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## Valorization of CTC tea waste through kombucha production and insight into GC-MS based metabolomics

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### Abstract

This research paper is aimed to deliver a novel approach to prepare probiotic formulation i.e., kombucha from infusion made of tea waste to valorize the by-product. In this study, a GC-MS based approach in combination with preliminary in vitro biochemical analyses was used to investigate the metabolic profiles, especially the antioxidant compounds during the fermentation of waste tea infusion. Waste tea infusion was found to be rich in xanthosine, the precursor of caffeine while the fermented sample was seen to be rich in more bioactive components derived from the substrate compounds i.e., xanthosine and sucrose. In addition, GC-MS based metabolomics and proposed biosynthesis pathway of metabolome was identified which suggest the later breakdown of xanthosine and other substrate components into microbial metabolites during post-fermentation process. These findings are expected to be useful for further studies on production of bioactive formulations from tea waste.

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## 1. Introduction

Over the past few decades, sustainability and the concept of sustainable development are considered as the main issues that have been underlined by scientists, researchers and policy makers at the national and international levels. Valorization of food waste by different methods and their effects on the sustainability of food production are today's main topic for the scientific community. Some focus on the recovery of the value-added compounds from food waste, while others try to convert the waste into processed materials to bring out an economy from it. Biovalorization of food waste is an economical and environmental approach, which can reduce the problems regarding conventional disposal.

Regarding tea waste, a large amount of bio-wastes are produced before production (agricultural or tea garden wastes) and during production (tea factory wastes), distribution (transport), processing, and consumption (made tea waste or infusion) across tea growing regions and the world. Previously, recycling of tea waste for extraction of caffeine was demonstrated by Shalmashi et al. and Khan et al. which is now considered as a method of waste recycling by many tea factories (1,2). Biochar of tea factory waste has been valorized previously by researchers. By using pyrolysis method, biochar is reported to be produced from tea which is used as a source of energy (3). Conversion of renewable resources into value-added products like bioenergy is one of the growing concerns of bioeconomy strategy where biomethane production from tea wastes by anaerobic digestion is also an important approach as reported by Gozde (4). Moreover, tea waste has been characterized as a novel adsorbent

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for toxic pollutants and metals from aqueous wastes. Removal of toxic pollutants such as copper and chromium ions, lead ions, other heavy metal ions, zinc ions, dyes, phenol (p-nitrophenol), antibiotics, benzene from wastewater by using black tea waste is reported (5,6).

This paper describes a novel use of food biotechnology to valorize wastes of tea manufacturing factories (commercially unused manufactured tea). Previous reports on richness of caffeine and other nutrients in tea waste and recovery of value-added compounds lead us to prepare a novel bio-based material through using food biotechnology. Kombucha fermentation process is dependent on symbiotic metabolic relationship between bacteria and yeasts (7–9). The beverage is reported to be fermented by a symbiotic consortium of acetic acid bacteria (*Acetobacteraceae*) and osmophilic yeasts (10). SCOBY or symbiotic colony of bacteria and yeasts is actually a biofilm that floats on the fermented broth of kombucha by forming a cellulose pellicle. This biofilm is produced by the microbial colony involved in kombucha fermentation (11). The dominant bacteria in the system are *Gluconobacter* and *Komagataeibacter* (or *Gluconacetobacter*) as reported by Marsh et al. with various other organisms identified as *Komagataeibacter xylinus*, *Komagataeibacter intermedius*, *Komagataeibacter rhaeticus*, *Komagataeibacter saccharivorans* and *Komagataeibacter kombuchae* (11). The yeast species in the system are even more variable, and can include yeasts like *Zygosaccharomyces*, *Candida*, *Torulasporea*, *Pichia*, *Brettanomyces/Dekkera*, *Schizosaccharomyces*, and *Saccharomyces* (12). All of these microorganisms involved in kombucha fermentation uses tea infusion and sugar as substrate from broth to produce secondary metabolites through microbial fermentation pathway. However, studying the microbial ecosystem was not a part of this research as it is already well established by various scientific communities. In this research, our intention was to produce a value-added probiotic health drink or by-product from tea waste. Moreover, after brewing kombucha from waste infusion, some biochemical analysis were assessed to characterize infusion and its fermented form where special insights was given on *in vitro* antioxidant activity and GC-MS analysis.

## 2. Materials and Methods

### 2.1. Collection of Tea Waste and Preparation of Infusion

Fresh and dry tea waste (Figure 1) was collected from the CTC (or crush-tear-curl, a type of processed black tea) tea manufacturing factory of Jayantika Tea Estate, Darjeeling (26°32'06.0"N, 88°16'18.0"E). Infusion of tea waste (WTI) was prepared following the conventional method of making black tea infusion. One litre of hot water (freshly boiled) was poured over 10 g of fresh CTC tea waste to steep and covered for 15 minutes and strained thereafter to prepare infusion of WTI (Figure 1).



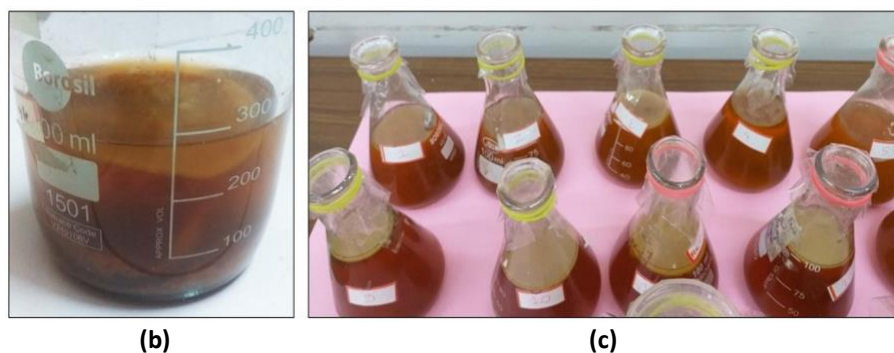


Figure 1. (a) Wastes of CTC tea factory; (b) Close view of CTC tea waste comprising of stalks, stem and other fibrous parts; (c) Layer of SCOBY on waste tea kombucha (WTK); (d) Replicas of healthy (without molds) WTK batches prepared for biochemical analysis.

## 2.2. Production of Waste Tea Kombucha

Following the conventional kombucha brewing method waste tea kombucha or WTK was prepared (with slight modifications) (8,9). Following the conventional methodology standardized by of Majumder et al. (9), for every one litre batch of kombucha (or WTK), 100 ml (or 10% v/v) of starter and 60 g (or 6% w/v) of white sugar (as nutrient for microbes involved in kombucha fermentation) were added in sterilized glass jars filled with freshly prepared and cooled (at room temperature) WTI. Mouths of the jars were covered with sterilized muslin cloth. Following the standardized protocols, jars containing kombucha fermentation broth were left undisturbed for fifteen days in a dark room at 25°C temperature for incubation (Figure 1). To brew the first batch of waste tea kombucha, fresh kombucha (normal black tea kombucha) was used as a starter. But, after that, continuous batches of waste tea kombucha (WTK) were produced (for generations) by using starter from the previous batch (WTK), just to decrease the exposure of black tea kombucha components in WTK prior for a genuine metabolomics. After brewing a total of five generations, WTK (for analysis) was collected from batches prepared at the last generation.

## 2.3. Preliminary Biochemical Analysis

Qualitative detection tests for bioactive components like tannin, coumarin, cardiac glycoside, terpenoid, flavonoid, phenol, protein, reducing sugar, starch, steroid, saponin, alkaloid and caffeine in WTI and WTK were assessed after following protocols demonstrated by Majumder et al. (13,14). pH was monitored on both samples to investigate the changes in acidity of the broth between initiation (WTI) and termination (WTK) of fermentation process. It was determined using a pre-calibrated pH meter (13). Result is given as means of a total of five replications.

