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Zooming into CTC black-tea wine metabolites: A GC-MS-based study

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Abstract. This research was designed to propose a report on the fermentation metabolomics of CTC (crush-tear-curl) tea wine (TW), a yeast-fermented broth of sugared CTC black tea infusion. The gas chromatography-mass spectrometry analysis of the tea wine revealed the presence of thirty-five metabolites, including the major compound glycerine with some potential antioxidant molecules and other bioactive agents (4H-pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-; furfural; furfuryl alcohol; succinic acid; levulinic acid; palmitic acid; tyrosol, pyruvaldehyde; and 1-hexadecanol). The role of metabolites in the physicochemical, biochemical, and medicinal properties of TW has been discussed. Biomolecules responsible for the flavour of TW were as follows: glycerine derivatives; pyruvaldehyde; furfural; furfuryl alcohol; acetic, levulinic, succinic, and palmitic acids, etc. – which might develop a sweet, caramel-like, astringent, slightly sour and wine-like flavour and taste. Furthermore, on the basis of yeast metabolism, possible biosynthesis pathways of metabolites were designed aiming for fermentation metabolomics. The outcome of this study cross-verified physicochemical, biochemical, and medicinal properties of TW suggesting its acceptability. As the fields of both wine research and tea science continue to evolve, the findings of this study may encourage fermentation technology for product development from tea that may also boost the growth of the tea industry.

Keywords and phrases: CTC tea, tea wine, fermentation, bioactive metabolites, biosynthesis pathway

1. Introduction

Wines contain several metabolites derived from the substrates and the fermenting yeast, which together determine its quality. Wine's bioactivities have been associated with reduced risk of cardiovascular and oxidative-stress-related diseases, as wines are rich sources of polyphenolic antioxidant substances such as flavonoids, flavonols, anthocyanins, leuco-anthocyanins, catechins, and resveratrol (*German & Walzem*, 2000; *Villano et al.*, 2006). Additionally, wines possess various cardioprotective and neuroprotective medicinal properties such as: promoting smooth blood circulation; providing antioxidants to prevent the degradation of cell walls in arteries and the brain; contributing to the breakdown of blood platelets and the balance of fibrinolysis, which is essential for coagulation; helping in the treatment of cancer and Alzheimer's disease (*Arranz et al.*, 2012). Fermentation not only depends on the type of substrate material, but also there are influences exerted by the starter's microflora. The fermentation of wine naturally occurs through the introduction of inoculated starters or yeasts, which consists of beneficial strains. The quality of commercial wine yeast is determined by its oenological properties and parameters such as storage, stability, osmotolerance, freeze-thaw resistance, and drying/rehydration resistance. *Saccharomyces cerevisiae* is mostly the principle agent of wine fermentation to convert hexose sugars to ethanol, carbon dioxide, and a variety of compounds, including sugar alcohols, esters, aldehydes and acids, which contribute to the sensory attributes of the beverage (*Viljoen*, 2006). The technological process involves a wide range of yeast species making different contributions to wine quality. *Saccharomyces cerevisiae* and *Saccharomyces bayanus* are "wine yeasts", the most desired agents of wine fermentation, which have the highest oenological potential and are commonly used in wine making (*Braschi et al.*, 2019). There is a universal agreement that *S. cerevisiae* predominates in fermenting broth after a few days of spontaneous fermentation (*Braschi et al.*, 2019; *Majumder et al.*, 2022a).

Generally, fermented alcoholic beverages from different raw materials are called "wine" added with the name of that material. Therefore, the one prepared from tea can be termed as tea wine. Tea is globally the second most popular and consumed low-cost beverage, second only to water, and is taken for rejuvenation, relaxation, and health benefits. The average global consumption of tea, which is around 100 ml/head/day, is much ahead of coffee, beer, wines, and carbonated soft drinks (*Trevisanato & Kim*, 2000). There are many types of tea produced in the tea-growing regions of the world such as black tea, green tea, white tea, oolong tea, Pu-erh tea, etc. Among them, black tea is a good fermentation medium because the infusion contains proteins, amino acids, volatile compounds, lipids, enzymes, and polyphenols in a good amount, which also gives it a unique flavour (*Kumar & Joshi*, 2016; *Majumder et al.*, 2022a). The development of the ancient ethnic beverage kombucha and its emerging popularity evidenced that brewed

tea, being the most popular drink with medicinal properties, can be used as a potential substrate in fermentation technology. Kombucha is a fizzy, sour, flavoury, and less alcoholic fermented tea infusion brewed by a starter called SCOBY (the symbiotic colony of bacteria and yeast) (*Jayabalan et al.*, 2007; *Majumder et al.*, 2020). After ages of research and development on kombucha, the term "tea wine" was coined by oenologists, which refers to the wine made from tea (*Li et al.*, 2020). The fermentation of sugared broth of tea using wine yeast (*Saccharomyces cerevisiae*) or koji (*Aspergillus oryzae*, traditional rice wine starter) to produce a low alcoholic "tea wine" has been reported (*Li et al.*, 2020; *Majumder et al.*, 2022a). However, there is very little information available on the production and characterization of tea wine (*Majumder et al.*, 2022a) in the scholarly world, unlike the case of kombucha, which is a constantly top-trending topic in food science and food technology. Among the various innovations and technologies, microbial fermentation is an option that can be utilized to increase biological activities and alter the flavour profile of a typical cup of tea. Recent reports on tea wine, tea-flower wine, and production of bioactive formulations like "tea haria" by using the indigenous starter "bakhar" also justified the importance of this issue (*Majumder et al.*, 2021; *Majumder et al.*, 2022b). *Aroyeun et al.* (2005) established that their tea wine preparation has an excellent sensory profile with good flavour characteristics. The tea wine could be expected to not only serve as a mild stimulatory drink but also a health supplement with low alcohol content.

