



## FUSION OF TEA INFUSION AND *BAKHAR* (STARTER OF ETHNIC LIQUOR *HARIA*) TO DEVELOP “TEA *HARIA*”: A NOVEL APPROACH TO FERMENT TEA WITH INSIGHT INTO *IN VITRO* BIOCHEMICAL ATTRIBUTES AND METABOLOMICS

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### ABSTRACT

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#### Keywords

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*Haria*, the traditional rice based liquor of eastern India and Nepal, is an indigenous alcoholic beverage prepared by different ethnic communities where *bakhar* is used as starter culture which is a tablet like mixed culture of various yeasts, filamentous molds, and lactic acid bacteria etc. This research work was designed to make a fusion of tea infusion and *bakhar* to produce “Tea *Haria*”. Tea infusion and *bakhar* were co-cultured for fermentation which was further characterized by different physicochemical and biochemical parameters. *In vitro* assessment of antioxidant, antidiabetic, anti-lipid peroxidation activity and antibacterial activity were assessed. Primary investigations for qualitative characters on this fermented broth or tea *haria* revealed the presence of steroids, tannin, flavonoids, phenol, cardiac glycosides, coumarin, caffeine etc. Tea *haria*, the fermented broth showed high free radical scavenging activity. The antioxidant activity, acidity and alcohol percentage were seen to be gradually increased while brix and specific gravity of broth were decreased during the first fifteen days of fermentation. Gas chromatography-mass spectrometry analysis revealed the presence of thirty-three compounds including many potential antioxidant molecules and other bioactive agents which validated the qualities detected and estimated through *in vitro* assays in tea *haria*. Presence of alcohol with medicinally active compounds and antioxidant, antibacterial and other biological activities have made this tea *haria* a bioactive probiotic-fermented formulation and, furthermore, brought out the ethnic knowledge of *haria* fermentation, specifically, ethnic starter *bakhar* as a useful biotechnological tool which can be utilized in research and development of fermentation technology.

**Contribution/Originality:** This study on production of Tea *Haria* is a novel approach to ferment tea infusion. This research has discovered a possible biotechnological application of ethnic starter *bakhar* where it has been used to produce bioactive formulation from tea using fermentation technology.

## 1. INTRODUCTION

During brewing of various wines, probiotic drinks or other fermented beverages, a wide range of coexisting microbes interact with each other and influence the dominance of fermenting microbes and analytical profiles of the beverage. The use of these microbes or starter culture is widely diffused in winemaking or food fermentation depending on nature of substrates, type of fermented foods or beverages, environment of fermenting cultures etc. other than the most important factor i.e., the traditional knowledge of people involved in the brewing methodology. Traditional or ethnic alcoholic beverage means a liquor that antecedes any similar commercial alcohol, has been made following centuries-old ethnobiological knowledge and methods, which is prepared to fulfill ethnoecological challenges by meeting needs and tastes of local people (Luu, Nguyen, & Newman, 2014). Elements of human lifestyle such as habitation, clothing, food habits etc. are components to represent ethnic identity of a society where traditionally brewed alcoholic beverages play an important role exhibiting traditional knowledge of that society (Majumder, Ghosh, Chakraborty, Saha, & Bhattacharya, 2021a). *Haria* is one of that kind of non-commercial and ethnic alcohol (a rice based alcoholic beverage) which is traditionally brewed and consumed by tribal population across Indian subcontinent. However, to refer *haria* in this paper, terms like “traditional” and “ethnic” have been chosen to prefix before the alcohol and its starter *bakhar*.

In this research, an experimental fusion has been made using two ethnoecological food elements i.e., the tea and ethnic starter *bakhar* (*haria*'s starter) collected from Terai, a tea growing region located in Himalayan foothills. *Haria* or *handia*, the traditional rice beer of eastern India and Nepal is an indigenous alcoholic-fermented beverage that is prepared from parboiled rice (*Oryza sativa* L.). Many ethnic tribes like Santal, Sabar, Bhumij, Paroja, Kondh, Kolh, Mundari, Juang, Oraon etc. prepare this rice based alcoholic beverage by using their own traditional knowledge. *Haria* in tribal life is used as an intoxicating beverage (Chaudhary, Sharma, & Arora, 2018); though, there are several scientific reports on medicinal uses of fermented rice based beverage *Haria* describing it as a beneficial probiotic beverage (Ray, Hor, Singh, & Mondal, 2017) with gastro-protective, immuno-stimulatory and antioxidant (Ghosh et al., 2015), anticancer (Phutthaphadoong et al., 2009), hepatoprotective (Dhal, Pattanaik, & Reddy, 2010) and cardio-protective (Das, Kundu, Singh, Rastogi, & Banerjee, 2017) properties. Preparation of this rice based alcoholic beverage *haria* are a part of traditional knowledge offered by tribal communities across West Bengal and East Central India including tea garden workers and other locals of Terai, Dooars<sup>1</sup> and other tea growing regions of North Bengal and Assam (Ghosh et al., 2014; Gupta, 2013; Roy, 2017). Moreover, regarding this scenario, reported documents are available which prove that, besides ethnicity, *haria* is also an important part of socio-economic profile of the concerned region. Tea garden workers are often seen to be engaged with the business of brewing and selling *haria*<sup>2</sup>.

To our knowledge, brewing of fermented tea infusion using indigenous traditional starter (like *bakhar*) is a novel approach that encouraged us to experiment on a fusion between tea- world's most popular beverage and *bakhar*- an indigenous traditional starter to prepare a tea based alcoholic beverage. However, kombucha was the only established fermented tea based alcoholic beverage that bears a history of Chinese ethnicity and its effect in Asia's cuisine. The main aim of this research was to prepare a bioactive formulation by fermenting tea infusion using *haria*'s starter *bakhar* and carry some experiments to characterize the properties of the formulation prior to set a collective impact towards ethnobiology, socio-economy and future research on this traditional knowledge. Furthermore, GC-MS based metabolomics was done to look into the metabolite profile of the formulation, probable medicinal properties and to study the biosynthesis of bioactive metabolites by microbes of *bakhar* involved in fermentation.