## 2.4. Antioxidant Activity (DPPH Assay)

Antioxidant activity by DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical scavenging assay was conducted following the protocol developed to analyze kombucha (13,14). To 2800  $\mu$ l of the methanol solution of 0.2 mM DPPH (SRL, India), 200  $\mu$ l of WTI and WTK samples were added and incubated at room temperature for 20 minutes in the dark. Absorbance was measured at 517 nm by UV-Vis spectrophotometer (Agilent). Ascorbic acid was used to prepare the standard curve to quantify the antioxidant value. Results of DPPH scavenging

activity were expressed as AAE or ascorbic acid equivalent (mg AAE/ ml). Result is given as means of a total of five replications.

### 2.5. GC-MS Based Metabolomics

Metabolite profiling of WTI and WTK were done following the method of GC-MS analysis of beverages as developed by Majumder et al. (15). Methanolic (50%) extracts were prepared by dissolving 1 ml (WTI and WTK) of each sample overnight in 1 ml of methanol (Merck). Methanol was used for extraction because it is the widely used solvent for extraction of bioactive substances and polarity of methanol as an organic solvent is very close to water and edible alcohols (primary solvent of any beverage). GCMS-QP2010 Plus (Shimadzu Co., Japan) was used in this analysis. DB-5 fused-silica capillary column (0.25  $\mu$ m thick, 0.25 mm of internal diameter and 30 m of length) was fitted. 1  $\mu$ l of each sample with a split ratio of 20:1 was injected (injection temperature was 260°C). Interface and source temperature was set to 270°C and 230°C respectively. Helium was used as carrier gas. Total flow rate was 16.3 ml/min and column flow rate were 1.21 ml/min. Mass spectra were recorded at 5 scan/sec with a scanning rate of 40–650 m/z. After comparing the spectral configurations obtained with that of available mass spectral databases (NIST08s.LIB and WILEY8.LIB), compounds were identified. The chromatogram (TIC or Total Ion Chromatogram) is based on the intensity of fragments produced by the ionization. Quantification of the amount (area %) of each compound was done on the basis of peak areas. The data obtained from GCMS analysis were further studied from available reported documents in search for their bioactivities. Study on biosynthesis pathway of the metabolome was conducted on components of WTI and WTK by following GC-MS based metabolomics (16,17). Available scientific literature was studied to find probable biosynthesis pathways of WTI and WTK metabolites. KEGG pathway database (18) was used as reference to study biosynthesis pathways of WTI and WTK metabolomes.

## 3. Results and Discussion

### 3.1. Preliminary Biochemical Analysis

Qualitative detection tests revealed presence of bioactive components like tannin, coumarin, cardiac glycoside, terpenoid, flavonoid, phenol, protein, reducing sugar, steroid, saponin, alkaloid and caffeine in samples. Interestingly, phenol, flavonoid, cardiac glycoside, protein and saponin were detected high in WTK compared to WTI while reducing sugar, alkaloid and caffeine which was found high in WTI but low in its fermented form (WTK). Starch was found absent in both samples. Slight presence of tannin, coumarin, and terpenoid were detected in both samples. Both samples were found to contain a huge amount of steroid as sharp and thick reddish brown rings were developed in both test tubes as a result of this qualitative detection test. Interestingly, decrease of caffeine correlates some previous hypothesis reported by researchers (7,9,13,19) where withdrawal of caffeine from tea during kombucha fermentation is suggested. A heat-map (Figure 2) regarding this experiment has been provided as a representative of the result.

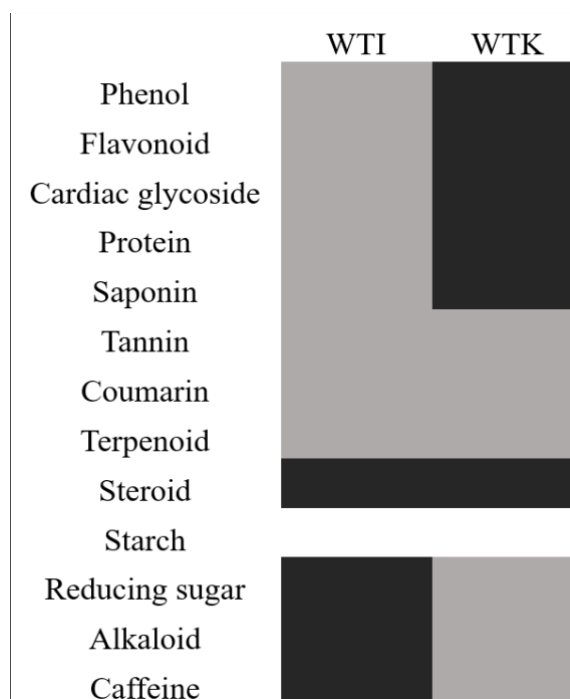


Figure 2. Heatmap (dark to light = high to low) showing levels of different qualities tested on different samples.

Acidity was also found to be increased after WTK fermentation. pH was determined as  $5.6 \pm 0.1$  for WTI while WTK was more acidic with pH  $3.9 \pm 0.1$ . Increase in acidity during kombucha fermentation was previously described in many research papers where production of organic acids like acetic acid by kombucha fermenting acetic acid bacteria (*Acetobacteraceae*, *Gluconobacter* and *Gluconacetobacter*) was marked as a reason behind it (10,12). Further GC-MS analysis was carried out to evaluate these differences observed between WTI and WTK through preliminary tests.

### 3.2. Antioxidant Activity (DPPH Assay)

DPPH assay for free radical scavenging activity is considered as the widely used and one of the most successful indicators of antioxidant property. It is used to explore the capability of scavenging or neutralizing free radicals exhibited by any biochemical extract that is dissolved in polar solvents. The result of this assay simply showed that fermented samples, i.e., WTK has a huge free radical scavenging activity compared to the control (WTI). Ascorbic acid standard curve was also prepared to quantify the antioxidant activity by comparing it with the equivalent amount of ascorbic acid needed to scavenge the same. In result, it was found that DPPH scavenging activity shown by WTK,  $0.459 \pm 0.0124$  mg AAE/ ml, was nearly double of that shown by WTI ( $0.288 \pm 0.015$  mg AAE/ ml). This difference in antioxidant property between both samples was previously reflected in results of qualitative tests (as bioactive group of molecules were detected more in WTK than WTI) and further justified by GC-MS analysis. Presence of many anti-oxidative molecules in the fermented sample was revealed after metabolite profiling which is discussed below.

### 3.3. Metabolite Profiling

As a result, chromatographs of both samples undergone GC-MS analysis have been provided in Figure 3. Chromatogram showing fourteen peaks for twelve different components for WTI (Table 1) was mainly occupied by purines like caffeine (5.19%) and its precursor xanthosine (68.89%) while the kombucha (WTK) was rich in derivatives of bioactive fermented metabolites (Table 2) like pyruvic acid; glycerol; acetic acid; valeraldehyde; 1,3-propanediol; desulphosinigrin; octose and  $\gamma$ -butyrolactone along with some amount of sugar derived non-fermented products (due to non-enzymatic browning or sugar caramelization) like 2-cyclopenten-1-one and 2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one (DDMP). Small amount of caffeine was also detected in WTK (with a peak area of 1.03% only) which was again correlated with the results of previous studies and preliminary tests of this research as described earlier. A total of twenty two different components were detected in WTK where, excluding fermented metabolites and non-fermented sugar derivatives, nine compounds, i.e., caffeine, sitosterols, derivatives long chain fatty acids and (Z,Z)-3,9-cis-6,7-epoxy-nonadecadiene were found to be directly derived from components of control (WTI) without major changes in chemical structure by fermentation or other reaction. Moreover, among the common compounds, (Z,Z)-3,9-cis-6,7-epoxy-nonadecadiene is reported as an insect pheromone which (derived from tea waste) can be a semiochemical directly derived from tea plant as it shows insect attracting properties (20).