Mainly two types of tea are consumed, i.e. green tea and black tea (orthodox hand-rolled whole-leaf tea and crush-tear-curl, or CTC tea), in the world, which are mostly produced and exported by leading tea-growing nations such as China and India (*Majumder et al.*, 2022a). CTC, or "crush-tear-curl", is a type of manufactured black tea that is the most commonly manufactured and the most consumed type of tea worldwide (*Pou et al.*, 2019; *Majumder et al.*, 2022c). In the Indian domestic market, CTC tea is by far the most popular choice in tea shops and domestic households (over 80% of tea production is of this type), which is less expensive and mostly sold loose in markets of India (*Solanki*, 2022).

Our research group has already developed different fermented tea infusions from both black tea (CTC tea and orthodox black tea) and green tea, using brewer's yeast (*Saccharomyces cerevisiae*) and the traditional kombucha starter SCOBY (*Majumder et al.*, 2022a) and carried out a comparative *in vitro* biochemical characterization. Moreover, research results suggested CTC black tea as the most potential substrate for showing the highest fermentation-led increased antioxidant property, unlike orthodox black tea and green tea that already contain notable levels of bioactives in their raw/crude states (*Majumder et al.*, 2022a). Interestingly, fermented CTC tea samples exhibited comparatively higher alterations in physicochemical properties due to fermentation than other tea samples (*Majumder et al.*, 2022a). Therefore, unlike expensive orthodox or hand-rolled whole-leaf black tea and green tea, cheaper or less costly CTC tea was validated as the ideal solution to be utilized

in fermentation technology where fermentation-driven quality enhancement was found to be the highest. After preliminary *in vitro* experiments, it was crucial to investigate the metabolite profile before further *in vivo* tests and consumption. Therefore, in this follow-up research work, GC-MS analysis was utilized to explore the volatile profile of CTC-tea wine (TW). The objectives of this study were to conduct the metabolite profiling of TW through GC-MS analysis, correlate the results with known biochemical characteristics and bioactivities of TW, and elucidate the biosynthesis pathways of the identified metabolites.

2. Materials and methods

Collection of CTC-tea wine (TW) and sample preparation for GC-MS analysis

Fermented TW sample (*Figure 1*) was collected from the fresh batch (fifteen days of fermentation period), which was used for physicochemical and biochemical tests and *in vitro* bioactivity analysis (as reported in: *Majumder et al.*, 2022a). The brewing process has been reported as follows: ten grams of CTC tea (or 1% w/v) were added in one litre of freshly boiled double-distilled sterile water (at 98 ± 1 °C) and left for fifteen minutes to prepare the tea infusion (generally 2–5 minutes are preferred; here, brewing time was extended for the proper extraction of biomolecules, including caffeine into the broth or substrate). The infusion was then filtered through sterile muslin cloth and poured into a fermentation jar followed by adding 100 g of sucrose (or 10% w/v) as nutrient or carbon source for fermenting microbes. The jar was then autoclaved properly for sterilization. After cooling at room temperature, 2 g of dried brewer's yeast – *Saccharomyces cerevisiae* – was added as starter in the jar. Sterile acetic acid, or synthetic white vinegar, was added (to maintain the pH at 5) in the jar to increase the acidity of the fermentation broth (considered as an ideal fermentation condition). The mouth of the jar was covered with polythene sheet having pores to facilitate the gas (CO_2) release. Muslin cloth, glass goods, polythene cover, etc. were autoclaved and/or sterilized with 70% ethanol before using. The jar was then incubated in dark conditions at a well-ventilated and airy room for fifteen days. A control wine batch (CW) was prepared by inoculating 2 g of dried brewer's yeast and other ingredients in broths where tea infusion was replaced by sterile double-distilled water.

This is a follow-up metabolomic research on CTC-tea wine. In this research, the best performing (having the highest bioactive potential) and most healthy (the most uncontaminated) replicas of TW and CW samples were chosen for GC-MS analysis. One millilitre of both TW (*Figure 1: B*) and CW (*Figure 1: C*) were taken

in separate test tubes, air dried completely, and dissolved in 1 ml of methanol (chromatography grade from SRL, India) to prepare methanolic extracts prior to the GC-MS analysis.

Figure 1. A – fresh batch set-up for fermentation (CTC/Y = CTC-tea wine batch and C/Y = control wine batch), B – fermented CTC-tea wine (TW) sample, C – fermented control wine sample

