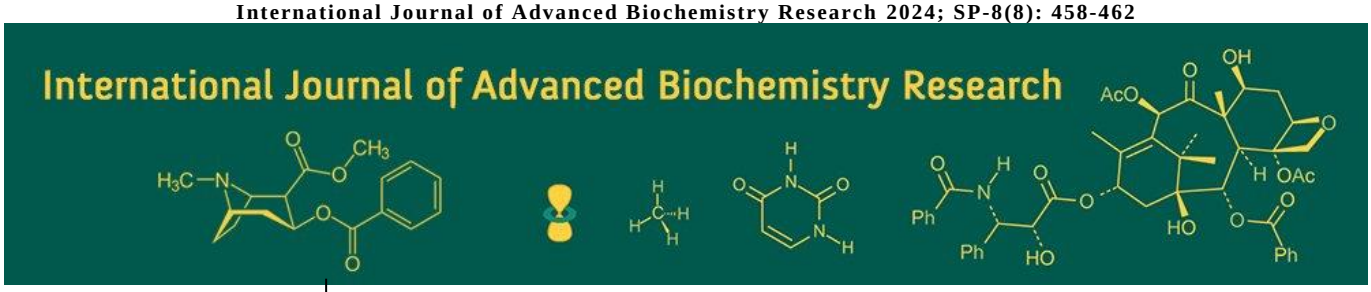

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GCMS elucidation of bioactive metabolites from fermented *Kombucha* tea

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Abstract
Kombucha or Manchurian tea is a popular fermented beverage produced by symbiotic growth of bacteria namely *Acetobacter xylinum* and some yeast strains such as *Saccharomyces cerevisiae* on sugared black tea extract. The *kombucha* cultures namely acetic acid producing *Acetobacter xylinum* (sju 1) isolated from sugarcane juice and yeast *Saccharomyces cerevisiae* was inoculated in 1:1 ratio in the black tea extract sweetened with white sugar and subjected to static fermentation for seven days. The flavoured broth produced during fermentation of substrates by *kombucha* cultures. The present study was carried out to study bioactive metabolites in chloroform and methanol (2:1) extract of *kombucha* tea and the compounds were identified using GCMS. About 18 different metabolites were eluted and several compounds possessing antibacterial properties were identified as dodecane, heptadecane, octadecane, hexadecane, octacosane, heneicosane, tricosane and nonane. Organic acid namely acetic acid and Thiophene-2-carboxylic acid were produced showing the least relative abundance in GCMS. The typical flavor of the extract was found to be produced by Neopentyl-2-oxobutanoate, Benzaldehyde dioctyl acetal and Santolin diacetylene. The water immiscible cellulosic mat was further purified and this can be used in food salads and other fruit based preparations. The various important metabolites identified serve as wholesome proof for the antibacterial propoerties of *kombucha* tea.

Keywords: *Kombucha*, fermentation, *Acetobacter xylinum*, yeasts, metabolites, GCMS

Introduction
Kombucha is a symbiotic growth of bacteria (such as *Acetobacter xylinum, A. xylinoides, Bacterium gluconicum*) and various yeast strains (including *Schizosaccharomyces pombe, Saccharomycodes ludwigii, Saccharomyces cerevisiae*) cultivated in sugared tea. The acetic acid bacteria and the yeast are in symbiotic association in which the yeasts by their invertases decompose sucrose and ferment its hexose units to ethanol and Carbondioxide. The Acetobacter genus oxidize ethanol through acetaldehyde to acetic acid. From glucose the Acetobacter synthesize gluconic acid and cellulose which occurs as a membrane on the surface of fermented liquid (Kersters, 2006) [9]. It is a popular beverage among many traditional fermented foods across the world. It was originated in North-East China (Manchuria) and later spread to Russia and rest of the world. It has sour, sweet and lightly carbonated taste. *Kombucha* tea or its extract show therapeutic properties, such as the ability to prevent cancers, decrease blood cholesterol level, reduce nephrotoxicity of pharmaceuticals, toxic metals and provide protection against the harmful effect of radiation. Several medical studies has revealed that *kombucha* has certain therapeutic value possessing antibiotic activity, positive effects on gastrointestinal activity, arthritis, gout, haemorrhoids, cholesterol value, arteriosclerosis and nervous system. Daily consumption of *kombucha* significantly reduces the risk of cancer. The synthesis of organic acids during fermentation protects the symbiotic colony from invasion by undesirable external microorganisms that are not associated with the tea fungus. In addition to tea and sugar components the beverage also contains acetic acid, gluconic acids, L-lactic acids, amino acids, biogenic amines, vitamins such as B-complex and vitamin C. One of the most important metabolite from therapeutical point of view is glucuronic acid, a carrier of detoxification activity of *kombucha*. Recent research on *kombucha* has proved that its antimicrobial activity against pathogenic microorganisms is largely attributable to acetic acid. Acetic acid is known to inhibit and destroy a number of Gram-positive and Gram negative microorganisms (Pinto *et al.*, 2008) [23].