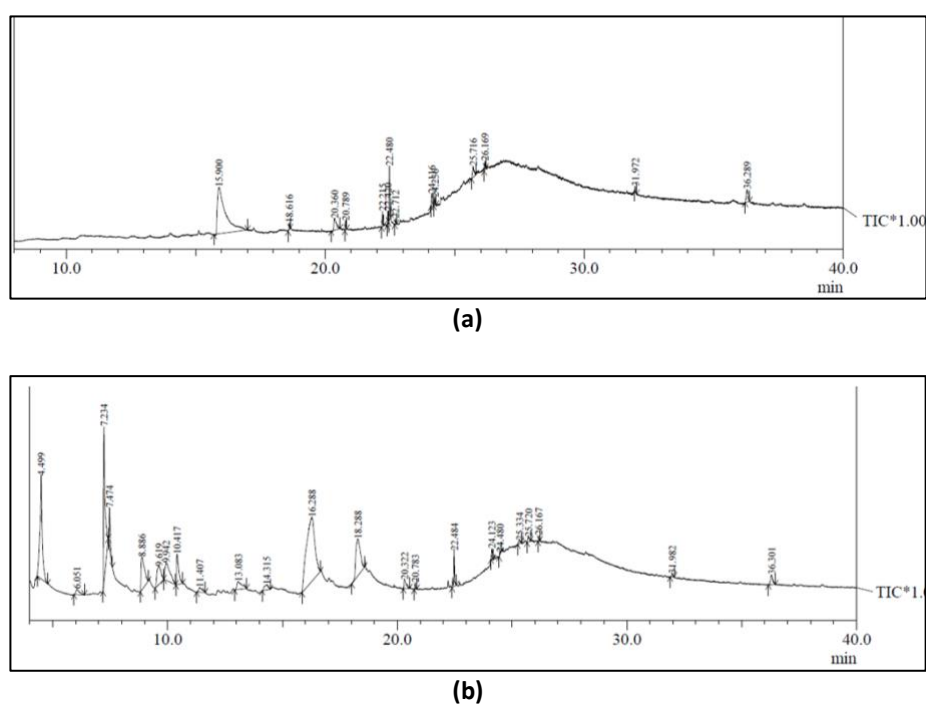


Figure 3. (a) GC-MS chromatographs of both waste tea infusion or WTI and (b) waste tea kombucha or WTK.

Table 1. Components of waste tea infusion (WTI) detected by GC-MS analysis.

Peak index	Retention time	Area%	Components of waste tea infusion (WTI)	Type of compound
1	15.9	68.89	Xanthosine	Precursor of alkaloids
2	18.616	1.04	Octadecanal	Fatty acid derivative
3	20.36	5.19	Caffeine	Alkaloid
4	20.789	1.09	Stearic acid methyl ester	Fatty acid derivative
5	22.215	2	13-Hexyloxacyclotridec-10-en-2-one	Enone
6	22.42	1.28	Linolelaidic acid, methyl ester	Fatty acid derivative
7	22.48	6.41	Petroselinic acid methyl ester	Fatty acid derivative
8	22.712	0.73	Stearic acid methyl ester	Fatty acid derivative
9	24.116	2.41	(Z,Z)-3,9-cis-6,7-epoxy-nonadecadiene	Semiochemical/pheromone
10	24.236	1.19	Lauric acid, chloride	Fatty acid derivative
11	25.716	3.35	1-oleoylglycerol	Fatty acid derivative
12	26.169	1.1	Dinonyl Phthalate	Phthalate contamination
13	31.972	0.81	beta-Sitosterol	Steroid (phytosterol)
14	36.289	4.53	beta-Sitosterol	Steroid (phytosterol)

Table 2. Components of waste tea kombucha (WTK) detected by GC-MS analysis.

Peak index	Retention time	Area%	Components of waste tea kombucha (WTK)	Type of compound
1	4.499	13.65	Pyruvic acid, methyl ester	Pyruvates
2	6.051	1.02	Pyruvic acid, ethyl ester	Pyruvates
3	7.234	13.52	2-Cyclopenten-1-one, 2-hydroxy-	Enone
4	7.474	2.17	2-Cyclopenten-1-one, 2-hydroxy-	Enone
5	8.886	6.18	2-Hydroxy-gamma-butyrolactone	Lactone
6	9.619	4.28	Glycerol	Triose Sugar Alcohols
7	9.942	5.06	Acetic acid, propyl ester	Monoarboxylic acid
8	10.417	3.63	Valeraldehyde	Saturated fatty aldehyde
9	11.407	0.91	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-	Glycoside (saponin)
10	13.083	2.15	Acetic acid, 2-methylpentyl ester	Monoarboxylic acid
11	14.315	1.09	Octose	Monosaccharide
12	16.288	30.86	1,3-Propanediol, 2-(hydroxymethyl)-2-nitro-	Glycol (glycerol derivative)
13	18.288	9.54	Desulphosinigrin	Glucoside

14	20.322	1.03	Caffeine	Alkaloid
15	20.783	0.22	Palmitic acid methyl ester	Fatty acid derivative
16	22.484	1.74	Petroselinic acid methyl ester	Fatty acid derivative
17	24.123	0.56	(Z,Z)-3,9-cis-6,7-epoxy-nonadecadiene	Semiochemical/pheromone
18	24.48	0.13	Palmitic acid, 16-hydroxy-, methyl ester	Fatty acid derivative
19	25.334	0.27	1-oleoylglycerol	Fatty acid derivative
20	25.72	0.44	1,3-dioleoylglycerol	Fatty acid derivative
21	26.167	0.16	Bis(tridecyl) phthalate	Phthalate contamination
22	31.982	0.2	beta-Sitosterol	Steroid (phytosterol)
23	36.301	1.17	gamma-Sitosterol	Steroid (phytosterol)

Regarding bioactive major compounds of WTK, derivatives of pyruvic acid, glycerol, 1,3-propanediol, acetic acid, valeraldehyde, 2-cyclopenten-1-one,  $\gamma$ -butyrolactone etc. are common fermented products of alcoholic beverages and other alcoholic beverages but desulphosinigrin (9.54%) is a very rarely reported fermented product whose metabolic pathway was needed to study from available documents to assure its biosynthesis as a WTK metabolite. Furthermore, GC-MS peak reports and other details (like chemotaxonomy) which are not completely described in the text, are given in Table 1 and Table 2.

GC-MS metabolite profiling strongly suggests that only xanthosine, detected major component of WTI (not detected in WTK) and added sugar (sucrose) were gone through several enzymatic breakdowns during kombucha production to produce fermented metabolites and non-fermented sugar derivatives in WTK. Complete utilization of the major compound by WTK's fermenting microbes and breakdown into bioactive molecules also increased the acceptability of waste tea infusion as a productive substrate for fermentation of beverages. Furthermore, detailed metabolomics through studying biosynthesis pathways of detected metabolites are discussed below.