GC-MS analysis

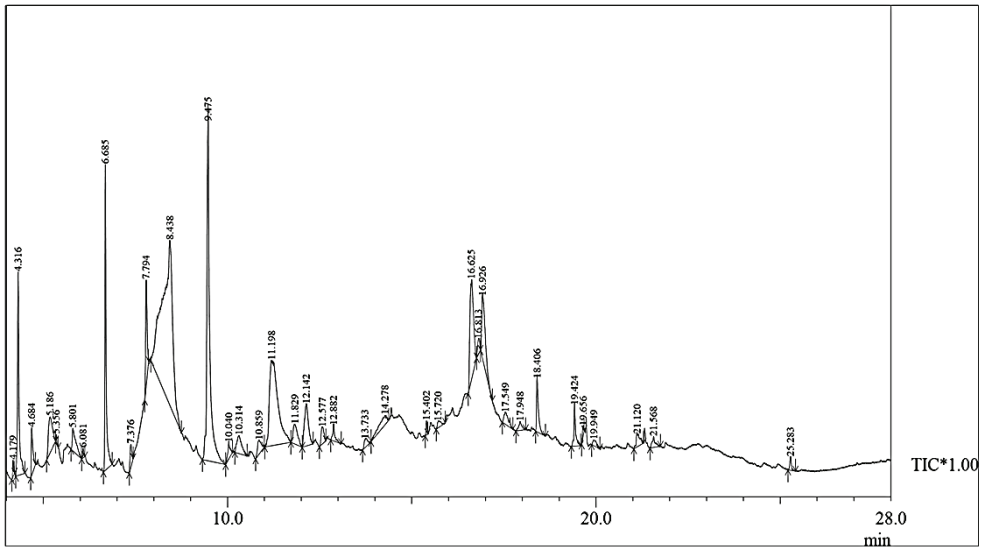
Samples (TW and CW) were subjected to GC-MS analysis following the pre-standardized research protocol for tea petal wine developed by *Majumder et al.* (2021). GCMS-QP2010 Plus (Shimadzu Co., Japan) with a DB-5 fused-silica capillary column (30 m × 0.25 mm × 0.25 µm) was used in this research. The split injection (with a ratio of 20:1) technique was adopted, where the injection volume was 1 µL. Column oven temperature: 80 °C, carrier gas: helium (99.9995% purity), injection temperature: 260 °C, total flow rate: 16.3 mL/min, and column flow rate: 1.21 mL/min were considered. Flow control mode was at a linear velocity of 40.5 cm/sec. The interface temperature and ion source temperature were set to 270 °C and 230 °C respectively. Total ion chromatogram (TIC) was based on the intensity of fragments produced by the ionization. A single-quadrupole mass spectrometer was used in this research. Mass spectra were recorded at 5 scan/sec with a scanning rate of 40-600 m/z. For compound identification, the probability-based matching method was used, and the obtained spectra were compared to the Wiley and the NIST databases. Data acquisition and control of the chromatograph were carried out using the GCMS solution software. According to the guidelines by Shimadzu Co., the peak area corresponds to the amount of compound present in a sample (https://www.shimadzu.eu.com), and previously researchers (*Acharyya et al.*, 2021; *Chakraborty et al.*, 2023) also considered the peak area for the quantification of detected compounds (relative area %). Based

on the results of the GC-MS analysis (peak report), the lists of metabolites were prepared. A further study was based on the available literature and databases. The results have been expressed in the following order: a. metabolite profile (with peak reports and chemical classification); b. role of metabolites in physicochemical and biochemical properties; c. medicinal properties of the metabolites; d. flavour-imparting molecules; e. metabolic pathways to study the metabolites' biosynthesis.

3. Results and discussions

Metabolite profile

The GC chromatograms of the sample TW and CW are presented in *Figure 2,* and the list of metabolites based on peak reports are included in *Table 1*. A total of thirty-five compounds have been detected in the TW sample, including control metabolite glycerol as a major component (glycerine- 26.6%; and glycerol, 1-acetate- 11.61%). Sugar alcohol glycerine derivatives occupied almost the whole chromatogram (95.72%) of the control or sample CW, which include triol-glycerine (86.43%), secondary alcohol- 2,3-butanediol (7.52%), and glycerol derivatives (cis-5-hydroxy-2-methyl-1,3-dioxane and 4-hydroxymethyl-2-methyl-1,3-dioxolane). The domination of compound glycerine was clearly reflected in the CW's chromatogram (*Figures 2B* and *3*), while the majority of the peaks of different molecules is visible in the sample TW (*Figures 2A* and *B*).



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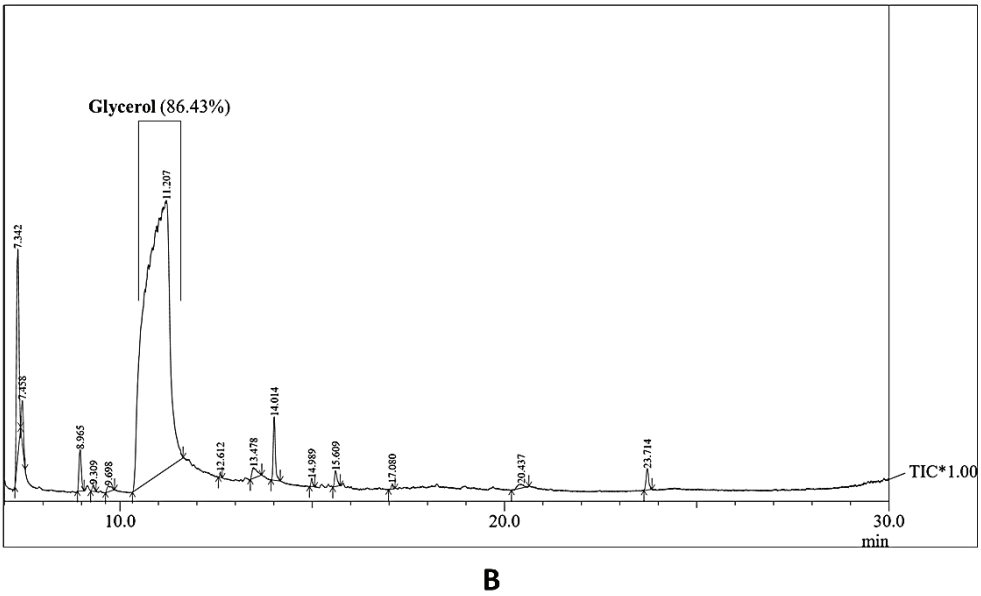


Figure 2. A – GC chromatogram of sample TW, B – GC chromatogram of sample CW (showing a large peak of glycerine)

Table 1. GC-MS peak reports derived list of metabolites for the sample CTC-tea wine (TW) and control wine (CW) and their peak area (%)

Name of the compound	Type of compound	TW	CW
3-Methyl-2(5H)-furanone	Furanone derivative	0.24	
Furfural	Furans	4.99	
Furfuryl alcohol	Furans	1.26	
Butanoic acid, 2-ethyl-, methyl ester	Carboxylic acid	2.31	
Pyruvaldehyde dimethyl acetal	Sugar aldehyde	0.12	
1,3-Cyclopentanedione	Cycloalkane	1.33	
(+-)-4-Amino-4,5-dihydro-2(3H)-furanone	Furanone derivative	0.18	
2,4-Dihydroxy-2,5-dimethyl-3(2H)-furan-3-one	Furanone derivative	6.23	
Succinic acid derivative	Carboxylic acid	0.65	0.11
Levulinic acid	Carboxylic acid	2.49	
Glycerine	Sugar alcohol (triol)	26.6	86.43
4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-	Pyrone (flavonoid fraction)	15.58	