<sup>1</sup> <http://sanhati.com/articles/236/>

<sup>2</sup> [https://wcd.nic.in/sites/default/files/stories%20behind%20a%20hot%20cup%20of%20Assam%20Tea1\\_n.pdf](https://wcd.nic.in/sites/default/files/stories%20behind%20a%20hot%20cup%20of%20Assam%20Tea1_n.pdf)

## 2. MATERIALS AND METHODS

### 2.1. Collection of Bakhar and Tea

Fresh samples of *bakhar* were collected from a potential *haria* brewer belongs to ethnic Mech community [one of the important indigenous communities of Siliguri subdivision with a concentration in Naxalbari block (Gupta, 2013; Sarkar, 2017). Samples of garden fresh quality of CTC black tea were also collected from Nuxalbari tea factory located in Terai (foothills of Darjeeling Himalaya) at an altitude of 700 feet (West Bengal, India) (26°40'00.2"N 88°12'06.4"E).

### 2.2. Preparation of Tea Haria

Fermentation of tea in this research was done following developed methodology of kombucha (only reported and popular fermented tea) production (Majumder, Ghosh, Chakraborty, & Bhattacharya, 2020a) with slight incorporated modification which has been described here with details. Ten grams of CTC black tea were added in one liter of sterile freshly boiled double distilled water (at  $98\pm 2^\circ\text{C}$ ) and left for ten minutes to prepare the infusion. The tea infusion was then filtered through sterile muslin cloth and kept in a jar followed by adding 100 g of sucrose (or 10% w/v) for nutrient or carbon source of fermenting microbes. The jar was then autoclaved properly. After cooling the jar of tea (at room temperature  $25\pm 1^\circ$ ), two grams of *bakhar* Figure 1 or the selected fermentation starter were powdered and added in the infusion. Sterile polythene cover with pores was used to facilitate releasing of evolved carbon dioxide during fermentation. Muslin cloth, glass goods, polythene cover etc. were either autoclaved or sterilized with 70% ethanol before using. After inoculation of starter, the jar was incubated under a dark condition following the methodology of *haria* production to provide the fermenting microbes of *bakhar* a familiar environment to grow (at  $25\pm 1^\circ\text{C}$ ) for fermentation of "Tea Haria" (TH). TH was collected at regular intervals up to fifteen days for further studies (Figure 1 depicts a batch of TH on 15<sup>th</sup> day of fermentation). Moreover, following the same procedure, jars were prepared as replicas and results of further experiments were expressed as mean  $\pm$  SD of three replications.



**Figure 1.** Bakhar, starter of haria (left side) collected from Naxalbari, West Bengal, India and fermented broth of TH (Tea Haria) after fifteen days of fermentation (right side).

### 2.3. Preliminary Biochemical Tests

Qualitative determination of terpenoids, steroids, tannin, flavonoids, phenol, proteins, cardiac glycosides, coumarin, reducing sugar, starch and caffeine were conducted following the protocol of Ghosh, Majumder, Saha, and Bhattacharya (2020) and Majumder et al. (2021b). TH samples were collected at different stages of fermentation (regularly up to 15<sup>th</sup> day) and stored at  $-20^\circ\text{C}$ . Tests were done on the same day for a better

comparison and to evaluate the changes in biochemical qualities. Before adding starter (or *bakhar*) and sucrose, same tests were performed on collected Tea infusion (TI) or unfermented sample also in aim for a comparison.

#### 2.4. Quantification of Total Phenol and Flavonoid Content

Quantification of total phenolic content (TPC) and flavonoid was done on samples i.e., TI (considered as control) and TH (at final day of fermentation or 15<sup>th</sup> day). TPC was quantified following the Folin–Ciocalteu method following (Blainski, Lopes, & De Mello, 2013). Total phenolic content (TPC) was measured against gallic acid standard curve ( $R^2 = 0.9975$ ;  $y = 0.0043x - 0.1672$ ). TPC was expressed as gallic acid equivalent per 100 ml of each sample (mg GAE/100 ml). Total flavonoid content was determined by aluminium chloride method demonstrated by Ghosh et al. (2020). Standard curve of quercetin was used as reference ( $R^2 = 0.962$ ;  $y = 0.207x - 0.204$ ). Flavonoid content was expressed as quercetin equivalent (QE) per 100 ml of sample (mg QE/100 ml). Experiments were carried out in triplicates.

#### 2.5. Analysis of Physicochemical Properties

Following protocol of Majumder et al. (2021c), monitorization of physicochemical properties like estimation of pH (for acidity), specific gravity (SG), percentage brix (% Bx) and alcohol by volume (%ABV) of the fermented samples were done on samples collected at regular intervals during fermentation to study time dependent alteration in those characteristics. Acidity of sample was recorded by measuring pH by using a pre-calibrated pH meter (LMPH10, Labman Co.) to investigate the changes in acidity of the broth. Specific gravity was determined mathematically by calculating the ratio of the specific weight of sample to the specific weight of water at 4°C<sup>3</sup>. Changes in brix (%Bx) of TH was measured using a pre-calibrated brix meter (0-90% Brix Refractometer, Labart, Japan). Percentage Brix (%Bx) is the sugar content of an aqueous solution. One degree Brix (1% Bx) is equivalent to one gram of sucrose in 100 grams of solution and represents the strength of the solution as percentage by mass. The %Bx is traditionally used in the wine, sugar, carbonated beverage, fruit juice, fresh produce, maple syrup and honey industries. In this research, %Bx was considered as a parameter to study the changes in physicochemical property of TH during fermentation. Alcohol percentage or %ABV of TH were estimated by online ABV calculator<sup>4</sup> using calculation method from brix readings regularly during fifteen days of fermentation. All the experiments were carried out in triplicates.

#### 2.6. Gas Chromatography–Mass Spectrometry Analysis

GC-MS analysis was done following the research protocol of Majumder et al. (2021c) developed for analysis of fermented beverages. The data obtained from GC-MS analysis were further analyzed by studying available literatures (Majumder, Ghosh, & Bhattacharya, 2020b).

#### 2.7. In Vitro Antioxidant Activity (DPPH Assay)

DPPH assay for antioxidant activity was performed on TI (for control) and TH (at regular intervals up to 15<sup>th</sup> day). Moreover, further DPPH scavenging activities were also measured on the sample TH on aging up to 90 days following the protocol of Majumder et al. (2021c). Results were expressed as the percentage of DPPH inhibition (%) occurred due to exposure of samples (Majumder et al., 2021c). Moreover, Different concentrations were also prepared from TI and TH at 15<sup>th</sup> day and assessed for the same experiment to quantify the IC<sub>50</sub> values. Ascorbic acid standard curve was used as reference in this experiment ( $R^2 = 0.9904$ ;  $y = 0.9088x + 12.331$ ). Result of this assay was expressed as means of three replications.