### 3.4. Biosynthesis Pathways of The Waste Tea Kombucha's Metabolome

Study of GC-MS based metabolomics on a developed food or beverage product is always challenging as different biological metabolic pathways get involved. To study biosynthesis pathway of WTK metabolomes from substrate or sugared WTI, a detailed background was investigated where biosynthesis or occurrence of metabolites present in substrate (here WTI) before fermentation, biosynthesis of secondary metabolites (here metabolites of kombucha microbes) during fermentation and changes of substrate components after fermentation as detected in WTK, were involved.

According to the results of metabolite profiling of WTI, xanthosine (68.89%) was detected as the major compound of substrate which was not further detected in WTK suggesting complete breakdown of the compound after kombucha fermentation. Moreover, xanthosine is the precursor of another substrate metabolite (also detected in WTK), i.e., caffeine. Xanthosine is produced through the purine metabolic pathway (21) and also through the pentose phosphate pathway. Presence of a good amount of purine (in the form of tea's signature purine caffeine) in tea waste was earlier established by many biochemists (1,2). So, detection of the compound in WTI brings no confusion regarding its occurrence in the tea



waste infusion. But, the presence of the alkaloid (xanthosine) as a major compound (in WTI) may depend on a lot of factors. The processed tea wastes are actually composed of stalks, hard leaves, stems, and other fiber rich parts of a tea plant whose detailed metabolomics has not been studied earlier (22). According to principle of metabolism, an organism's metabolism is the sum of all the chemical reactions that occur within the organism. There are two types of such reactions i.e., anabolism (building large molecules that cells need) and catabolism (breakdown of large molecules). Similarly, a specific metabolic pathway of an organism is comprised of both anabolism and catabolism reactions occurring in different organs or regions of an organism. So, if any specific organ of an organism biosynthesizes metabolites of a specific metabolic pathway, then it is natural when those metabolites or other metabolites of the same pathway are found in another organ of that organism. This is why the occurrence of huge amount of alkaloid precursor xanthosine in a fibrous part of tea plant is valid because tea is a major source of another metabolite of the same pathway, i.e., methylxanthine alkaloid caffeine. However, compound xanthosine is a nucleoside which is basically made up of xanthine (a purine base) and ribose (a 5-carbon sugar) and has the possibility to be broken down into those compounds. Results of metabolite profiling (complete breakdown of xanthosine in WTK) also supports this because all the fermentation derived components have possibilities to be derived from both xanthine and ribose as described below.

Based on metabolomics, components derived from GC-MS analysis were divided into three sub-groups based on their occurrence in samples (Table 2). These are, fermented products or probable metabolites of kombucha fermenting microbes (derivatives of pyruvic acid, glycerol, acetic acid, valeraldehyde, 1,3-propanediol octose, desulphosinigrin and  $\gamma$ -butyrolactone); non-fermentation sugar derived compounds (derivatives of 2-cyclopenten-1-one and DDMP) and substrate derived metabolites (derivatives of caffeine, petroselinic acid, palmitic acid, Z,Z-3,9-cis-6,7-epoxy-nonadecadiene, 1-oleoylglycerol, 1,3-dioleoylglycerol,  $\beta$ -sitosterol,  $\gamma$ -sitosterol).

#### 3.4.1. Substrate Derived Metabolites

In this subgroup, common components of both samples have been arranged that are directly derived only from the substrate WTI (with or without necessary chemical changes during extraction). These are derivatives of caffeine; petroselinic acid; palmitic acid; (Z,Z)-3,9-cis-6,7-epoxy-nonadecadiene; 1-oleoylglycerol and 1,3-dioleoylglycerol and sitosterol, where caffeine; petroselinic acid; (Z,Z)-3,9-cis-6,7-epoxy-nonadecadiene; and 1-oleoylglycerol were completely identical. Among these compounds, caffeine was a major compound of the substrate WTI (5.19%) whose precursor has also been detected as described above. Furthermore, derivatives of even chain fatty acids like stearic acid ( $C_{18}$  fatty acid), petroselinic acid ( $C_{18}$  fatty acid), linolelaidic acid ( $C_{18}$  fatty acid), lauric acid ( $C_{12}$  fatty acid), octadecanal (derived from  $C_{18}$  fatty acid) and 1-oleoylglycerol (a oleic acid/  $C_{18}$  fatty acid derivative) etc. were clearly detected in WTI. So, presence of derivatives of even chain fatty acids like petroselinic acid ( $C_{18}$  fatty acid), palmitic acid ( $C_{18}$  fatty acid), 1-oleoylglycerol or 1,3-dioleoylglycerol (oleic acid/  $C_{18}$  fatty acid derivatives) in the fermented broth (WTK) brought no confusion regarding metabolomics. According to GC-MS based metabolomics, these compounds arrived in the tea factory's post-manufacturing waste as phytochemicals from the tea leaf itself because caffeine, fatty acid and steroid biosynthesis pathways are very common for tea plants.

### 3.4.2. Biosynthesis of Fermented Products (Probable Metabolites of Kombucha Fermenting Microbes)

Fermented products or metabolites of kombucha fermenting microbes detected in GC-MS analysis are known to be biosynthesized during glucose or ribose fermentation (mainly glycolysis and pentose phosphate pathway) however other microbial metabolic pathways (18) were also involved in biosynthesis of WTK major bioactive compounds like desulphosinigrin, valeraldehyde and octose. Derivatives of glycerol i.e., glycerol and 1,3-propanediol, 2-(hydroxymethyl)-2-nitro-; acetic acid (acetic acid, propyl ester and acetic acid, 2-methylpentyl ester); pyruvic acid (methyl pyruvate and ethyl pyruvate); valeraldehyde; and  $\gamma$ -butyrolactone (2-Hydroxy- $\gamma$ -butyrolactone) etc. were simply produced from sucrose (substrate) derived glucose and WTI (substrate) derived ribose by microbial sugar fermentation following glycolysis and pentose phosphate pathway respectively (23) as shown in Figure 4. Following the steps of glycolysis pyruvic acid (major WTK compound occupying a total of 14.97% peak area) was produced which further produced acetic acid (total 7.21%) and entered into the TCA cycle. In the TCA cycle, succinic acid is an intermediate which is converted into  $\gamma$ -butyrolactone by the enzyme  $\gamma$ -lactonase. Derivative of  $\gamma$ -butyrolactone (2-Hydroxy- $\gamma$ -butyrolactone) is another major compound (6.18%).  $\gamma$ -Butyrolactone is a very common major wine component responsible for the unique flavour of red wine (24). Glycolysis derived triose sugar alcohol or glycerol and its derivative 1,3-propanediol, detected as 1,3-propanediol, 2-(hydroxymethyl)-2-nitro- (30.86%), the major bioactive compound of WTK, which is also reported as sugar fermentation components. 1,3-propanediol which was previously reported as a fungal fermentation metabolite (25). So, this compound of WTK was definitely derived from sugar fermentation and produced as a secondary metabolite of kombucha fermenting microbes. Components like desulphosinigrin (a glucosinolate) and valeraldehyde are reported to derive from amino acids and there were two probable sources in the substrate from where amino acids (precursors of desulphosinigrin and valeraldehyde) were biosynthesized, these are; amino acid biosynthesis pathway derived from 3-phosphoglycerate (glycolysis intermediate) and glycine, serine and threonine metabolism derived from substrate compound xanthosine. Both of the pathways are clearly depicted in Figure 4. Interestingly, occurrence of desulphosinigrin as a fermented product is rare and which is though previously reported in some fungal fermentation broths (26–28). According to Vinothkanna et al., this is found as a major component in ayurvedic fermented medicine (balarishta) which is used to treat arthritis (29). Previously, Al-Gboory has described desulphosinigrin as the major fermented volatile of camel milk and component of a yogurt starter as well (30). However, along with biosynthesis pathways, the above reports has helped desulphosinigrin to be established as a fermented product of waste tea kombucha. According to Pires et al., compound valeraldehyde is a fermenting yeast metabolite which is generally biosynthesized in beer as a flavour imparting compound from amino acids during fermentation (31). Following the Ehrlich pathway, these types of aldehydes are formed from amino acids after transamination and decarboxylation which finally reduces to form higher alcohols as end product. However, detection of this common fermentation compound in WTK has confirmed the occurrence of the Ehrlich pathway. WTK compound octose is an eight carbon monosaccharide which is probably derived from sugars (glucose and ribose) present as substrates in this fermentation broth as previously demonstrated by Zhang and Bantels (32). GC-MS analysis based biosynthesis pathways of the waste tea kombucha metabolome has been designed as depicted in Figure 4.

