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RESEARCH ARTICLE

Improved functionality of roselle (*Hibiscus sabdariffa)* calyx extract blended *Kombucha*, a fermented beverage

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Abstract

Kombucha is a fermented drink with a range of medicinal benefits prepared from sweetened tea infusion (*Camellia sinensis*), which is cultured symbiotically with yeast and acetic acid bacteria. In the present investigation, *kombucha* was prepared from sugared black tea extract blended with aqueous calyx extract of roselle (*Hibiscus sabdariffa*) @15% and fermented with cultures *viz*., *Komagataeibacter rhaeticus* (NAIMCCTB-3976) and *Brettanomyces bruxellensis* (CAP9) at 35°C. The floating water insoluble mat of *kombucha* was observed under a scanning electron microscope, which revealed the cellulosic nanofibrils secreted by *K. rhaeticus.* The total phenolic and flavanoid content, DPPH and ABTS activity of roselle calyx blended *kombucha* were significantly higher than black tea *kombucha*. Further, the compounds present in *kombucha,* when analyzed by fourier transform infra-red spectroscopy, denoted the presence of carbonyl compounds, aromatic olefinic compounds, ketones, aldehydes and esters. The different bioactive metabolites formed during fermentation were elucidated using gas chromatography-mass spectrometry and the major compounds excited within the retention time of 45 min with maximum peak area were 13-hexyloxacyclotridec-10-en-2-one (37.64%), palmitins such as 1,3 dipalmitin (6.42%), glycidyl palmitate (3.30%), organic acids such as undecanedioic acid, linoleic acid, acetic acid (3.88%), etc. The results proved that blending black tea extract with 15% roselle calyx extract as a substrate for *kombucha* fermentation was highly accepted with an organoleptic score of 95% and improved functional properties compared to black tea extract *kombucha* alone.

Keywords

bioactive compounds; black tea; Kombucha; roselle calyx extract; starter cultures

Introduction

Roselle (*Hibiscus sabdariffa*), commonly called 'roselle' or 'red sorrel' belonging to the family of Malvaceae, is used in various edible foods, salads, colorants and beverages due to its nutraceutical and phytochemical qualities. Calyces of roselle are used as a natural pigment and are rich in antioxidants, which are largely used in the production of functional foods. Dry roselle calyces extract and must were used for the production of the naturally coloured beverage by fermenting with baker's yeast, *Saccharomyces cerevisiae,* to produce a clear, tasty wine in Nigeria (1). In traditional medicine, the calyces are used in the preparation of several medications for hypertension, liver damage, pyrexia and illness prevention (2, 3).

Kombucha is a ubiquitous fermented sour beverage that emerged in the Northeast of China (Manchuria) and spread to Russia and the rest of the world. *Kombucha* is prepared by fermenting sweetened black or green tea extract with static fermentation with scoby (Symbiotic Colonies of Bacteria and Yeast), which tastes tart, sweet, and softly carbonated. *Kombucha* has therapeutic capabilities as well as favourable effects on gastrointestinal activity, is an anti-hyperglycemic agent in humans and exhibits bactericidal effects (4, 5). The drink also has amino acids, acetic acid, gluconic acid, L-lactic acid, biogenic amines, vitamin C and B complex. Jakubczyk *et al.* (6) reported the antioxidant properties of *kombucha* as a rich source of flavonoids. Accumulating research findings with literature evidence and according to the Normative Instruction (NI) No. 41 of Brazil, 2019 *kombucha* is defined as a sugared infusion or extract of *Camellia sinensis* (pure or in combination with other herbs) made by symbiotic cultures of yeast and bacteria through anaerobic fermentation and aerobic respiration. Good manufacturing practices and guidance involving preparation procedures and risk analysis with *kombucha* fermentation pertaining to worldwide regulatory requirements were elaborately corroborated. Also, it established evidence that implicated the addition of various substances to the beverage to aid in the improvement of fermentation (7). The succinct review of Jayalakshmi *et al.* (8) on the application of cellulose of *kombucha* in the field of medicine as supra-adsorbent pads for third-degree burns and wounds, development of foods, namely *nata-de-coco*, fermented probiotic *kombucha* tea, foliar bio-stimulants in agriculture and permaculture, biosorbents in heavy metal removal, waste management and other industries significantly implies the utility of *kombucha* cultures on tea broth fermentation. The *kombucha* market is expanding and becoming well-known around the world, with family-owned businesses gaining significant traction in this sector. There are an estimated 235 *kombucha* companies dispersed throughout Europe, North America and Asia. The market is expected to grow to USD 3.5-5 billion by 2025 (9). With an estimated growth rate of 23.2% by 2027, the global *kombucha* market is expected to have grown significantly in the last few years, reaching USD 1.84 billion in 2019 (10). In India, Borecha has teamed with 'Akasa Air', rapidly developing airline, to provide *kombucha* on their flights, making Akasa the first Indian airline to offer this probiotic refreshing, nutritious and delicious beverage as part of their in-flight beverage menu. Borecha's probiotic mango *kombucha*, offers a pleasant and nourishing taste, as well as stomach-friendly features (8).