<sup>3</sup> [https://scetcivil.weebly.com/uploads/5/3/9/5/5395830/fluids\\_chap01.pdf](https://scetcivil.weebly.com/uploads/5/3/9/5/5395830/fluids_chap01.pdf)

<sup>4</sup> <https://www.petedrinks.com/abv-calculator-refractometer-hydrometer/>

### 2.8. *In Vitro* Antidiabetic Activity (Determination of Glucose Uptake Capacity by Yeast Cells)

Glucose uptake assay using yeast cells was used to determine *in vitro* antidiabetic activity following the protocols developed by Shettar, Sateesh, Kaliwal, and Vedomurthy (2017). Metronidazole was used as standard drug ( $R^2 = 0.9862$ ;  $y = 0.0119x - 0.0003$ ), thus, results were expressed as mg MetE/ml (milligram Metronidazole Equivalent per milliliter).

### 2.9. *In Vitro* Lipid Peroxidation Inhibition Assay

The *in vitro* lipid peroxidation inhibition assay was determined following the protocol developed by Rahman, Islam, Biswas, and Alam (2015). Value of estimated lipid peroxidation inhibition activity were measured from a standard ( $R^2 = 0.9973$ ;  $y = 0.0077x + 0.0321$ ) curve prepared using tocopheryl acetate. Results have been expressed as mg TAE/ml (milligram tocopheryl acetate equivalent per milliliter) of each sample.

### 2.10. *In Vitro* Antibacterial Activity

Antibacterial assay of the samples (TH and TI) were performed by well diffusion method described by Ghosh et al. (2020). Overnight grown cultures of two Gram-positive (*Staphylococcus aureus* and *Bacillus subtilis*) and two Gram-negative (*Escherichia coli* and *Klebsiella pneumoniae*) bacteria were used for this experiment. Antibacterial activity was measured from diameter of the inhibition zones formed surrounding the well containing samples.

## 3. RESULTS AND DISCUSSION

According to the field visit for sample collection, it was learned that *haria* starter *bakhar* is produced by mixing culture of yeasts, filamentous molds, lactic acid bacteria etc. with different medicinal plant ingredients which may differ from community to community. The culture enriched with beneficial fermenting microbes and phytochemicals of medicinal plants are then mixed with rice powder and finally made into small round balls. Then balls shaped and wrapped in banana leaves, are allowed to ferment and dry continuously in shade for three days. According to Roy, Khanra, Bhattacharya, Mishra, and Bhattacharyya (2012), around forty-two species of ethnobotanically important plants have been reported to be used as an ingredient of *bakhar*.

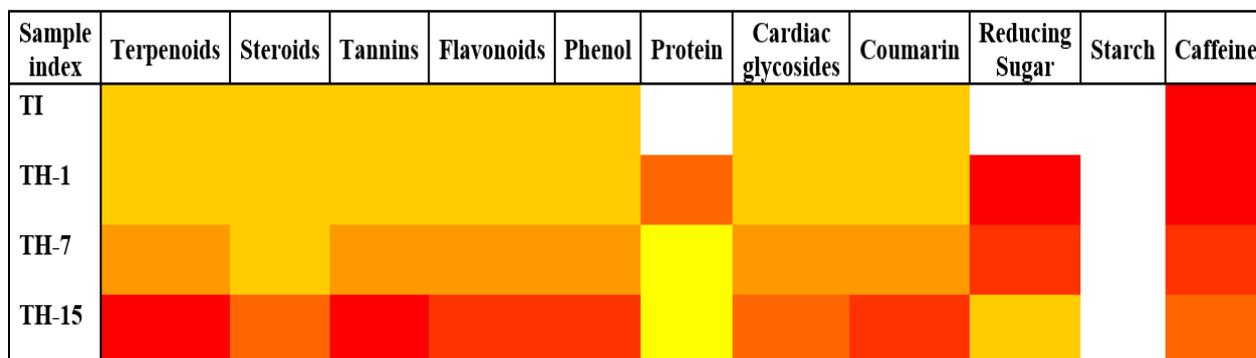
### 3.1. Qualitative Biochemical Properties

Qualitative tests revealed presence of terpenoids, steroids, tannins, flavonoids, phenol, coumarin, cardiac glycosides, coumarin, and caffeine in both control of TI and fermented sample or TH collected on different stages during fermentation i.e., day 1 (TH-1), day 7 (TH-7) and day 15 (TH-15). Steroid and caffeine were observed high in control sample of TI and TH of day 1 which were further seen to be decreased during fermentation. Moreover, qualities like presence of flavonoids, terpenoids, phenol, tannin, cardiac glycosides, coumarin etc. were detected increasingly high in fermented sample (TH). These changes in qualities surely reflected the fermentation process and biosynthesis of fermentation derived secondary metabolites in TH. Starch was found to be completely absent at all brought no confusion. However, reducing sugar was found expectedly high in fermented sample at day 1 (due to addition of sugar in broth) and was further observed to be reducing during fermentation which was definitely a clear indicator of progress in fermentation process. Tea infusion is already known to be rich in flavonoids and phenolic compounds, steroids, caffeine, terpenoids etc., so, presence of these qualities in TI or control sample was also justified as well. A heatmap is given in Figure 2 to depict the results of qualitative biochemical tests.

### 3.2. Total Phenol and Flavonoid Content

Total phenol was found to be  $54.697 \pm 2.63$  mg GAE/ 100 ml for TH while control (TI) resulted only  $21.326 \pm 1.22$  mg GAE/ 100 ml. Flavonoid content similarly was significantly high for TH ( $23.2 \pm 1.41$  mg QE/ 100 ml) compared to TI ( $12.3 \pm 0.98$  mg QE/ 100 ml). Results of this quantification assays were compatible with results

qualitative biochemical tests where both phenol and flavonoids were found to be high in fermented samples. However, both fermented beverages (wines) and tea infusion are already famous for flavonoids and other phenolic contents so presence of such molecules in TH or control (TI) was absolutely justified, but results of this research revealed that fermented tea infusion contain comparatively high amount of phenolics compared to that of the non-fermented broth which was quite remarkable. Results of this assay clearly suggested that fermentation definitely inputs more quality.



**Figure 2.** Heatmap (white = absent; yellow-red = low-high) showing levels of different qualities tested on TI and TH (taken at different stages of fermentation).