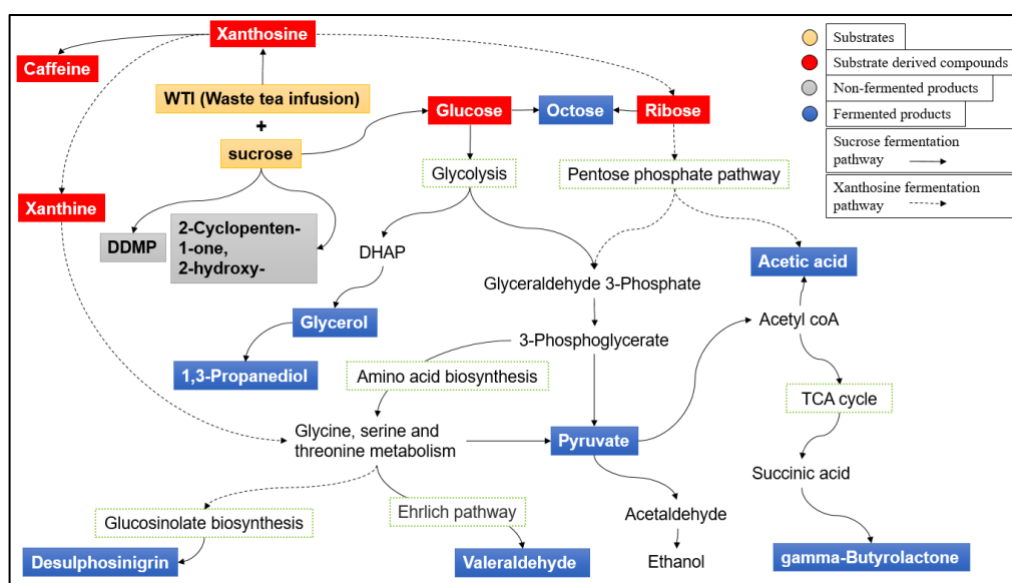


Figure 4. Probable biosynthesis pathway of waste tea kombucha's (WTK) metabolome.

### 3.4.3. Non-fermented Sugar Derived Compounds

Regarding non-fermented products, 2-cyclopenten-1-one, 2-hydroxy- (15.69%) and DDMP (0.91%) are actually sugar derived components known to be formed due to caramelization of certain sugars present in the fermentation broth or any other reaction happened during extraction. However, the Maillard reaction is also known to produce these lactones and other caramelized products from sugar and amino acid rich substrates. This is not fermentation or other metabolism, but a chemical reaction that occurred between amino acids and reducing sugars (both were either intermediates of fermentation pathways or present as substrate in high quantities) which gives browned colour and a distinctive flavour in fermented broths. However, the substrate of WTK was very rich in sugar content (ribose and sucrose) and synthesis of such caramel compounds is very natural during fermentation process different wines. These two compounds were previously reported in kombucha as well (9).

### 3.5. Biological Activities of WTI and WTK Components

Several literatures have been studied for the components detected by GC-MS analysis to presume benefits of using WTI and WTK as a beverage. And results of this study helped increase the quality of waste tea infusion and developed probiotic health drinks from it. So, this waste can be used in many projects by the scientific community. Moreover, results of preliminary biochemical tests for qualitative analysis and antioxidant activity were found to be interrelated with the components which are also described below.

#### 3.5.1. Bioactive Substrate Derived Metabolites

Not only by-products like kombucha production was the only objective of this research but also detection of reported bioactive molecules in tea waste itself was a major part of the research which have been described here. Xanthosine, the major component of WTI is not only the precursor of caffeine but also an antioxidant and radio-protective agent (33). Caffeine, detected in both samples, is well-known as a stimulant drug which is a signature

molecule found in tea and coffee. It has many important pharmacological activity like stimulant of central nervous system, diuretic and cardio-protective activity (34). All of the even chain fatty acids, which have already been referred as to be originated from tea plant, are highly bioactive. Stearic acid, petroselinic acid and palmitic acid are previously reported as cardio-protective (15). Antimicrobial, anticancer and neuroprotective effects of the major WTI fatty acid i.e., petroselinic acid (also detected in WTK) are previously reported by many scientists (15,35). WTI's stearic acid has antidepressant, antimicrobial, anticancer and hepatoprotective properties linoleic acid is an apoptosis inducer and lauric acid is a potential cardio-protective and antimicrobial (36–41). Lauric acid is used to treat viral infections, common cold, genital, genital warts etc. It is also used for preventing the transmission of HIV from mothers to children (42). There are lots of other uses for lauric acid that include treatment of bronchitis, gonorrhoea, yeast infections, chlamydia, and intestinal infections (*Giardia lamblia* and ringworm). Similarly, WTK's palmitic acid is a potential vasodilator, antioxidant, anticancer, antimicrobial and anti-inflammatory (pain reliever) agent (15). Oleic acid derived 1-oleoylglycerol (detected in both WTI and WTK) has anti-diabetic property (43). Octadecanal is also reported as antibacterial anticancer and antioxidant (44,45). Another prime bioactive substrate derived components are  $\beta$ -sitosterol and  $\gamma$ -sitosterol. Interestingly, both compounds have potential antibacterial properties and antidiabetic properties as reported previously (46,47). And presence of antidiabetic components like sitosterols and oleoylglycerols in by-product or tea waste's infusion is a significant finding because tea specially black tea, as a beverage (without sugar), is praised for its antidiabetic property. Moreover, bioactivities of  $\beta$ -sitosterol are more profound as it possesses antioxidant, immunomodulatory, anticancer, anti-inflammatory, lipid lowering effect, hepatoprotective, and respiratory-protective and wound healing properties (46). Another substrate (WTI) derived compound was 13-Hexyloxacyclotridec-10-en-2-one (2% peak area) which is the only compound that was neither detected in WTK nor converted due to fermentation. Still, the compound has some reported biological activities like antimicrobial activity and potential antitumor activity (48,49). Overall, presence of such bioactive molecules in tea waste extraction is definitely an important finding of this research.