Name of the compound	Type of compound	TW	CW
Cyclobutanecarboxylic acid, octadecyl ester	Carboxylic acid	0.61	
Isosorbide Dinitrate	Sugar alcohol (sorbitol) derivative	2.11	
Pyruvic acid ethyl ester	Carboxylic (keto) acid (etserified)	0.82	
Glycerol, 1-acetate	Acetic acid ester of sugar alcohol (triol)	11.61	
Acetic acid, hexyl ester	Carboxylic acid, fatty acid ester	1.41	
Octyl octanoate	Fatty acid derivative	2.68	
Tridecyl acrylate	Fatty acid derivative	0.53	
Tyrosol	Phenol	0.46	
1-Oxa-4-azaspiro[4.5]dec-4-yl-6,6,10,10-D4-oxy, 3,3-dimethyl		1.25	
1-Hexadecanol	Fatty alcohol	0.2	
Myrtanol	Monoterpenoid	0.35	
Pyrimidine-2,4(1H,3H)-dione, 6-hydroxy-5-methyliminomethyl-	Pyrimidine	5.7	
Octanoic acid, 4-pentadecyl ester	Fatty acid	0.48	
1-Isobutyl-7,7-dimethyl-1,3,4,5,6,7a-hexahydroisobenzofuran-3a-ol	Furans	3.57	
3-Butyl-4-nitro-pent-4-enoic acid, methyl ester	Carboxylic acid	0.86	
Oxalic acid, allyl octadecyl ester	Carboxylic acid (fatty acid) derivative	0.43	
Caffeine	Purine alkaloid	1.63	
Palmitic acid	Fatty acid	1.16	
Furfuryl heptanoate	Furan (fatty acid ester)	0.24	
trans-Ascaridol glycol	Monoterpenoid	0.32	
(3Z,9Z)-cis-6,7-epoxy-3,9-nonadecadiene	Fatty acid derivative	0.75	
Palmitic acid, ethyl ester	Fatty acid	0.51	
Phthalic acid derivative	Phthalate	0.33	0.71
2,3-Butanediol	Secondary sugar alcohol		7.52
cis-5-Hydroxy-2-methyl-1,3-dioxane	Glycerin derivative		1.27

Name of the compound	Type of compound	TW	CW
4-Hydroxymethyl-2-methyl-1,3-dioxolane	Glycerin derivative		0.5
Eucalyptol	Monoterpenoid		0.08
5-Acetyldihydrofuran-2(3H)-one	Furanone derivatives		0.66
Phenylethyl Alcohol	Primary alcohol		1.73
L-Camphor	Monoterpenoid		0.18
4-(1-hydroxyethyl)-gamma-butanolactone	Furanone derivatives		0.51
Phenylethylene glycol	Glycol		0.3

Role of metabolites in CTC-tea wine's physicochemical and biochemical properties

Previously, *Majumder et al*. (2022a) analysed the physicochemical and biochemical properties of TW in comparison with control wine, where they highlighted the differences between the tea wine and the control wine sample and demonstrated the impact of the various groups of phytochemicals present in tea as substrate metabolites. The pH (one of the most important physicochemical properties of wine) was found to be very low in the TW compared to the control wine. Generally, a number of organic acids are produced during the fermentation of carbohydrates as common yeast metabolites following the glycolysis and further Kreb's cycle (as shown below in *Figure 4*), which increase the acidity of the fermented broth. Here, the GC-MS analysis revealed many such organic acid derivatives. The total peak area of such acid products may account for the amount or ratio of total organic acid content present in the tested wine samples. A total of 21.19% peak area in TW's chromatogram comprised various organic acid (carboxylates) derivatives (acetic acid, butanoic acid, succinic acid, levulinic acid, pyruvic acid, oxalic acid, etc.), while CW contained only 0.11%. TW also contained fatty acid products (5.36% total peak area) unlike CW, which might also influence the pH. Furthermore, qualitative and quantitative biochemical tests were carried out by *Majumder et al.* (2022a). Reportedly, total phenol content, terpenoids, and alkaloids were found to be comparatively high in TW, while the glycerol content was found to be higher in control wine compared to TW, aligning with this GC-MS result, as shown in *Table 1*. TW contains various bioactive metabolites belonging to phenol, terpenoids, and alkaloids, in contrast to CW, where approximately 96% of the total peak area was attributed to glycerol and its derivatives. Thus, the reported physicochemical and biochemical properties have been validated through this metabolite profiling. *Figure 3* exhibits a 3-D area chart showing the percentage shares of different groups

of metabolites based on chemotaxonomy and biosynthesis pathways. The graphs in *Figure 3* are symmetrical to the chromatograms of TW and CW, where the peak of sugar alcohol products in CW corresponds to glycerine present in it, unlike the TW rich in various phytochemical components.