### 3.3. Time Dependent Alteration in Physicochemical Properties

Determination of alteration in physicochemical properties like pH, brix, specific gravity and alcohol percentages were important to judge fermentation rate and acceptability of a beverage as wine. Acidity (pH), specific gravity, brix (%Bx) and alcohol by volume (%ABV) were generally considered as quality parameters of physicochemical properties of any beverage, especially alcoholic ones which have been incorporated in this research. By following research methodology of [Majumder et al. \(2021c\)](#), changes of these properties in replicas of the fermenting broth have been recorded during different stages of fermentation (daily up to fifteenth day) which not only validated the quality of fermentation but also ensured the acceptance of TH as an alcoholic beverage. Moreover, results of these experiments also suggested stabilization of experimenting parameters after fifteen days based on which sample was collected for metabolomics. [Figure 3](#) has been given to depict the results of physicochemical properties as graphical representations. Acidity was found to be increased during fermentation as a decreasing pH was appeared in the graph. Typically, pH of wine ranges between 3 to 4 and increasing acidity or decreasing pH is a characteristic of any wine of beverage fermentation process ([Majumder et al., 2021c](#)). pH of TH was found to be  $3.1 \pm 0.1$  on 15<sup>th</sup> day (final day as considered) which was very acceptable. Generally, during fermentation, yeast or other microbes cause acidification by their metabolic process and the broth is turned into acidic as a result which (an increasing amount of organic acids) further accelerates the fermentation rate as well. Increasing acidity is a factor which indirectly assures the progress in the fermentation process. Results of this experiments demonstrated that the rate of fermentation was at its peak during the first week of fermentation. Previously, [Majumder et al. \(2021c\)](#) have described this decreasing pH along with increasing antioxidant activity as a cumulative parameter that judges the quality of fermented beverages. In this research, TH also exhibited the same to confirm an excellent fermentation process. The graphs of specific gravity and brix were completely opposite to that of alcohol by volume (%ABV). At first six days of fermentation brix was seen to be sharply decreased about 8% (from  $12 \pm 0.1\%$  to  $4 \pm 0.5\%$ ) and specific gravity was also seen to be decreased from  $1.0488 \pm 0.0015$  to  $1.0157 \pm 0.0021$  and, after that a gradual decrease was seen for further six days followed by a steady (parallel to x axis) graph up to the fifteenth day or final day ( $3.1 \pm 0.1\%$  Bx and specific gravity of  $1.0115 \pm 0.002$ ). Drop in specific gravity and brix actually confirmed the loss of sugars which apparently converted into alcohol and carbon-dioxide due to fermentation. Mean %ABV were calculated from brix which was calculated to be  $6.17 \pm 0.65\%$  after eleven days of

fermentation, further, no notable change has been detected. The results reflected by pH, specific gravity, brix and %ABV Figure 3 and free radical scavenging property (DPPH assay) also Figure 4 were clear indicatives of a fact that fermentation process dependent metabolic changes took place till a certain period of fermentation and then stopped gradually. Majumder et al. (2021c) have described a brief reason for this result i.e., depletion of carbon source and occurrence of stationary or death phase attained by the yeast cells are the causes behind conclusion of a fermentation process.

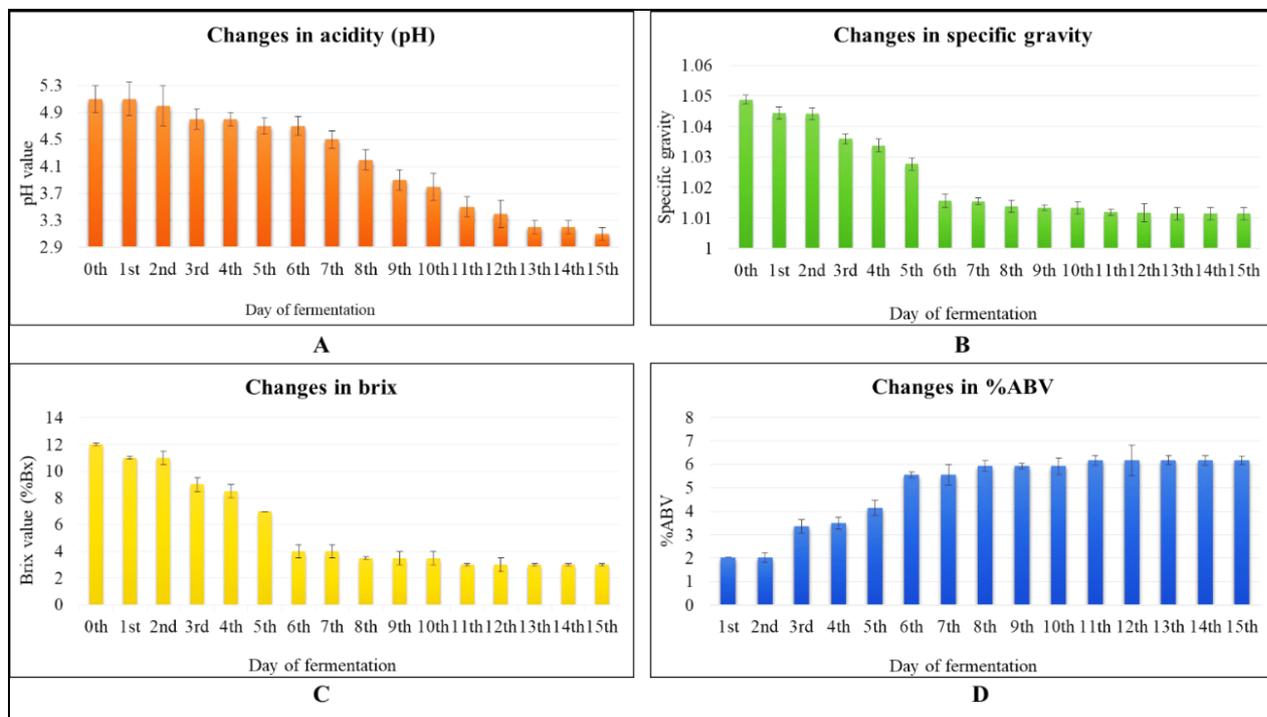


Figure 3. Changes in physicochemical properties during fermentation of TH. A- Acidity, B- Specific gravity, C- Brix, D- ABV (alcohol by volume).