### 3.5.2. Bioactive Fermented Products

Major fermented product 1,3-Propanediol, 2-(hydroxymethyl)-2-nitro- (30.86%) is a glycerol derivative which has potential antimicrobial (anti-staphylococcal) and antioxidant activity (50,51). The compound is reported to be useful in treatment of metabolic acidosis (52). Glycerol, precursor of this major compound, has many bioactive properties and this component is an essential sugar alcohol, very commonly found in fermented beverages like beer, wine, rum etc. It is most commonly used to treat constipation, it improves hydration and reduces skin problems (53). Pyruvic acid (total 14.67%) is another major component as well as common fermentation metabolite present in WTK, which (ethyl pyruvate) is a potential antioxidant and anti-inflammatory compound (54). Fermented beverages like dark beer and red wine are rich sources of pyruvic acid. Recent research suggests pyruvate in high concentrations may have a role in cardiovascular therapy, as an inotropic agent (55). Another organic acid i.e., acetic acid, detected as a major fermented metabolite also exhibits antibacterial and antifungal properties however, the mechanism of action is not known. It's the second simplest carboxylic acid (after formic acid) and a common kombucha component.  $\gamma$ -Butyrolactone (6.18%) is a commonly found fermented beverage

product which was detected as one of the major compounds of WTK. This component has potential antiviral and anti-thyroid properties which was also identified as a prodrug for  $\gamma$ -hydroxybutyric acid (GHB), a recreational CNS depressant with effects similar to those of barbiturates. Fermenting microbial metabolite valeraldehyde is reported for its antimicrobial activity (56). Desulphosinigrin (9.54%) is a bioactive glucosinolate. Despite being rarely found in fermented beverages, it is one of the major WTK compounds established as a fermented product in this study by considering metabolomics. Desulphosinigrin was mentioned earlier as an ayurvedic fermented medicinal component that is used to treat arthritis. Moreover, antibacterial, strong antioxidant and anticancer properties of this rare fermented product helped increase the pharmacological importance of this probiotic fermented beverage as a waste by-product (57).

### 3.5.3. Bioactive Non-Fermented Sugar Derived Compounds

Two non-fermented sugar derived kombucha components, i.e., DDMP and 2-cyclopenten-1-one, 2-hydroxy- are also responsible for probable high biological activities of WTK. Flavonoid fraction DDMP has anti-inflammatory, antimicrobial, anti-proliferative, antioxidant and antidiabetic properties while the major non-fermented product 2-cyclopenten-1-one, 2-hydroxy- (15.69%) is also an antimicrobial, anti-inflammatory, diuretic and antioxidant component (26,58–60). However, DDMP is previously reported as fungal metabolite also but there is an absence of strong evidences (26).

### 3.6. Interrelations Between Results of Biochemical Assays and GC-MS Based Metabolomics

Results of GC-MS analysis clearly showed that waste tea kombucha contains a huge amount of components responsible for antioxidant and bioactivities. Waste tea infusion, containing xanthosine as the major compound further produced several bioactive components in WTK after fermentation (described earlier designing of the biosynthesis pathways for WTK metabolome) which was also reflected in results of qualitative tests. Interestingly, results of GC-MS analysis became completely complementary with the results of *in vitro* antioxidant assay as WTK was found scavenge significantly DPPH compared to WTI. Moreover, WTK contains glycosides like DDMP and desulphosinigrin while DDMP is a saponin and a flavonoid fraction (17) too. So, qualitative detection of high saponin, flavonoid and glycosides in WTK is valid, if GC-MS metabolite profiling is considered. Similarly, alkaloid (caffeine and xanthosine) rich WTI resulted high for alkaloids in the preliminary detection test. Interpretation on decreased reducing sugar, alkaloids and caffeine after fermentation also leaves an interesting conclusion as those got utilized by kombucha fermenting microbes to produce secondary metabolites which were too bioactive to exhibit significant results for WTK in specific biochemical tests and antioxidant assay. Caffeine was previously reported to enhance the speed of SCOBY formation in kombucha (7,9,19). So, presence of alkaloid or caffeine rich substrate (including its precursor) and results of biochemical tests showing utilization of those components also characterized the fermentation process and widened the acceptability of waste tea as a perfect substrate of kombucha. Preliminarily determined decrease of pH during fermentation is also harmonizing with GC-MS results. Previously, in depth research on physicochemical characteristics of kombucha has revealed that acetic acid and other organic acid formation by microbes was the main cause behind this increasing acidity (8). Likewise, major peaks for derivatives of organic acids (acetic acid and pyruvic acid) were also detected in WTK which brought significance towards increasing acidity of WTK.

## 4. Conclusions

Our aim of this research was to study the possibilities to produce a probiotic health drink or kombucha from tea factory wastes to make it economically useful in a novel way where qualitative analysis, antioxidant assay and GC-MS based metabolomics were considered as parameters for characterization. Production of high antioxidant and bioactive broth from unutilized wastes was the key outcome of this research. Detection of xanthosine, caffeine and bioactive fatty acids in waste tea's infusion or hot water extraction (WTI); bioactive fermented components like desulphosinigrin; 1,3-propanediol, 2-(hydroxymethyl)-2-nitro-; 2-cyclopenten-1-one, 2-hydroxy-; 2-hydroxy-gamma-butyrolactone in WTK; establishment of biosynthesis pathways for fermentation derived metabolites; and discussion on biological activity definitely brought significance in this research. Hopefully, the concept and outcomes of this research have shown a possible and biotechnologically novel way to valorize tea waste which will help the waste to be commercially and scientifically more useful.

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## Author Contributions

S.M. and M.B. conceived and designed the experiments; S.M., A.G., S.S., S.C. and S.D.S. performed the experiments; S.M. and S.A. analyzed the data; M.B. and S.S. contributed materials; S.M. wrote the paper.

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## Data Availability Statement

Not applicable.

## Conflicts of Interest

Authors may declare no conflict of interest.

## References

1. Shalmashi A, Abedi M, Golmohammad F, Eikani MH. Isolation of caffeine from tea waste using subcritical water extraction. *J Food Process Eng* [Internet]. 2010;33(4):701–11. Available from: <https://doi.org/10.1111/j.1745-4530.2008.00297.x>
2. Khan Z, Kakkar S, Ghag S, Shah S, Patil S, Gupta AD. Recycling of tea waste for extraction of caffeine and production of a transdermal patch. *World J Pharm Res* [Internet]. 2018;7(17):1511–21. Available from: <https://doi.org/10.20959/wjpr201817-13494>
3. Akgül G, Iglesias D, Ocon P, Moreno Jiménez E. Valorization of tea-waste biochar for