Dutta and Gachhui, 2007 [2] has indicated that *kombucha* is frequently called "tea fungus", although no fungus is actually involved in the fermentation process. Fermentation using *kombucha* colonies is composed of two portions: a floating cellulosic pellicle layer and sour liquid broth below the cellulosic layer formed by *A. xylinum* and yeasts. This fungus-like mixture of microorganisms and cellulose is likely the reason why *kombucha* is also called "tea fungus". Klemm *et al.,* 2005 [10] has indicated that the cellulosic mat is an organic high dietary fiber rich food product, low in fat and calories and contains no cholesterol and further the cellulose is recognized by the FDA as edible and the *Acetobacter* is a non-pathogenic cellulose-producing food grade bacterium.

Legaz *et al*., [13] has developed a non-edible biomaterial called Bioskin using *Acetobacter xylinum*. Cellulose formed from the culture medium was mechanically disrupted, filtered through a multi-layered cheese-cloth, pressed and dried. The final dry product was not dissolved in water or organic solvents, but when rewetted in distilled water absorbed about 250 per cent of its dry weight. Ultra structural studies by TEM reveal that Bioskin was an anastomous structure composed by fibers surrounding concrete bodies and was able to fix osmium tetroxide. The bioskin was lipidic or lipoproteic nature. SEM analysis shows that Bioskin is apparently formed by crossed fibers with very large interfibrillar spaces. Fibers showed a very regular structure without superficial adherences. Interfibrillar spaces were the basis of the water-absorbing property of bioskin. Some low molecular weight compounds, such as sucrose, dissolved in water, also enter the void spaces and they crystallize on the surface of fibers to which they remain adhered. Considering all the above, a research was carried to develop fermented organic extract from *kombucha* and the structural bioactive compounds was elucidated by GCMS.

Materials and Methods

Production of *kombucha* cultures

The *Acetobacter xylinum* strain (sju-1) used in this study was previously isolated and identified in our laboratory from fermenting sugarcane juice (Coimbatore, Tamil Nadu, India). The yeast namely *Saccharomyces cerevisieae* was obtained from MTCC 6507.The culture was resuscitated by incubation on YPM (25 g/L mannitol, 5 g/L yeast extract, 3 g/L peptone, and 15 g/L agar) at 30 °C for 2 days. Working cultures were routinely prepared on YPM and stored at 4 °C until use. The basic growth medium used for the *Acetobacter* strain was Hestrin and Schramm (HS) medium (20 g/L glucose, 5 g/L peptone, 5 g/L yeast extract, 2.7 g/L Na_2HPO_4, 1.15 g/L citric acid.H_2O) (Hestrin *et al.,* 1954) [6]. A traditional tea-based medium (80 g/L sucrose and 3 g/L tea) was also used to culture the organism. Inoculum was prepared by transferring a single colony from the YPM working culture plates into 100 ml of HS medium in 500 ml bottles and incubating the culture without agitation at 30 °C for 2 days. The broth was shaken vigorously to release the attached cells from the cellulose pellicle and then aseptically filtered through sterilized gauze. The resulting cell suspension was used for all subsequent experiments. The experiments were carried out by adding 10 ml of inoculum to a 500 ml bottle that contained 90 ml of medium. The bottle was then shaken at 30 °C for seven days either statically at 60 oscillations per minute.

Preparation of crude extract of *kombucha* culture

The fermentation media containing the cellulosic pellicle and the broth was homogenized well in a blender homogenizer and the cellulose was allowed to sediment and settle down. The crude extract of *kombucha* was obtained as per the procedures of Ibrahim *et al*., 2011 [7]. The supernatant was mixed with equal quantities of chloroform and methanol (2:1) and shaken well vigorously in a separating funnel and incubated overnight (Parliament, 2020) [22].

Structural elucidation of bioactive compounds by GCMS

The Gas chromatography Mass Spectrometry (GCMS) analysis of the samples isolated from the chloroform and methanol (2:1) extract of fermented broth was performed using Thermo GC - trace ultra VER: 5.0,Thermo MS DSQ II equipped with DB 5 - MS capillary standard non-polar column of 30 Mts dimension, ID: 0.25 mm, film: 0.25 µm, for GC-MS detection, an electron ionization system with ionization energy of 70 eV was used. Helium was used as carrier gas with a flow rate of 1.0 ml/min and oven temperature 70 °C raised to 250 °C at 6 °C/min. The chromatographic peak identification was carried out by comparing their mass spectra with those of the bibliography data of unknown compounds from the NIST library mass spectra database on the basis of the criterion similarity (SI)>800 (the highest value is 1000). The approximate quantification of volatile compounds was estimated using the Wanakhachornkrai and Lertsiri, 2003 [28] method, which involved using the Xcalibur software (Vienna, VA) to integrate the peaks on the total ion chromatogram. The peak area normalized (%) is used to display the results.