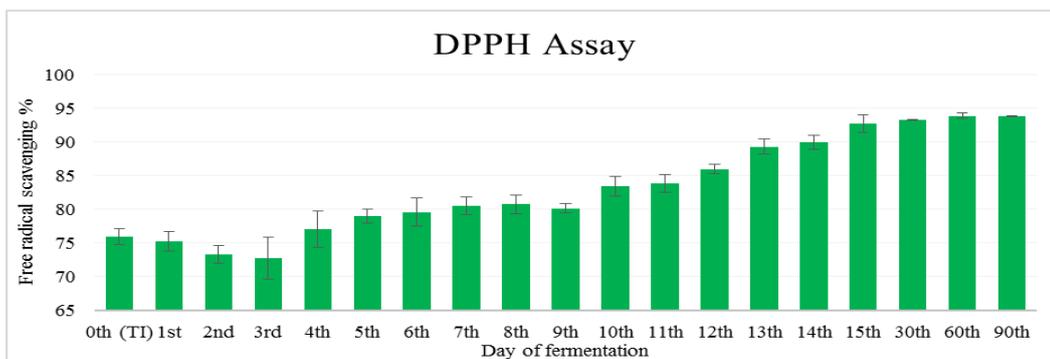


Figure 4. DPPH free radical scavenging activity exhibited by TH (Tea *Haria*) sample collected on regular intervals during fermentation.

### 3.4. Gas Chromatography-Mass Spectrometry Analysis

GC-MS analysis was done on fermented tea sample or TH collected on the 15<sup>th</sup> day of fermentation. A total of fifteen days were considered for fermentation process based on the results of preliminary biochemical and physicochemical experiments where those properties inside the fermenting broth were seen to be sharply changing at first 6-7 days (a week approximately), thereafter, up to 15<sup>th</sup> day, recorded graphs of every parameter was found to be more gradual to steady. Moreover, the sole established probiotic fermented tea infusion kombucha is generally incubated for more or less two weeks (Majumder et al., 2020a) and the same methodology was used to brew TH. So, after fifteen days of fermentation, sample was considered as final product and used for GC-MS analysis. A total of

thirty-seven peaks were recorded in the chromatogram Figure 5 where chemotaxonomically thirty-three different compounds belong various biosynthesis pathways were identified from the mass spectra. Table 1 represents the peak reports of GC-MS where identifiable components of TH have been enlisted.

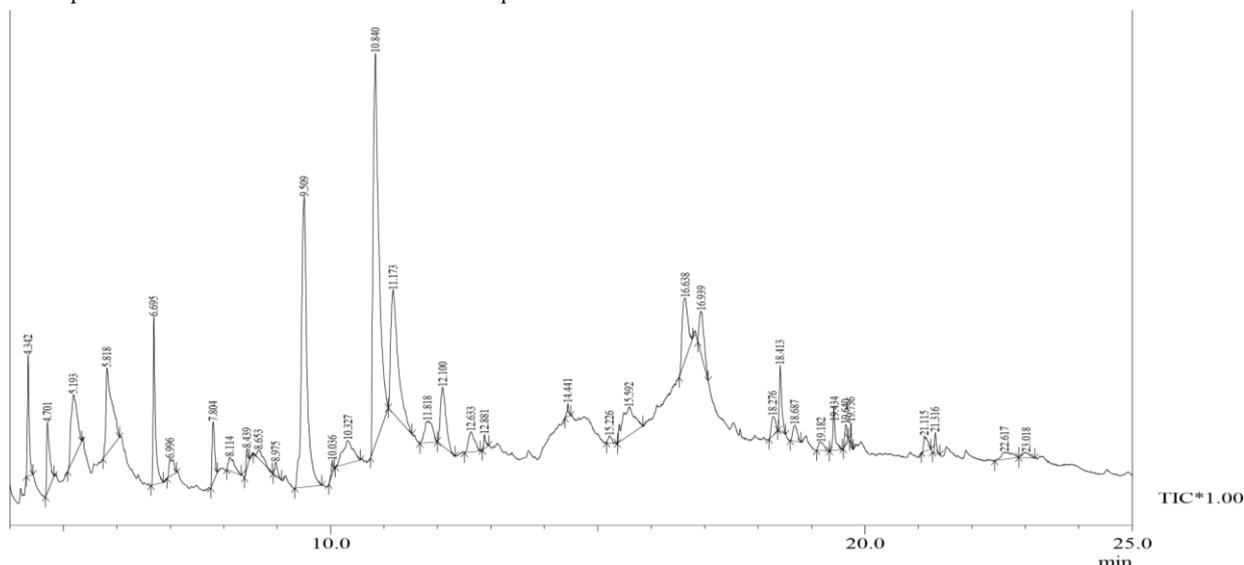


Figure 5. GC-MS chromatogram of the sample TH (Tea Haria) showing peaks of different components (listed in Table 1).

Table 1. Components of TH (Tea Haria) detected by GC-MS analysis.

Peak Index	Area %	Name of compounds
1	2.25	Furfural
2	2.18	Furfuryl alcohol
3	4.16	Methyl 2-ethylacetoacetate
4	5.37	2-Hydroxy-2-cyclopenten-1-one
5	3.91	2,4-Dihydroxy-2,5-dimethyl-3(2H)-furan-3-one
6	0.86	Glutaconic acid
7	1.67	Levulinic acid
8	0.86	Furaneol
9	0.42	4-Hydroxypyridine
10	0.51	1-Acetyl-1,2-epoxy-cyclopentane
11	0.41	2R,3S-9-[1,3,4-Trihydroxy-2-butoxymethyl]guanine
12	16.75	Hydroxydihydromaltol (4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-)
13	0.27	1-Undecanol
14	2.48	1,2,6-Hexanetriol
15	23.63	5-Hydroxymethylfurfural
16	9.4	Glycerol monoacetate
17	1.84	Glycerol monoacetate
18	3.42	4-Pentenoic acid, 3-hydroxy-, ethyl ester
19	1.33	Citramalic acid
20	0.28	Palmityl alcohol (1-Hexadecanol)
21	0.25	2,6-Di-tert-butylphenol
22	0.27	Isobutyl acetoacetate
23	3.69	4-butyl-3-methoxy-2-Cyclopenten-1-one
24	3.71	5-Butyl-3-methoxy-2-cyclopenten-1-one
25	2.19	Hydrazinecarboxamide, 2-(2-methylcyclohexylidene)-
26	0.84	5-Butyl-3-methoxy-2-cyclopenten-1-one
27	1.61	Caffeine
28	0.75	5-Butyl-3-methoxy-2-cyclopenten-1-one
29	0.48	Succinic acid, tridec-2-yn-1-yl trans-4-methylcyclohexyl ester
30	1.09	Palmitic acid
31	0.45	2-Propenylcyclopropanecarboxylic acid, ethyl ester
32	0.2	Oxalic acid, monoamide, N-(3,4-dimethylphenyl)-, heptyl ester
33	0.63	cis-9-Hexadecenal
34	0.39	Stearic acid
35	0.8	Unidentified
36	0.38	Sclareol glycol
37	0.27	Bis(2-ethylhexyl) phthalate