- energy storage. *BioEnergy Res* [Internet]. 2019;12(4):1012–20. Available from: <https://doi.org/10.1007/s12155-019-10025-6>
4. Gozde Ozbayram E. Waste to energy: valorization of spent tea waste by anaerobic digestion. *Environ Technol* [Internet]. 2021;42(22):3554–60. Available from: <https://doi.org/10.1080/09593330.2020.1782477>
  5. Hussain S, Anjali KP, Hassan ST, Dwivedi PB. Waste tea as a novel adsorbent: a review. *Appl Water Sci* [Internet]. 2018;8(6):1–16. Available from: <https://doi.org/10.1007/s13201-018-0824-5>
  6. Kabir MM, Mouna SSP, Akter S, Khandaker S, Didar-ul-Alam M, Bahadur NM, et al. Tea waste based natural adsorbent for toxic pollutant removal from waste samples. *J Mol Liq* [Internet]. 2021;322:115012. Available from: <https://doi.org/10.1016/j.molliq.2020.115012>
  7. Jayabalan R, Malbaša R V, Lončar ES, Vitas JS, Sathishkumar M. A review on kombucha tea-microbiology, composition, fermentation, beneficial effects, toxicity, and tea fungus. *Compr Rev food Sci food Saf* [Internet]. 2014;13(4):538–50. Available from: <https://doi.org/10.1111/1541-4337.12073>
  8. Villarreal-Soto SA, Beaufort S, Bouajila J, Souchard J, Taillandier P. Understanding kombucha tea fermentation: a review. *J Food Sci* [Internet]. 2018;83(3):580–8. Available from: <https://doi.org/10.1111/1750-3841.14068>
  9. Majumder S, Ghosh A, Chakraborty S, Bhattacharya M. Withdrawal of stimulants from tea infusion by SCOBY during kombucha fermentation: A biochemical investigation. *Int J Food Ferment Technol* [Internet]. 2020;10(1):21–6. Available from: <https://doi.org/10.30954/2277-9396.01.2020.5>
  10. De Filippis F, Troise AD, Vitaglione P, Ercolini D. Different temperatures select distinctive acetic acid bacteria species and promotes organic acids production during Kombucha tea fermentation. *Food Microbiol* [Internet]. 2018;73:11–6. Available from: <https://doi.org/10.1016/j.fm.2018.01.008>
  11. Marsh AJ, O’Sullivan O, Hill C, Ross RP, Cotter PD. Sequence-based analysis of the bacterial and fungal compositions of multiple kombucha (tea fungus) samples. *Food Microbiol* [Internet]. 2014;38:171–8. Available from: <https://doi.org/10.1016/j.fm.2013.09.003>
  12. May A, Narayanan S, Alcock J, Varsani A, Maley C, Aktipis A. Kombucha: a novel model system for cooperation and conflict in a complex multi-species microbial ecosystem. *PeerJ* [Internet]. 2019;7:e7565. Available from: <https://doi.org/10.7717/peerj.7565>
  13. Bhattacharya M, Majumder S, Saha S, Ghosh A, Chakraborty S, Acharyya S, et al. Comparative in vitro biological characterization of black and green tea infusions fermented with brewer’s yeast and SCOBY with special emphasis on antioxidant activity. *Int J Nutraceuticals, Funct Foods Nov Foods*. 2022;
  14. Majumder S, Acharyya S, Ghosh A, Chakraborty S, Sarkar S, Saha S, et al. Insights into low biological activity of wax apple (*Syzygium samarangense*) juice by in vitro phytochemical investigation with special reference to metabolomics. *Asian J Nat Prod Biochem* [Internet]. 2021;19(1). Available from: <https://doi.org/10.13057/biofar/f190106>
  15. Majumder S, Ghosh A, Chakraborty S, Saha S, Bhattacharya M. Metabolomics affirms traditional alcoholic beverage raksi as a remedy for high-altitude sickness. *J Ethn Foods* [Internet]. 2021;8(1):1–10. Available from: <https://doi.org/10.1186/s42779-021->

- 00094-4
16. Majumder S, Ghosh A, Bhattacharya M. Natural anti-inflammatory terpenoids in *Camellia japonica* leaf and probable biosynthesis pathways of the metabolome. *Bull Natl Res Cent*. 2020;44(1):1–14.
  17. Chakraborty S, Majumder S, Ghosh A, Saha S, Bhattacharya M. Metabolomics of potential contenders conferring antioxidant property to varied polar and non-polar solvent extracts of *Edgaria darjeelingensis* CB Clarke. *Bull Natl Res Cent* [Internet]. 2021;45(1):1–12. Available from: <https://doi.org/10.1186/s42269-021-00503-3>
  18. KEGG PATHWAY. Pathway Maps [Internet]. 2022. Available from: <https://www.genome.jp/kegg/pathway.html>
  19. Greenwalt CJ, Steinkraus KH, Ledford RA. Kombucha, the fermented tea: microbiology, composition, and claimed health effects. *J Food Prot* [Internet]. 2000;63(7):976–81. Available from: <https://doi.org/10.4315/0362-028X-63.7.976>
  20. Luo ZX, Li ZQ, Cai XM, Bian L, Chen ZM. Evidence of premating isolation between two sibling moths: *Ectropis grisescens* and *Ectropis obliqua* (Lepidoptera: Geometridae). *J Econ Entomol* [Internet]. 2017;110(6):2364–70. Available from: <https://doi.org/10.1093/jee/tox216>
  21. Pathway K. Biosynthesis of alkaloids derived from histidine and purine - Reference pathway [Internet]. 2012. Available from: <https://www.kegg.jp/pathway/map01065+C01762>
  22. İçen H, Gürü M. Effect of ethanol content on supercritical carbon dioxide extraction of caffeine from tea stalk and fiber wastes. *J Supercrit Fluids* [Internet]. 2010;55(1):156–60. Available from: <https://doi.org/10.1016/j.supflu.2010.07.009>
  23. McLeod A, Zagorec M, Champomier-Vergès M-C, Naterstad K, Axelsson L. Primary metabolism in *Lactobacillus sakei* food isolates by proteomic analysis. *BMC Microbiol* [Internet]. 2010;10(1):1–10. Available from: <https://doi.org/10.1186/1471-2180-10-120>
  24. Vose J, Tighe T, Schwartz M, Buel E. Detection of gamma-butyrolactone (GBL) as a natural component in wine. *J Forensic Sci* [Internet]. 2001;46(5):1164–7. Available from: <https://doi.org/10.1520/JFS15116J>
  25. Tabah B, Varvak A, Pulidindi IN, Foran E, Banin E, Gedanken A. Production of 1, 3-propanediol from glycerol via fermentation by *Saccharomyces cerevisiae*. *Green Chem* [Internet]. 2016;18(17):4657–66. Available from: <https://doi.org/10.1039/C6GC00125D>
  26. Hameed IH, Hamza LF, Kamal SA. Analysis of bioactive chemical compounds of *Aspergillus niger* by using gas chromatography-mass spectrometry and fourier-transform infrared spectroscopy. *J Pharmacogn Phyther* [Internet]. 2015;7(8):132–63. Available from: <https://doi.org/10.5897/JPP2015.0354>
  27. Kadhim MJ, Mohammed GJ, Hussein H. Analysis of bioactive metabolites from *Candida albicans* using (GC-MS) and evaluation of antibacterial activity. *Int J Pharm Clin Res*. 2016;8(7):655–70.
  28. Giannoukos K, Giannoukos S, Lagogianni C, Tsitsigiannis DI, Taylor S. Analysis of volatile emissions from grape berries infected with *Aspergillus carbonarius* using hyphenated and portable mass spectrometry. *Sci Rep* [Internet]. 2020;10(1):1–11. Available from: <https://doi.org/10.1038/s41598-020-78332-z>
  29. Annadurai V, Paramasivan M, Muralitharan G, Sekar S. In silico probing of anti-arthritis