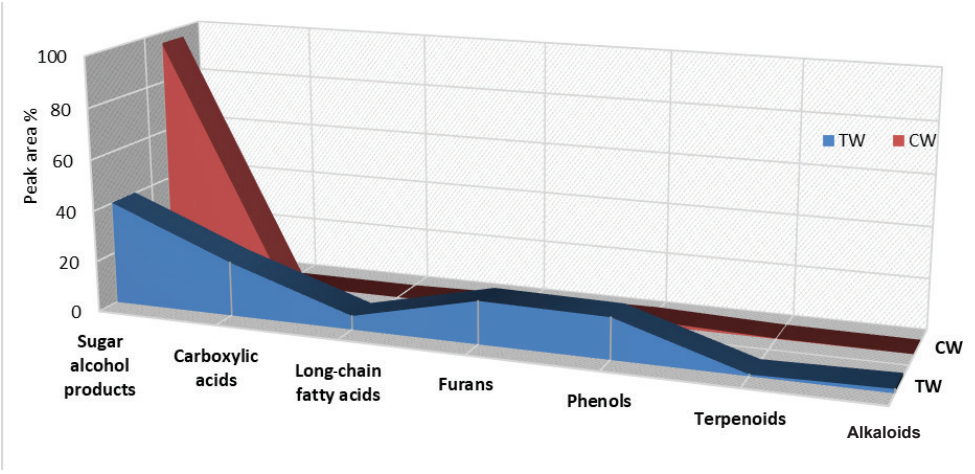


Figure 3. Peak area percentage shares by different classes of metabolites in the chromatograms

Medicinal properties of CTC-tea wine metabolites

The medicinal properties of exclusive TW metabolites have been described here under different sub-subheadings prepared aiming at different bioactivities, i.e. antioxidant, hepatoprotective and antidiabetic, antimicrobial and other medicinal properties, including anti-inflammatory, cardioprotective, anticancer, neuroprotective properties, etc. The reported medicinal properties of bioactive TW's metabolites are listed in *Table 2*.

Table 2. Bioactive compound of CTC-tea wine (TW) and reported medicinal properties

Name of the compound	Reported medicinal properties*
4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-	Antioxidant, antimicrobial, anti-inflammatory, anti-diabetic, anti-proliferative, and hepatoprotective
Furfural	Antioxidant, hepatoprotective, antimicrobial

Name of the compound	Reported medicinal properties*
Palmitic acid derivatives	Antioxidant, anti-inflammatory, antimicrobial, cardioprotective (hypocholesterolaemia), anticancer properties
Levulinic acid	Antioxidant
Tyrosol	Antioxidant, hepatoprotective (anti-lipidperoxidation), antidiabetic, antimicrobial, anti-inflammatory
Furfuryl alcohol	Antioxidant, antimicrobial
1-hexadecanol	Antimicrobial (anti-staphylococcal)
Methylglyoxal or pyruvaldehyde	Antimicrobial, anticancer
Isosorbide dinitrate (sorbitol derivative)	Preventative against chest pain (angina), vasodilator, cardioprotective (treats achalasia, chronic painful diabetic neuropathy, ischemic cardiovascular diseases, congestive heart failure, oesophageal spasms, etc.)
Caffeine	Central nervous system stimulant, cardioprotective (induces diuresis), promotes secretion of gastric acid, alleviates migraine

Note: References are given in the text below.

Antioxidant compounds

Enhancement of food antioxidant properties during fermentation corresponds to the changes in physicochemical and biochemical attributes, which are interrelated with the metabolic actions of the employed starter – here: wine yeast *Saccharomyces cerevisiae*. Exclusive TW major compounds 4H-pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl- (15.58%) and furfural (4.99%) are reported as potential antioxidant molecules having several other biological activities (*Majumder et al.*, 2021). Antioxidant fatty acid- palmitic acid and its derivatives were detected in TW, which were previously reported as antioxidative fatty acids of red wine by *Yunoki et al.* (2004). *Yi* and *Kim* (1982) reported on the role of levulinic acid and furfural behind the antioxidant activity of wine. *Covas et al.* (2003) reported on the antioxidant activity of detected phenolic tyrosol. *Osada* and *Shibamoto* (2006) determined the antioxidant properties of furfuryl alcohol. Previously, TW was reported to exhibit significantly high antioxidant activity (*Majumder et al.*, 2022a) and, likewise, in this GC-MS analysis the sample was found to contain such antioxidative wine volatiles as 4H-pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-, furfural, furfuryl alcohol, levulinic acid, palmitic acid, and tyrosol.

Hepatoprotective and antidiabetic compounds

A high lipid peroxidation inhibition property was reported for the sample TW (*Majumder et al.*, 2022a). The presence of the hepatoprotective compound tyrosol is the possible reason behind this result. *Kalaiselvan et al.* (2016) reported the attenuation of hepatic oxidative stress by hydroxytyrosol and tyrosol, which corresponds to the anti-lipidperoxidation activity of TW. *Chandramohan et al.* (2015) reported the antidiabetic activity of tyrosol, which is in line with the *in vitro* antidiabetic activity reported for TW (*Majumder et al.*, 2022a). Furfural has the potential to inhibit alcohol dehydrogenase, aldehyde dehydrogenase, and pyruvate dehydrogenase (*Modig et al.*, 2002), which could be useful in preventing poisoning from alcohols that metabolize into toxic products and also in preventing alcohol-induced inflammatory responses and in playing a role as a hepatoprotective agent.

Antimicrobial compounds

Antioxidant 4H-pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl- is a yeast secondary metabolite that possesses antifungal properties (*Majumder et al.*, 2021). *Togashi et al.* (2007) demonstrated the potential antibacterial activity of a TW metabolite, 1-hexadecanol, against *Staphylococcus aureus*. This also corresponds with our previous finding, where crude TW sample produced inhibition zone against *Staphylococcus aureus*. Potential antibacterial properties of detected furan derivatives – furfural and furfuryl alcohol – were also reported (*Chai et al.*, 2013; *Kalt* & *Cock*, 2014). Furfural has been reported as mutagenic against *Salmonella typhimurium* (*Zdzienicka et al.*, 1978). Methylglyoxal or pyruvaldehyde (detected as pyruvaldehyde dimethyl acetal) also exhibits antimicrobial activity (*Atrott* & *Henle*, 2009). Therefore, the presence of the antimicrobial compounds 4H-pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-, furfural, furfuryl alcohol, tyrosol, pyruvaldehyde, and anti-staphylococcal 1-hexadecanol might be responsible for the reported antibacterial properties of TW against Gram-positive *Staphylococcus aureus* and *Bacillus subtilis*, as well as Gram-negative *Klebsiella pneumoniae* (*Majumder et al.*, 2022a).