### 3.5. *In Vitro* Antioxidant Activity (DPPH Assay) and Antioxidant Compounds in TH

DPPH assay for antioxidant activity was carried out in this experiment where mean result for DPPH scavenging activity was found to be  $75.86 \pm 1.2\%$  ( $69.90 \pm 1.02 \mu\text{g AE/ml}$ ) for control sample, i.e., tea infusion or TI while the fermented sample or TH on 15<sup>th</sup> day (considered as final product to use in other assays) scavenged DPPH  $92.63 \pm 1.3\%$  ( $88.35 \pm 1.1 \mu\text{g AE/ml}$ ), significantly higher than that of control. Moreover, same test was done on samples at different stage stages of fermentation where a gradual increase was seen up to 15<sup>th</sup> day of fermentation (Figure 4) and further (on aging up to 90<sup>th</sup> day) no significant change in antioxidant activity has been noticed. Figure 4 depicts the DPPH free radical scavenging % or antioxidant activity of samples (TH samples collected during different stages of fermentation) and control (TI). This result drew a probable conclusion regarding time dependent biosynthesis of antioxidant molecules as secondary metabolites of fermenting microbes (of TH) responsible for high antioxidant activity of TH. Moreover, no significant change in DPPH scavenging activity was seen between 15<sup>th</sup> day and 90<sup>th</sup> day, which also indicated that after two weeks of fermentation, the metabolism (production or development of secondary metabolites) was stabilized in broths. To calculate the IC<sub>50</sub> value for this assay, different strengths of samples (TH and TI) were prepared using distilled water for dilution. After calculation, near one-fourth dilution of TI (21.288% strength) was found to scavenge 50% of DPPH solution while 10.655% strength resulted as the IC<sub>50</sub> for TH. These results clearly suggest that the fermented tea sample of TH shows remarkably higher antioxidant activity compared to the unfermented infusion.

According to GC-MS analysis, major components of TH were found to be antioxidant as well as fermentation derived metabolites. Components with majorities in peak area, like 5-hydroxymethylfurfural (23.63%), hydroxydihydromaltol (16.75%) are reported as potential anti-oxidative metabolites (Majumder et al., 2021c) derived from fermented broth. Regarding antioxidant activity, a detailed literature study has thrown light upon some other compounds, specially fermented products, which might worked as TH possessed remarkable outcomes in anti-radical assay and other biochemical tests. Out of thirty-three different identified compounds, twenty one components are reported to possess antioxidant activity. Except major compounds 5-hydroxymethylfurfural and hydroxydihydromaltol; other furfural derivatives such as, furfural and furfuryl alcohol; and furanone derivatives, such as furaneol (or strawberry furanone) and 2,4-dihydroxy-2,5-dimethyl-3(2H)-furan-3-one detected in TH have antioxidative properties (Majumder et al., 2021c; Rath, Panigrahi, Kar, & Maharana, 2018). Other fermentation derived components, i.e., 4-hydroxypyridine (Stětinová & Grossmann, 2000); glycerol derived microbial metabolites i.e., glyceryl monoacetate (Dodson, Avellar, Athayde, & Mota, 2014) and citramalic acid (Luís et al., 2016; Wu et al., 2020); pharmaceutically high valued 2,6-di-tert-butylphenol (Milaeva et al., 2008; Muhammad, Pandian, & Hopper, 2020); fatty acid derivatives i.e., palmityl alcohol, palmitic acid, stearic acid (Majumder et al., 2021a) and cis-9-hexadecanal (Qadir et al., 2018), fermentation derived organic acids i.e., levulinic acid (Majumder... et al., 2021c), succinic acid (Zarubina, Lukk, & Shabanov, 2012) and oxalic acid (Kayashima & Katayama, 2002) are also reported antioxidant molecules. Ianaro et al. (2003) has described antioxidant potential of cyclopentenone derivatives (namely 2-cyclopenten-1-one). Interestingly, this GC-MS analysis has revealed number of 2-cyclopenten-1-one's peaks Table 1 in TH which may also possess antioxidant activity, however, further *in vitro* biochemical experiments or *in silico* approaches are welcome. Besides fermentation derived compounds, tea infusion derived stimulant molecule caffeine is also quite known as an antioxidant as Shi, Dalal, and Jain (1991) reported its hydroxyl radical scavenging efficacy.

### 3.6. *In Vitro* Antidiabetic Activity and Antidiabetic Agents in TH

Glucose uptake capacity is considered as one of the parameter to evaluate antidiabetic activity. In this research, glucose uptake by yeast cells was spectrophotometrically estimated to quantify the antidiabetic activity of samples, TH and TI. Metronidazole was used to prepare the standard curve where 1 ml of TI or the control showed a glucose uptake capacity that was equivalent to the capacity shown by  $10.42 \pm 1.65$  mg of metronidazole.

Interestingly, the fermented sample or tea *haria* (TH) exhibited approximately double glucose uptake capacity or antidiabetic activity than that shown by TI or the control. TH's antidiabetic activity was quantified to be  $21.613 \pm 1.99$  mg MetE/ ml.

Tea metabolite caffeine possesses potential antidiabetic activity (Fu et al., 2017) and this could be a reason behind exhibition of high *in vitro* antidiabetic activity by samples analyzed through this assay. However, two of the major biologically active compounds of TH as well as fermentation derived metabolites i.e., 5-hydroxymethylfurfural (23.63%), hydroxydihydromaltol (16.75%) are the causes behind high *in vitro* antidiabetic activity of TH if metabolomics is concerned. In recent studies have described antidiabetic or antihyperglycemic activity of 5-hydroxymethylfurfural (Essien, Thomas, Ekanem, & Choudhary, 2021; Ge et al., 2020) and hydroxydihydromaltol (Hameed, Hussein, Kareem, & Hamad, 2015).

### 3.7. In Vitro Lipid Peroxidation Inhibition Assay and Hepatoprotective Components in TH

Lipid peroxidation is the chain of reactions of oxidative degradation of lipid molecules. It is the process in which free radicals collect electrons from the lipids in cell membranes, resulting in cell damage. In this assay, liver cells (goat liver homogenate) were used to evaluate *In vitro* lipid peroxidation inhibition activity. Thus, objectives of this experiment were to study the liver cell protective efficacy of crude samples and find out probable responsible components protecting liver cells from oxidative damages or having hepatoprotective activity. Activity for TI or tea infusion was estimated to be  $4.558 \pm 0.53$  mg TAE/ ml while fermented tea *haria* or TH was found to be quite significant, about five times more efficient lipid peroxidation inhibitor than TI as the result was quantified to be  $21.389 \pm 1.02$  mg TAE/ml.