Results

GCMS Analysis of fermented *kombucha* extract

The result of GCMS analysis of *kombucha* extract is presented in table 1 and Figure 1. The result revealed the presence of seven volatiles compounds dominated by thiophene-2-carboxylic acid, santolin diacetylene (24.8%), 1,5-dihydroxyl-3,3-dimethyl-7-acetylaminomethyl-tricyclododecane (10.75%), 1-(2-hydroxyethylthio)-1-phenyl-2-butanone (12.33%), Nonane, 4- hepatanol (6.53%) and 3-(4-nitrobenzoyl) cinnoline (5.8%).

Table 1: Bioactive metabolites in fermented *kombucha* tea elucidated using GCMS

S. No.	Retention time (min)	Peak Area (%)	Compounds Elucidated	Molecular Weight
1.	3.04	1.25	Acetic acid	76
2.	4.31	1.68	Buyladamantan-1-ol	208
3.	6.18	0.19	Illimaquinone-epoxide	374
4.	6.77	0.77	2-(5-chloromehoxyphenyl)pyrrole	207
5.	8.98	0.59	3-ethylaminosulpholane, 2-propenoic acid	163
6.	9.44	1.86	Dodecane, heptadecane, octadecane, hexadecane, octacosane, tricosane, nonane	170, 254, 254, 226, 394, 324, 128
7.	9.72	1.02	Heneicosane, 1-pentanol	296, 130
8.	11.29	6.68	2,2,6-trimethyl-5-hepten-3-one	154
9.	13.13	0.45	Benzaldehyde dioctyl acetal	348
10.	16.07	0.37	1-piperazine	267
11.	18.54	0.30	Crocusatin B	184
12.	22.56	24.80	Thiophene-2-carboxylic acid, santolin diacetylene	153, 204
13.	25.59	10.75	1,5-dihydroxyl-3,3-dimethyl-7acetylaminomethyl-tricyclododecane	295
14.	30.22	0.13	Lavanduyl acetate, 1-heptanol	196, 116
15.	31.17	12.33	1-(2-hydroxyethylthio)-1-phenyl-2-butanone	224
16.	34.85	6.53	Nonane, 4-hepatanol	244, 232
17.	37.48	5.80	3-(4-nitrobenzoyl) cinnoline	279
18.	41.71	0.78	Methylsilylpropyne	222