Metabolites with other bioactivities

Overall, the detection of 4H-pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl- as major compound was an important finding of this research because this molecule can possess a wide range of bioactivities, including antimicrobial, anti-inflammatory, antidiabetic, antioxidant, anti-proliferative, and hepatoprotective properties (*Teoh & Don*, 2015; *Hameed et al.*, 2015; *Chakraborty et al.*, 2023), which could help the novel TW to show promising medicinal properties. The major fatty acid palmitic

acid and its derivatives also have anti-inflammatory, antioxidant, antimicrobial, cardioprotective (hypocholesterolaemia), and anticancer properties (*Majumder et al.*, 2021). Isosorbide dinitrate, a probable sugar-fermented product (sorbitol derivative), exhibits a range of medicinal properties to be celebrated as a life-saving drug. It plays a role as preventative against chest pain (angina), and it also functions as potential vasodilator. Further, it prevents anti-cardiovascular diseases, treats achalasia, chronic painful diabetic neuropathy, ischemic cardiovascular diseases, congestive heart failure, oesophageal spasms, etc. (https://go.drugbank.com/drugs/DB00883). The phenolic antioxidant metabolite tyrosol exhibits anti-inflammatory properties and exerts its beneficial effects against hypertension, atherosclerosis, coronary heart disease, chronic heart failure, insulin resistance, and obesity as well (*Karković Marković et al.*, 2019). Antimicrobial methylglyoxal or pyruvaldehyde has also been reported as an anticancer compound by *Talukdar et al.* (2009). The bioactivity of the signature tea-leaf compound caffeine as a central nervous system stimulant is well established and its presence in CTC-tea wine is one of the useful findings of this research. Caffeine induces diuresis, which shows positive effects on the cardiovascular system, and it also promotes the secretion of gastric acid and alleviates migraine as well (*Yang et al.*, 2010).

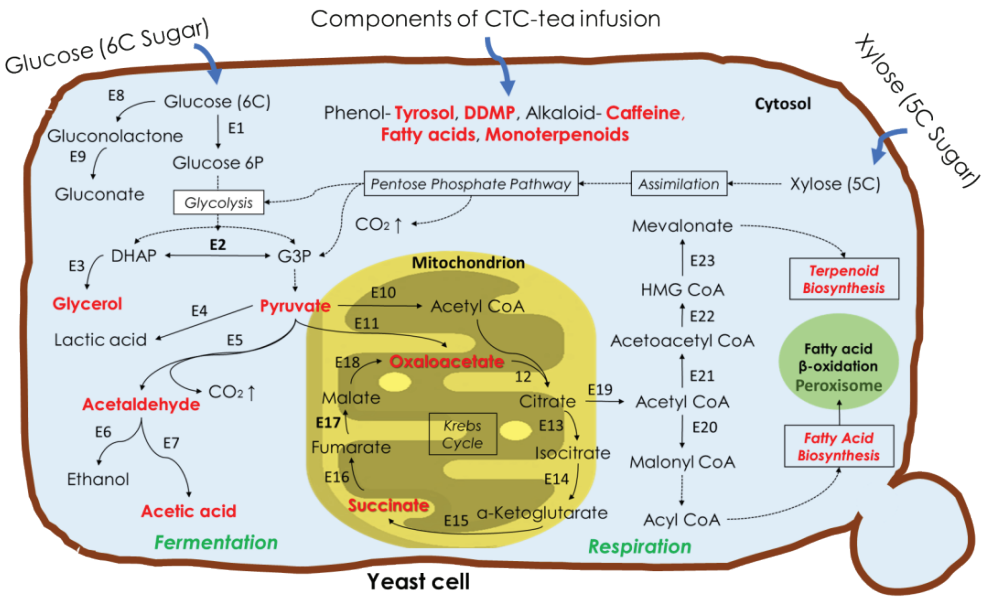
Flavour-imparting molecules of CTC-tea wine

According to The Good Scents Company database (http://www.thegoodscents company.com/), compounds like glycerine derivatives, acetic acid, pyruvaldehyde, furfural, furfuryl alcohol, levulinic acid, succinic acid, palmitic acid, etc. probably have a synergistic contribution towards the development of a sweet, caramel-like, astringent, slightly sour, and wine-like flavour as well as taste of the CTC-tea wine sample. The major compound 2,4-dihydroxy-2,5-dimethyl-3(2H)-furanone (6.23%) has been documented as an aroma-imparting component (potential fruity and wine-like aroma in wines) (*Chukwu et al.*, 2017). Butanoic acid, 2-ethyl-, methyl ester (2.31%) is also reported to exhibit wine-aroma-imparting properties (*Wei et al.*, 2019). Moreover, butanoic acid and furfural are reported as volatile aroma compounds of some famous Chinese liquors, which are probably considered as aging markers of some wines (*Xu et al.*, 2017). Butanoic acid and succinic acid are yeast metabolites and are reported as fermentation products (*Wilson et al.*, 2021) responsible for a distinct cidery flavour. So, TW is rich in both sugar alcohols and organic acids, and together these would produce a sweet and sour taste. (+-)-4-Amino-4,5-dihydro-2(3H)-furanone is a gamma-butyrolactone (major red wine aroma compound) derivative, a yeast metabolite that has been recognized for the unique flavour of wine and medicinal properties (*Vose et al.*, 2001; *Majumder et al.*, 2022b).