Fermentation using *kombucha* colonies is composed of two portions: a floating cellulosic pellicle layer and sour liquid broth below the cellulosic layer formed by *Acetobacter xylinum* and yeasts. Harrison and Curtin (11) reported that the microbial community structure comprising the co-cultures such as *Komagataeibacter* and *Brettanomyces* produced well-accepted *kombucha* with acidic taste, flavor, and aroma. The fungus-like mixture of microorganisms and cellulose is likely the reason why *kombucha* is also called "tea fungus". This microbial biofilm consists of a multi-layered matrix of entangled bacterial cellulose nano and microfibrils and represents an extremely resistant protection structure for the bacterial colony. The liquid phase of the fermentation process, or the "soup", contains high amounts of antioxidants, B-vitamin complex, D-saccharic acid-1, 4-lactone, glucuronic acid, phenols and polyphenols (12). According to Su *et al.* (13), several alternate substrates and blends with tea extracts have been reported for *kombucha* fermentation, including cinnamon, cardamom, Shirazi thyme, pineapple peel and core, exfoliated coffee cherry husk/pulp and peel waste. Additionally, probiotic drinks have been prepared using extracts from butterfly pea (Clitoria) flowers, and kombucha's chemical properties were enhanced by blending with Indian gooseberry extract. Other novel beverages include kombucha made with red seaweed/ laver extracts, Rooibos tea, Chinese jasmine tea, cherry leaves and a blend of Olympus Mountain tea, thyme and honey.

From the practical point of view, the original colour of the tea extract is reduced due to the transformation of phenolics and tannins in tea by the action of enzymes secreted by scoby that resulted in an acidic pH of the finally brewed *kombucha*. Hence, the final appealing colour and also functional compounds of the fermented *kombucha* may be improved by incorporating roselle calyx extract in the growing substrate. Hence, the present study was conducted to prepare roselle-blended *kombucha* using native strains previously isolated from sugarcane juice and to characterize the various functional bioactive compounds. The graphical abstract of the entire experiment is depicted in Fig. 1.

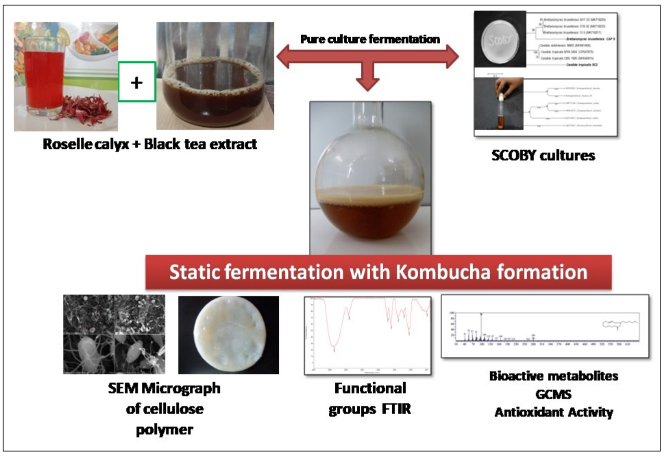

Fig. 1. Graphical abstract of the manuscript.

Materials and Methods

Kombucha fermentation and starter cultures

The *Komagataeibacter rhaeticus* (NAIMCC TB-3976), cellulose-producing bacterial strain and *Brettanomyces bruxellensis* (CAP9) yeast strain used in this investigation were isolated and identified earlier in our laboratory from fermenting sugarcane juice. The bacterial strain was activated in Hestrin and Schramm (HS) medium and yeast culture in yeast peptone mannitol (YPM) broth by incubating it for two days at 30°C. Experiments were carried out by inoculating and growing the strains by incubating at 30°C for 7 days without agitation.