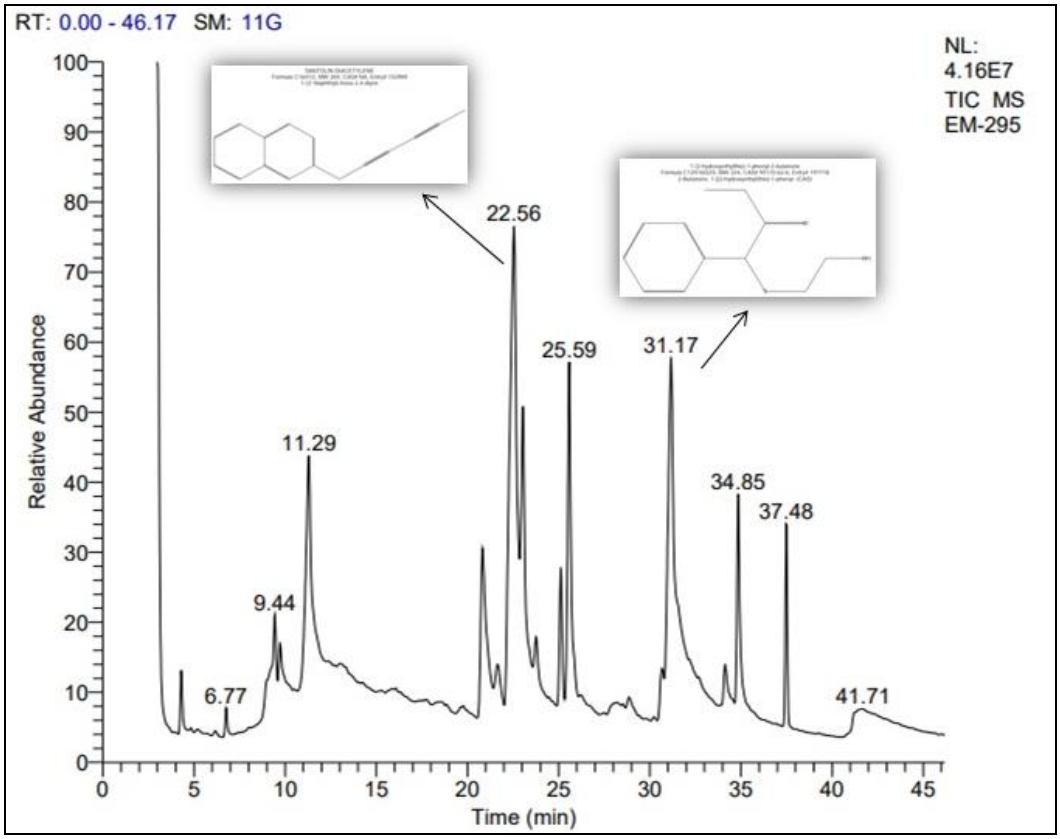

Fig 1: Relative abundance of bioactive metabolites elucidated by GCMS in *kombucha*

Discussion

The relative abundance of alkane hydrocarbon or fatty acid molecules like dodecane, heptadecane, octadecane, hexadecane, octacosane, heneicosane, tricosane and nonane were higher in the methanolic extract of *kombucha* tea. Haigh *et al.*, 1973 [5] has illustrated that induction of bifringence (orientation of microfibrils) were due to the liberation of 1:1 mixture of saturated and monounsaturated tetra-hydroxy terpene. Previous publications reported that the compounds such as 1-octadecene, 1-heptadeceane found in algae, bacteria and plants show anticancer, antioxidant and antimicrobial activity (Lee *et al.*, 2007; Mishra, 2007) [12, 19]. According to Santos *et al.*, (2009) [25], active fatty acids and lipids with antimicrobial properties are found in high concentrations in a variety of Gram-negative bacteria. These compounds kill microorganisms by rupturing the cellular membranes of bacteria, fungi, and yeasts. They accomplish this by penetrating the thick layer of peptidoglycan in the cell wall without leaving visible traces and reaching the bacterial membrane, which causes it to disintegrate. Kombucha was utilized to valorize tea wastes, and compounds such as long chain fatty acids and dienes, which were similar to the metabolites identified in the current findings, were found (Majumder *et al.*, 2022) [16]. Majumder *et al.*, (2020) [15] revealed the removal of caffeine in coffee kombucha and identified various alkaloids and

undesirable metabolites, including amides, during fermentation. Their findings indicated that hexadecane had the most significant peak area. In a similar experiment conducted by Rinaldi, 2022 [24] about 65 different volatile compounds, including acids, alcohols, aromatic hydrocarbons, esters, phenols, terpenes, and others, were identified.

The GCMS results revealed the presence of seven important volatile compounds essential for antibacterial activity in crude extract of *kombucha*. The typical flavour of the end product was due to the prevalence of thiophene-2-carboxylic acid, santolin diacetylene, acetic acid, neopentyl alcohol, lavanduyl acetate, 1-heptanol and 3-(4-nitrobenzoyl) cinnoline. Neopentyl-2-oxobutanoate is yet another flavour compounds present in crude extract. Benzaldehyde dioctyl acetal being an important ketone derivative is a prominant flavour compound that are normally present in most of the distilled beverages. Santolin diacetylene is an hepatoprotective agent and an important antioxidant compound. Compounds like acetamide and diacetamide are also present in the extract which are essential for stabilisation of cellulose derivatives which are formed as floating pellicle by the bacterium *A. xylinum* in the *kombucha* extract. Some other medically important compounds identified were piperazine, leucinol, crocusatin, sarracine, haliclorensin and quinoxaline.

In earlier investigatons carried out by Czaja *et al*., 2006 [1] it has been indicated that bacterial cellulose obtained from *A. xylinum* can be used for making chronic and acute wound care products. Toda *et al*., 1997 [27] as reported that cellulose produced by acetic acid tolerant *A. xylinum* DA strain has an excellent moisture retaining capacity and continous production stability suited for industrial scale production. Because of the unique properties, resulting from the ultrafine reticulated structure several application such as skin grafts, face peeling, collagen, blood vessels and granulation have been proposed for this cellulosic layer by Lynd *et al*., 2002 [14]. From the present investigation, the pure cellulose obtained as a floating pellicle can better be applied for developing a wide array of rewettable supra - adsorbent pads.

Conclusion

The relative abundance of antibacterial metabolites expressed in *kombucha* extracts might be due to the occurance of both hydrophobic and hydrophilic bioactive compounds. The various important metabolites identified serve as wholesome proof for the antibacterial propoerties o*f Kombucha* tea. An improved knowledge of the composition, analysis and properties of *A. xylinum* with respect to antimicrobial compounds and bacterial cellulosic membrane would assist in efforts for the pharmaceutical application of this bacteria.

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