. Time	Name	R. Time
(2R,4R)-(-)-Pentanediol	4.785	9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)-	19.294
Diglycerol	6.825	11-Octadecenoic acid, methyl ester	19.342
1,2,4-Triazino[5,6-E][1,2,4]-triazine-3,6-dione, hexahydro-	7.534	1H-Indene, 2,3,3a,4-tetrahydro-3,3a,6-trimethyl-1-(1-meth	19.554
1,5-ANHYDRO-6-DEOXYHEXO-2,3-DIULOSE	8.319	ETHYL (9Z,12Z)-9,12-OCTADECADIENOATE #	19.841
L-Alanine, TMS derivative	9.137	9,12,15-Octadecatrienoic acid, 2,3-dihydroxypropyl ester,	19.900
2-Oxabicyclo[2.2.2]octan-6-ol, 1,3,3-trimethyl-	9.296	2,6,9,11-DODECATETRAEN-1-CARBONSAEURE, MET	21.230
2-FURANCARBOXALDEHYDE, 5-(HYDROXYMETHY	9.930	5-(PROPENYL-2)-1,3,7-NONATRIENE	21.510
1,2-Cyclohexanediol, 1-methyl-4-(1-methylethenyl)-	11.079	1,2-BENZENEDICARBOXYLIC ACID	22.998
4-HEPTANONE, 2,6-DIMETHYL-	12.346	(1S,4R,5R)-1,3,3-Trimethyl-2-oxabicyclo[2.2.2]octan-5-yl	24.367
1,6-CYCLODECADIENE, 1-METHYL-5-METHYLENE-	12.717	1-(HYDROXYMETHYL)-2,5,5,8A-TETRAMETHYLDE	25.138
1,3-Propanediol, 2-(hydroxymethyl)-2-nitro-	13.056	1-(HYDROXYMETHYL)-7,7-DIMETHYLBICYCLO[2.2	25.830
6-Ethoxy-6-methyl-2-cyclohexenone	13.589	1-Hydroxymethyl-7,7-dimethylbicyclo[2.2.1]heptan-2-one	26.024
3-(2-HYDROXY-2-METHYL-PROPYL)-CYCLOHEX-2-	13.862	NAPHTHALENE, 1-[1-(3-CYCLOHEXYLPROPYL)UND	26.408
2-BUTOXYETHANOL	15.368	NAPHTHALENE, 1-[1-(3-CYCLOHEXYLPROPYL)UND	26.528
9-Hexadecenoic acid, methyl ester, (Z)-	17.384	19-D-TORULOSOL	26.601
HEXADECANOIC ACID, METHYL ESTER	17.595	(3S,8S,9S,10R,13R,14S,17R)-17-((2R,5R)-5-Ethyl-6-meth	27.244
Palmitoleic acid	17.952	6,10,14,18,22-Tetracosapentaen-2-ol, 3-bromo-2,6,10,15,1	28.157
Ethyl 9-hexadecenoate	18.055	PAULOWNIN	28.247
9-OCTADECENOIC ACID (Z)-	18.128	PAULOWNIN	28.416
Hexadecanoic acid, ethyl ester	18.259	1H,3H-FURO[3,4-C]FURAN, 1,4-BIS(3,4-DIMETHOXY	28.743
(4,4-Dimethyl-2,4,5,6-tetrahydro-1H-inden-2-yl)acetic acid	19.188	STIGMAST-5-EN-3-OL, (3.BETA.)-	29.486
9,12-Octadecadienoic acid (Z,Z)-, methyl ester	19.232		

CONCLUSION

Zanthoxylum acanthopodium DC. is an important fruit in Darjeeling Himalayas. Fermentation is one of the ancient ways to preserve. The article depicted the ethnomedicinal values of Timbur *raski* as a high-altitude stress-releasing beverage. From GC-MS analysis, TR showed several bioactive compounds which play a crucial role in an antiinflammation, antimicrobial, and antioxidative agent. TR also acts as a potent reactive oxygen species scavenger. Moreover, molecular docking studies of the TR bioactive compound reflected possible relations with intracellular targets to provide cytoprotection during oxidative stress. However, further study is needed to commercialize this ethnic beverage.

Authors' contributions

AS conceived the idea. AS, SB, ML, and CG designed the experiment. SB and PG did the in-silico analysis. All the authors have contributed to drafting the manuscript and approved it.

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