Biosynthesis pathways of bioactive compounds

Fermentation metabolomics depends on both substrate (here sugared CTC-tea infusion) and starter (here brewer's yeast or *Saccharomyces cerevisiae*). *Saccharomyces cerevisiae* is the principle agent for commercial wine fermentation that converts hexose and pentose sugars to fermented products like ethanol, glycerol, carbon dioxide, and a variety of compounds: alcohols, esters, aldehydes, and acids, which contribute to the sensory attributes of the beverage (*Viljoen*, 2006). The fundamental yeast metabolic pathway has been given in *Figure 4*, which is self-explanatory showing the enzymatic steps responsible for the glycolysis of six-carbon sugars, the assimilation and pentose phosphate pathway for five-carbon sugars, the production of ethanol, glycerol, and acetic acid (major fermented beverage composition), the Krebs cycle, fatty acid biosynthesis, and beta-oxidation, terpenoid biosynthesis, etc. In this regard, the Yeast Metabolome Database (http://www.ymdb.ca/) and the KEGG pathway database (https://www.genome.jp/kegg/pathway.html) were accessed, and numerous research papers on *Saccharomyces* yeasts were reviewed to design these possible pathways involved in the biosynthesis of TW metabolites (*Figure 4*).

During yeast fermentation, hexose (i.e. glucose) and pentose (i.e., xylose) sugars present in the substrate or broth entered the glycolysis pathway and were further converted into dihydroxyacetone-phosphate (DHAP) and glyceraldehyde-3-phosphate. Enzyme glycerol-3-phosphate dehydrogenase produced glycerol (the major control wine metabolite detected in this research) from DHAP, while glyceraldehyde-3-phosphate was converted into pyruvic acid, which was the precursor of ethanol, acetaldehyde, and organic acids (lactic acid, acetic acid, succinic acid, etc.) (*Figure 4*). Pyruvate decarboxylase converted pyruvic acid into acetaldehyde and carbon dioxide. Acetaldehyde was further converted into ethanol and acetic acid, the major fermented beverage metabolites (*Figure 4*). On the other hand, mitochondrial pyruvate dehydrogenase complex and pyruvate carboxylase biosynthesised other organic acids and acetyl CoA, which was an intermediate to terpenoids' and fatty acids' biosynthesis and further to the beta oxidation of fatty acids in peroxisome following the respiration process (*Figure 4*). Moreover, phenolic-tyrosol (*Liu et al.*, 2023), 4H-pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl- (*Ma et al.*, 2023), purine alkaloid caffeine (*Majumder et al.*, 2020), detected fatty acids and terpenoids (*Wei et al.,* 2023) could be possibly introduced into tea wine product as tea infusion components, being reported as secondary metabolites of tea.



Notes: Metabolites written in red were involved in TW's metabolome.
(DHAP: dihydroxyacetone phosphate; G3P: glyceraldehyde-3-phosphate; DDMP: 4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-)
[E1: Hexokinase; E2: Triose-phosphate isomerase; E3: Glycerol-3-phosphate dehydrogenase; E4: Lactate dehydrogenase; E5: Pyruvate decarboxylase; E6: Alcohol dehydrogenase; E7: Acetaldehyde dehydrogenase; E8: Glucose oxidase; E9: Lactonase; E10: Pyruvate dehydrogenase complex; E11: Pyruvate carboxylase; E12: Citrate synthase; E13: Aconitase; E14: Isocitrate dehydrogenase; E15: Alpha-Ketoglutarate dehydrogenase/ Succinyl-CoA synthetase; E16: Succinate dehydrogenase; E17: Fumarase; E18: Malate dehydrogenase; E19: ATP citrate (pro-S)-lyase / ATP citrate synthase; E20: Acetyl-CoA carboxylase; E21: Acetyl-CoA C-acetyltransferase; E22: Hydroxymethylglutaryl-CoA (HMG CoA) synthase; E23: HMG-CoA reductase]

Figure 4. The proposed yeast metabolic pathway to understand TW's metabolomics

Glycerine was detected as the major compound in both CW (86.43%) and TW (26.6%). Glycerine is produced by fermenting yeast during beverage fermentation when ethanol and CO_2 (main by-products) formation stops. Yeast metabolism is divided into two major pathways: firstly where ethanol, acetic acid, and carbon dioxide are produced and secondly where glycerol is produced. In the case of fermented alcoholic beverages, both ethanol and glycerol are produced by fermentation of sugars (*Majumder et al.*, 2022d). Glycerol, 1-acetate (11.61%) was a glycerol derivative in TW sample, where the fermented product glycerol was esterified with another common sugar fermented product: acetic acid.

The plant cuticle is composed of cutin (a polymer of cross-esterified hydroxy-fatty acids) and a mixture of long-chain hydrocarbons (long-chain fatty acids,

fatty alcohols, alkanes, etc.), known collectively as waxes. Fatty acid derivatives, i.e. octyl octanoate, tridecyl acrylate; octanoic acid, 4-pentadecyl ester; palmitic acid derivatives; furfuryl heptanoate; the phenolic compound tyrosol, a strong antioxidant of tea (*Liu et al.,* 2023), monoterpenoids, i.e. myrtenol and trans-ascaridol glycol, etc. are common plant metabolites which might be present as tea/substrate metabolites in the fermented broth. However, yeast fermentation can also be the reason behind the production of fatty acids as designed on the pathway (*Figure 4*). Previously, caffeine and steroids were qualitatively determined in this particular TW sample (*Majumder et al.*, 2022a). Therefore, the detection of caffeine in its metabolite profile is valid.

Furfural and other furan derivatives detected in TW are reported as metabolites of fermenting microbes and common aroma components of fermented beverages as wine, brandy, whiskey, rum, etc. (*Majumder et al.*, 2021). Furfuryl alcohol occurs mainly due to the enzymatic or chemical reduction of furfural during the aging of a wine (*Majumder et al.*, 2021) and is a major volatile compound of beer (*Wei et al.*, 2001). Butanoic and succinic acids are yeast metabolites; therefore, their biosynthesis in tea wine is valid. Succinic acid is also the precursor of levulinic acid; these metabolites are reported as glucose/fructose fermentation products and are mainly found in aged wine and beer samples (*Majumder et al.*, 2021).