Antioxidant activity and responsible of antioxidant compounds have already been discussed before which might be a reason behind this significant anti-lipid peroxidation status of fermented sample TH as lipid peroxidation is a kind of oxidative damage. A huge number of fermented antioxidant metabolites have been detected in TH but, more precisely, some compounds identified in this study are reported to be namely hepatoprotective, such as, major compound 5- hydroxymethylfurfural (Ding, Wang, Yao, Li, & Cai, 2010); fatty acids like palmitic acid and stearic acid (Makni et al., 2008); Hydrazinecarboxamide, 2-(2-methylcyclohexylidene)- (Gopalakrishnan, Kalaiarasi, & Gnanendra, 2016) and caffeine (Cachón, Quintal-Novelo, Medina-Escobedo, Castro-Aguilar, & Moo-Puc, 2017).

### 3.8. In Vitro Antibacterial Activity and Antimicrobial Compounds in TH

Inhibition of bacterial growth by TH and TI samples was observed and the result is depicted in Table 2. All the samples showed remarkably better antibacterial activity against both Gram-negative bacterial samples by inhibiting their growth compared to Gram-positive ones. Among the samples, TH or the fermented tea *haria* sample showed better antibacterial activity than the control or TI. TH showed the best result by creating the maximum inhibition zone of 10 mm against *Escherichia coli* among all the four bacterial samples.

**Table 2.** Inhibition zones against bacteria produced by TH (Tea *Haria*) and TI (Tea Infusion or control) to exhibit antibacterial activity.

Sample	Gram-positive bacteria		Gram-negative bacteria	
	<i>Staphylococcus aureus</i>	<i>Bacillus subtilis</i>	<i>Escherichia coli</i>	<i>Klebsiella pneumoniae</i>
TH	0 mm	0 mm	10 mm	4 mm
TI	0.5 mm	0.25 mm	4 mm	1 mm

GC-MS analysis also revealed antimicrobial components present in TH. Among total of thirty three identified components twenty were reported to possess antimicrobial activity, these are, furfural derivatives i.e., furfural, furfuryl alcohol and 5-hydroxymethylfurfural (Chai et al., 2013); hydroxydihydromaltol (Ab Rahman, Sijam, & Omar, 2014); furanone derivative furaneol (Sung, Jung, Lee, Kim, & Lee, 2006); fermentation derived organic acids

like glutaconic acid (Kovanda, 2020), levulinic acid (Hawkins, 2014), citramalic acid (Luís et al., 2016; Wu et al., 2020) and oxalic acid (Kwak, Lee, Lee, Yun, & Kang, 2016); caffeine (Nonthakaew, Matan, Aewsiri, & Matan, 2015) and its analogue 2R,3S-9- $\beta$ -[1,3,4-trihydroxy-2-butoxymethyl]guanine (Peng & Don, 2013); 4-hydroxypyridine (Derikvand et al., 2010); fatty acid and alcohol derivatives such as palmitic acid, stearic acid, palmityl alcohol, cis-9-hexadecenal, 1-undecanol (Hoda et al., 2019; Togashi et al., 2007; Zheng et al., 2005); and some other components like glycerol derived glycerol monoacetate (Singh, 2014); antiseptic agent 2,6-di-tert-butylphenol (Milaeva et al., 2008; Muhammad et al., 2020) and hydrazinecarboxamide, 2-(2-methylcyclohexylidene)- (Vinaykumar et al., 2021).

### 3.9. Other Bioactive Components Present in TH

Scientific documents have disclosed that besides antioxidant, antidiabetic, hepatoprotective and antimicrobial activity, a number of other biological activities were found to be linked with components of TH. Ab Rahman et al. (2014) have reported that TH's major component hydroxydihydromaltol is a flavonoid fraction which can exhibit anticancer, anti-proliferative and potential anti-inflammatory activity. 5-Hydroxymethylfurfural, the TH component with highest peak area, is also an anti-proliferative compound (Zhao et al., 2013). Bioactive fatty acids of TH i.e., palmitate and stearate are reported to possess anticancer, antitumor, cardio-protective and anti-inflammatory activities (Majumder et al., 2021a). Pharmaceutically praised component and TH's metabolite 2,6-di-tert-butylphenol also exhibits anticancer (Milaeva et al., 2015) and anti-inflammatory activity (Murakami, Kawata, Katayama, & Fujisawa, 2015). Antioxidant and antimicrobial component citramalic acid has antiaging properties (Wu et al., 2020). A number of 2-cyclopenten-1-one derivatives have been detected in TH Table 1. 2-Cyclopenten-1-ones possess anti-proliferative and anti-inflammatory activity (Ianaro et al., 2003) and being an Hsp70, can function as a chaperone and protects neurons from protein aggregation and toxicity, protects cells from apoptosis, thus, helps to prevent Parkinson's disease, Alzheimer's disease, polyglutamine diseases, amyotrophic lateral sclerosis etc. Moreover, it is a stress marker (temporal lobe epilepsy) that can also protect cells from inflammation and is involved in the immune response in autoimmune disease (multiple sclerosis) being an adjuvant (Turturici, Sconzo, & Geraci, 2011). These cyclopentenones can also exhibit potential anticancer, antiviral, anti-ulcer activities. According to Simeonov, Nunes, Guerra, Kurteva, and Afonso (2016), sugar (d-glucose, d-galactose etc.) derived cyclopentenones are powerful synthon for the synthesis of a variety of bioactive target molecules. Last but not least caffeine, a stimulant of tea and a substrate derived metabolite of TH exhibits wide range of bioactivities. Köroğlu et al. (2014) have described its anti-inflammatory and respiratory-protective (lung protective) activities and, beside a stimulant this compound is often praised for cardio-protective activities.