- potential of traditionally fermented ayurvedic polyherbal product balarishta reveals lupeol and desulphosinigrin as efficient interacting components with urec. *Int J Pharm Pharm Sci.* 2014;6:469–75.
30. Al-Gboory HL. Determination of volatile compound in fermented camel milk by GC-MS. Lublin, Pol [Internet]. 2017; Available from: <http://dx.doi.org/10.24326/fmpmsa.2017.4>
  31. Pires EJ, Teixeira JA, Brányik T, Vicente AA. Yeast: the soul of beer's aroma—a review of flavour-active esters and higher alcohols produced by the brewing yeast. *Appl Microbiol Biotechnol* [Internet]. 2014;98(5):1937–49. Available from: <https://doi.org/10.1007/s00253-013-5470-0>
  32. Zhang Q, Bartels D. Octulose: a forgotten metabolite? *J Exp Bot* [Internet]. 2017;68(21–22):5689–94. Available from: <https://doi.org/10.1093/jxb/erx367>
  33. Popova NR, Gudkov S V, Bruskov VI. Natural purine compounds as radioprotective agents. *Radiatsionnaia Biol Radioecol.* 2014;54(1):38–49.
  34. Yang A, Palmer AA, de Wit H. Genetics of caffeine consumption and responses to caffeine. *Psychopharmacology (Berl)* [Internet]. 2010;211(3):245–57. Available from: <https://doi.org/10.1007/s00213-010-1900-1>
  35. Placek LL. A review on petroselinic acid and its derivatives. *J Am Oil Chem Soc.* 1963;40(8):319–29.
  36. Jubie S, Ramesh PN, Dhanabal P, Kalirajan R, Muruganantham N, Antony AS. Synthesis, antidepressant and antimicrobial activities of some novel stearic acid analogues. *Eur J Med Chem* [Internet]. 2012;54:931–5. Available from: <https://doi.org/10.1016/j.ejmech.2012.06.025>
  37. Pan P-H, Lin S-Y, Ou Y-C, Chen W-Y, Chuang Y-H, Yen Y-J, et al. Stearic acid attenuates cholestasis-induced liver injury. *Biochem Biophys Res Commun* [Internet]. 2010;391(3):1537–42. Available from: <https://doi.org/10.1016/j.bbrc.2009.12.119>
  38. Lerata MS, D'Souza S, Sibuyi NRS, Dube A, Meyer M, Samaai T, et al. Encapsulation of variabilin in stearic acid solid lipid nanoparticles enhances its anticancer activity in vitro. *Molecules* [Internet]. 2020;25(4):830. Available from: <https://doi.org/10.3390/molecules25040830>
  39. Li J, Rao H, Bin Q, Fan Y, Li H, Deng Z. Linolelaidic acid induces apoptosis, cell cycle arrest and inflammation stronger than elaidic acid in human umbilical vein endothelial cells through lipid rafts. *Eur J Lipid Sci Technol* [Internet]. 2017;119(7):1600374. Available from: <https://doi.org/10.1002/ejlt.201600374>
  40. Eyres L, Eyres MF, Chisholm A, Brown RC. Coconut oil consumption and cardiovascular risk factors in humans. *Nutr Rev* [Internet]. 2016;74(4):267–80. Available from: <https://doi.org/10.1093/nutrit/nuw002>
  41. Dayrit FM. The properties of lauric acid and their significance in coconut oil. *J Am Oil Chem Soc* [Internet]. 2015;92(1):1–15. Available from: <https://doi.org/10.1007/s11746-014-2562-7>
  42. WebMD. Lauric Acid - Uses, Side Effects, and More [Internet]. 2020. Available from: <https://www.webmd.com/vitamins/ai/ingredientmono-1138/lauric-acid>
  43. Chin YX, Chen X, Cao WX, Sharifuddin Y, Green BD, Lim PE, et al. Characterization of seaweed hypoglycemic property with integration of virtual screening for identification of bioactive compounds. *J Funct Foods* [Internet]. 2020;64:103656. Available from: <https://doi.org/10.1016/j.jff.2019.103656>

44. Pratap GK, Rather SA, Shantaram M. Anticholinesterase activity and mass spectral analysis of olea dioica Roxb., an in vitro study. Indian J Pharm Sci [Internet]. 2020;82(4):601–11. Available from: <https://doi.org/10.36468/pharmaceutical-sciences.686>
45. Kalita BC, Gupta D Das, Das AK, Hui PK, Tag H. Gas Chromatography-Mass Spectrometry of Methanol Extract of Urtica dioica L. from Arunachal Pradesh, India. J Clin Tri Cas Rep. 2018;1(1):52000111.
46. Babu S, Jayaraman S. An update on  $\beta$ -sitosterol: A potential herbal nutraceutical for diabetic management. Biomed Pharmacother [Internet]. 2020;131:110702. Available from: <https://doi.org/10.1016/j.biopha.2020.110702>
47. Sirikhansaeng P, Tanee T, Sudmoon R, Chaveerach A. Major phytochemical as  $\gamma$ -sitosterol disclosing and toxicity testing in Lagerstroemia species. Evidence-Based Complement Altern Med [Internet]. 2017;2017. Available from: <https://doi.org/10.1155/2017/7209851>
48. Malayaman V, Sheik Mohamed S, Senthilkumar RP, Ghouse Basha M. Analysis of phytochemical constituents in leaves of Bhumyamalaki (Phyllanthus debilis Klein ex Willd.) from Servaroy hills, Tamil Nadu. India J Pharm Phytochem. 2019;8:2678–83.
49. Mettupalayam Kaliyannan Sundaramoorthy P, Kilavan Packiam K. In vitro enzyme inhibitory and cytotoxic studies with Evolvulus alsinoides (Linn.) Linn. Leaf extract: A plant from Ayurveda recognized as Dasapushpam for the management of Alzheimer's disease and diabetes mellitus. BMC Complement Med Ther [Internet]. 2020;20(1):1–12. Available from: <https://doi.org/10.1186/s12906-020-02922-7>
50. Rane Z, Anish-Kumar P, Bhaskar A. Phytochemical evaluation by GC-MS and in vitro antioxidant activity of Punica granatum fruit rind extract. J Chem Pharm Res. 2012;4(6):2869–73.
51. Ololade ZS, Kuyooro SE, Ogunmola OO, Oyelese OJ. Physicochemical, volatile organic composition, phenolic, flavonoid and ascorbic acid contents, antioxidant, anti-Arthritic and anti-inflammatory properties of cocos nucifera juice. Glob J Med Res Pharma, Drug Discov Toxicol Med. 2017;17(2):43–9.
52. Kallet RH, Jasmer RM, Luce JM, Lin LH, Marks JD. The treatment of acidosis in acute lung injury with tris-hydroxymethyl aminomethane (THAM). Am J Respir Crit Care Med [Internet]. 2000;161(4):1149–53. Available from: <https://doi.org/10.1164/ajrccm.161.4.9906031>
53. WebMD. Glycerol - Uses, Side Effects, and More [Internet]. 2020. Available from: <https://www.webmd.com/vitamins/ai/ingredientmono-4/glycerol>
54. Himmelfarb J, Sayegh MH. Chronic kidney disease, dialysis, and transplantation E-book: a companion to Brenner and Rector's The Kidney. Philadelphia: Elsevier Health Sciences; 2010.
55. Drugbank Online. Pyruvic acid [Internet]. 2022. Available from: <https://go.drugbank.com/drugs/DB00119>.
56. Muniandy K, Gothai S, Tan WS, Kumar SS, Mohd Esa N, Chandramohan G, et al. In vitro wound healing potential of stem extract of Alternanthera sessilis. Evidence-based Complement Altern Med [Internet]. 2018;2018. Available from: <https://doi.org/10.1155/2018/3142073>
57. Sosa AA, Bagi SH, Hameed IH. Analysis of bioactive chemical compounds of Euphorbia lathyris using gas chromatography-mass spectrometry and fourier-transform infrared

- spectroscopy. *J Pharmacogn Phyther* [Internet]. 2016;8(5):109–26. Available from: <https://doi.org/10.5897/JPP2015.0371>
58. Alghamdi SS, Khan MA, El-Harty EH, Ammar MH, Farooq M, Migdadi HM. Comparative phytochemical profiling of different soybean (*Glycine max* (L.) Merr) genotypes using GC–MS. *Saudi J Biol Sci* [Internet]. 2018;25(1):15–21. Available from: <https://doi.org/10.1016/j.sjbs.2017.10.014>
59. Wang J, Ding W, Wang R, Du Y, Liu H, Kong X, et al. Identification and bioactivity of compounds from the mangrove endophytic fungus *Alternaria* sp. *Mar Drugs* [Internet]. 2015;13(7):4492–504. Available from: <https://doi.org/10.3390/md13074492>
60. Pavani P, Naika R. Evaluation of antibacterial activity and GCMS analysis of *Zanthoxylum ovalifolium* fruit extracts. *J Pharm Res Int*. 2021;3:7–17.