The major bioactive compounds of TW, i.e. 4H-pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl- or hydroxydihydromaltol (15.58%) and furfural derivatives, are strong antioxidants, Maillard reaction products of sugar (formed due to the thermal dehydration of sugars) found in fermented beverages, which might be derived from sugar fermentation or storage (*Majumder et al.*, 2021). Preparation of CTC-tea infusion (fermentation substrate) was done at high temperature (98 ± 1 °C) that might possibly induce Maillard reaction (*Kchaou et al.*, 2019). *Idowu et al.* (2017) and *Ma et al.* (2023) reported the occurrence of 4H-pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl- in different green teas and sun-dried Pu-erh tea. Previously, 4H-pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl- was reported as a sugar derivative, which is a possible derivative of a flavonoid biosynthesised during fermentation by yeast (*Majumder et al.*, 2021) because it is reported as a compound with flavonoid fraction and as a fungal secondary metabolite having antifungal properties (*Teoh & Don*, 2015). The production of furfural derivatives and levulinic acid has been reported from a rich carbohydrate source, i.e. lignocellulosic biomass (*Li et al.*, 2019). Therefore, furan derivatives (furfural, furfuryl alcohol, and furfuryl heptanoate) and levulinic acid may be derived from sugar during the fermentation process. *Velaga & Peela* (2022) showed the thermal decomposition of furfural and reported the green synthesis of levulinic acid. The high temperature of sugared black tea infusion (substrate) (98 ± 1 °C) could induce the production of levulinic acid. *Maharramov et al.* (2020) reported the production of furfurals in processed Azerbaijan Tea. *Parveen et al.* (2023) also identified furfurals as major compounds in black tea produced by

Camellia sinensis and *Camellia assamica*. *Maldonado et al.* (2012) demonstrated the acid-catalysed conversion of furfural alcohol to levulinic acid. Therefore, inside an acidic fermenting broth (TW) rich in organic acids, the detection of furfural and furfuryl alcohol alongside levulinic acid is also valid, and they belong to the same pathway. *Wang et al.* (2022) reported the dehydration of monosaccharides, such as fructose and glucose, to produce levulinic acid. Hence, infusion-induced non-enzymatic physicochemical reactions (like the Maillard reaction) and enzymatic biochemical changes due to fermentation or storage both steered the production of these components in TW. Further, working with tea-flower wine, *Majumder et al.* (2021) reported antioxidant agent levulinic acid and its precursor succinic acid (also detected in TW) as products of glucose fermentation, which are mainly found in aged wine and beer samples. Pyruvaldehyde dimethyl acetal is a sugar-fermented product either biosynthesised from pyruvic acid (also detected in TW) and acetaldehyde during alcoholic fermentation or produced as a result of the oxidative degradation of dihydroxyacetone (or glycerone, the precursor of glycerine) (*Lip et al.*, 2013) during glycerine production. Caffeine detected in TW occurred as tea metabolite, as CTC-tea infusion was the fermentation substrate. Glycerine; glycerol, 1-acetate; furfural, furfuryl alcohol, 2,4-dihydroxy-2,5-dimethyl-3(2H)-furan-3-one; organic acids (butanoic acid, succinic acid, levulinic acid, cyclobutanecarboxylic acid, pyruvic acid, acetic acid and oxalic acid); 4H-pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl- or hydroxydihydromaltol; 3-methyl-2(5H)-furanone, etc. belonging to sugar derivatives metabolome are directly associated with carbohydrate fermentation, while, through detection of compounds like phenolic- tyrosol; alkaloid- caffeine; flavonoid fraction 4H-pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-; fatty acid-palmitic acid; fatty acid derivatives, i.e. octyl octanoate, tridecyl acrylate; octanoic acid, 4-pentadecyl ester; palmitic acid derivatives; heptanoate; monoterpenoids-myrtenol and trans-ascaridol glycol, etc., the effect of CTC-tea in the fermentation has also been reflected. Altogether, the metabolites – whether biosynthesised or generated through non-enzymatic reactions – derived from sugar and tea fermentation or directly from the infusion have validated the bioactive potential and acceptability of this tea wine.

4. Conclusions

A major part of our regular diet, particularly green vegetables and beverages, such as tea and wine, serves as substantial sources of antioxidants. Given their richness in bioactive components, fusion products like tea wine hold the potential to align seamlessly with consumer acceptability. As the fields of wine research and tea science continue to evolve, the findings of this study can serve as a foundation for future investigations and innovations in the realm of product development

from tea. Researchers, policymakers, and industry professionals are encouraged to continue exploring innovative solutions based on tea, particularly focusing on CTC tea. Despite its relatively lower economic value compared to premium orthodox black tea and green tea, CTC tea exhibits substantial production potential and has a high demand in both the national and the global market. In the Indian scenario, some portion of manufactured tea (mostly CTC tea) remains unsold, and the brewing industry may utilize this unsold tea for its value-added form, i.e. tea wine. These untapped possibilities have the potential to significantly boost the growth of the tea industry. Moreover, the physicochemical, biochemical, and medicinal properties of TW have been successfully evaluated through this follow-up research work on GC-MS-based metabolomics. Alterations in the polyphenolic profile of black tea (i.e. theaflavins and thearubigins) should be analysed by using HPLC/LC-MS techniques. Further sophisticated instrumentations, scientific research trials, and value additions are needed to uphold the acceptability of this wine.

Authors' contribution

S. M. and M. B. designed the research. S. M., A. G., and S. C. carried out the experiment. S. M. analysed the data and wrote the draft manuscript. M. B. and S. M. revised the manuscript. M. B. supervised the research work.

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