### 3.10. Biosynthesis of GC-MS Detected Metabolites of TH

TH was analyzed through GC-MS based metabolomics where biosynthetic pathways of metabolites were studied by reviewing established reports on them. Among the thirty-three compounds, 5-hydroxymethylfurfural (the most abundant) is a prime wine flavonoid and a sugar derived fermented product. This could be biosynthesized during fermentation by microbes of *bakhar* because the compound is reported as a fungal secondary metabolite or fermented product found in aged samples of different wines (Majumder et al., 2021c). So, TH this could probably be synthesized due to breakdown or fermentation or caramelization of saccharides present in it. Other furfural derivatives detected in TH (furfural, furfuryl alcohol) are also reported as metabolites of fermenting microbes and common aroma components of wine and other alcoholic liquors like brandy, whiskey, rum etc. Furfuryl alcohol occurred mainly due to enzymatic or chemical reduction of furfural during fermentation (Majumder et al., 2021c). TH components i.e., methyl 2-ethylacetoacetate, furfural derivatives 2,4-dihydroxy-2,5-dimethyl-3(2H)-furanone etc. are reported volatile aroma imparting components (potential fruity and wine like aroma in wines) (Majumder et al., 2021c). Antioxidant agent levulinic acid and its precursor succinic acid, both are reported as products of glucose fermentation and mainly found in aged wine and beer samples (Majumder et al., 2021c), interestingly both have

been detected in TH validating fermentation process. Interestingly, compounds like furfural; furfuryl alcohol; levulinic acid; 5-hydroxymethylfurfural; palmityl alcohol; palmitic acid; stearic acid etc. detected in TH, might contribute a sweet (caramel like), astringent and wine like flavour (Majumder et al., 2021c) towards the development of wine like taste in tea *haria*. Previously, Rackemann and Doherty (2011) demonstrated biosynthesis of levulinic acid from sucrose during fermentation. However, production of 5-hydroxymethylfurfural and levulinic acid are reported in many fermented beverages and pathways are also reported regarding conversion of 5-hydroxymethylfurfural into levulinic acid (Zhang & Weitz, 2012). Glutaconic acid, another component of TH, could be synthesized from levulinic acid as these organic acids can be easily formed in any sugar fermentation broth. Kobayashi, Hattori, Honda, and Kirimura (2014) reported formation of oxalic acid as fermentation by-product (metabolite of *Aspergillus niger*). 1,2,6-Hexanetriol (a replacement of glycerine) is a sugar derived alcohol, just like glycerol, which was probably synthesized from available sugars of tea *haria* broth due to fermentation. Another two glycerol derived compounds are glyceryl monoacetate and 4-pentenoic acid, 3-hydroxy-, ethyl ester. 4-pentanoic acid is also known as allyl acetate which is actually the acetic acid ester of allyl alcohol (dehydrated form of glycerol) (Liu et al., 2010) while acetic acid is also a common microbial (fermentation) metabolite (Vidra & Németh, 2018). This metabolomics revealed more compounds conjugated with acetic acid i.e., glyceryl monoacetate and methyl 2-ethylacetoacetate which also validates acetic acid as fermentation metabolite. 2,4-Dihydroxy-2,5-dimethyl-3(2H)-furan-3-one is a compound present in both could be a tea component or sugar compound as it is reported as a plant derived flavour compound. Citramalic acid (1.33%) is bacterial and yeast metabolite. It derives from a succinic acid (<https://pubchem.ncbi.nlm.nih.gov/compound/Citramalic-acid>). Interestingly, citramalic acid and succinic acid (detected as succinic acid, tridec-2-yn-1-yl trans-4-methylcyclohexyl ester) both are present in TH. Some notable peaks of cyclopentenone derivatives have been detected in TH, i.e. 2-Hydroxy-2-cyclopenten-1-one (5.37%); 4-butyl-3-methoxy-2-Cyclopenten-1-one (3.69%) and 5-Butyl-3-methoxy-2-cyclopenten-1-one (5.29%). Cyclopentenones may be either biosynthesized by fermenting microbes (Lin et al., 2005) or can be derived from tea. Caffeine is tea's signature molecule, thus, detection of caffeine in its fermented broth brought no confusion at all. Another components named 2R,3S-9-[1,3,4-Trihydroxy-2-butoxymethyl]guanine is a structural analogue of caffeine, and, the functional group i.e., guanine is also known as a precursor of caffeine and a tea metabolite too (Majumder et al., 2020a). Fermented beverages like wines are well known to obtain different antioxidant fatty acids and their derivatives i.e., palmitic acid, stearic acid, palmityl alcohol, 1-undecanol and cis-9-hexadecanal which have been detected in TH also. However, fatty acids are common metabolites of tea and other plants, so, those might have occurred in TH due to their presence in substrate (tea infusion) or starter (used herbs for preparation of bakhar). Yi and Kim (1982) reported antioxidant activity of some wine compounds (levulinic acid, furfural, 5-hydroxymethylfurfural, and pyrazines) which were identically detected in our sample Table 1 and definitely support the fermentation metabolomics of TH. The above metabolomics discussion suggests components i.e., furfural; furfuryl alcohol; furaneol; 2,4-dihydroxy-2,5-dimethyl-3(2H)-furan-3-one; levulinic acid; hydroxydihydromaltol; glyceryl monoacetate; cyclopentenones; glutaconic acid; 1,2,6-hexanetriol; 5-hydroxymethylfurfural; 4-pentenoic acid, 3-hydroxy-, ethyl ester; citramalic acid; succinic acid; oxalic acid etc. are in a metabolome relationship with each other as all were directly or indirectly derived from sugared tea broth due to fermentation or from sugar derived non-fermented products. Moreover, GC-MS based metabolomics also validated the biochemical and physicochemical properties of the fermented sample TH as those qualities found in preliminary analysis were found harmonious with the results of GC-MS analysis.

#### 4. CONCLUSIONS

The aim of this research was to study the possibilities to produce a fermented health drink from tea where traditional liquor or *haria* starter *bakhar* was used. Production of highly biological active formulation from underutilized traditional brewing knowledge was the key outcome of this research. Detection of different groups of

bioactive biochemicals, *in vitro* biological activities (antioxidant, antidiabetic, hepatoprotective and antibacterial activity), detection of various bioactive fermented components; study on biosynthesis of fermentation derived metabolites; and discussion on biological activity definitely brought significance in this research. Hopefully, the concept and outcome of this research have shown a possible and biotechnologically novel way to use traditional knowledge where application of indigenous starter and tea is involved. Moreover, a major part of our regular diet, mainly, green foods and beverages like tea and wines are major sources of antioxidants where application of this type of formulations will be significant being rich bioactive components. But, further sophisticated instrumentations for scientific research and value additions are needed to uphold the acceptability of this fermented formulation as a consumable drink.

### List of Abbreviations

TH: Tea Haria.

GC-MS: Gas chromatography-mass spectrometry.

DPPH: 2,2-diphenyl-1-picrylhydrazyl.

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