Preparation of tea and roselle calyx extract for *kombucha*

A modified method formulated by Malbasa *et al*. (14) was performed to execute the preparation of tea (*Camelia sinensis*) extract using 2.0 g black tea powder in 1000 mL of water sweetened with sucrose of 70 g L^{-1}. Freshly harvested roselle calyx obtained from Tamil Nadu Rice Research Institute, Aduthurai, Tamil Nadu, India, was air dried in a cabinet dryer with initial primary drying for 2 h at 40°C and secondary drying at 60°C for 6 h. The dried calyx was peeled off and stored in an airtight container. About 2 g of the dried calyx was boiled in 100 mL of boiled water to obtain the extract. Tea extract was blended with different percentages (5%, 10%, 15%, 20%, 25% and 30%) of roselle calyces extract and the treatment combinations include tea extract (100%) as substrate were subjected to inoculation with two cultures (*K. rhaeticus* (NAIMCC TB-3976) and *B. bruxellensis* (CAP9) in the ratio of 8:2 (v/v) as suggested by Sutthiphatkul *et al*. (15).

Assessment of antioxidant activity in roselle calyx extract blended *kombucha*

Total phenolic and flavonoid content and the activity of antioxidants such as DPPH and ABTS were assessed for the tea extract-based *kombucha* and also in *Hibiscus sabdariffa* calyx extract blended *kombucha*. Using 96% ethanol, the extract from the *kombucha* samples was obtained as per the procedures of Yilmaz-Ersan *et al*. (16).

Determination of total phenolic and flavonoid content, 2,2-Diphenyl-1-Picrylhydrazyl (DPPH) and 2,2′-Azino-bis 3-Ethylbenzothiazoline-6-Sulfonic Acid (ABTS) activity in Roselle calyx extract blended *kombucha*

The total phenolic and flavonoid content was determined by the method described by Sultana *et al*. (17). The DPPH radical scavenging activity was carried out using a modified method of Huh *et al*. (18). The ABTS radical scavenging ability of *kombucha* was measured by referring to the method evaluated by Re *et al*. (19).

Sensory analyses of roselle calyx extract blended *kombucha*

The roselle calyx blended *kombucha* treatments were evaluated by panel members for colour, clarity, flavor, aroma, taste and overall acceptability. The assessment was conducted under fluorescent illumination inside isolated booths within an air-conditioned sensory laboratory. 100% tea extract-based *kombucha* served as a reference (control) for the assessment. The assessment was carried out using a 9-point hedonic scale and samples were served in coded transparent drinking glasses using the methodology described by Ihekoronye and Ngoddy (20).

Characterization of roselle calyx extract blended *kombucha* by scanning electron microscope (SEM)

Scanning electron microscope (SEM) (Model: S-3400 HITACH Co., Japan) was used to examine the morphology of the roselle blended *kombucha* broth for the presence of cellulose fibrils, bacteria and yeast. The sample was aseptically extracted and freeze-dried in a sterile bottle. They were individually gold-plated using an ion sputter (Fisons Instruments, UK). Magnification at 2000 X was used to examine and photograph the gold-coated samples.

FTIR spectroscopy analysis of roselle calyx extract blended *kombucha*

The type of fermentative chemicals contained in the roselle blended *kombucha* was identified using FTIR spectroscopy using the potassium bromide (KBr) pelleting method. A small droplet of fermented roselle blended *kombucha* was placed on the top of KBr plates. The second plate was placed on top and a quarter turn was given to obtain a fine, thin, even film. The plates were placed into the sample holder and the spectrum was run for eight scans to obtain a unique spectroscopic vibration. At the temperature of 308 Kelvin, the FTIR spectra were acquired in transmission mode using an FTIR spectrophotometer (Thermo Nicolet Model-6700, CIF, Pondicherry University, Puducherry, India). The sample's FTIR spectra were examined at wave numbers ranging from 4000 to 400 cm^{-1} in the mid-IR band. After passing through the plates, the infrared beam impinges on the sample and the percent transmittance is measured against wave numbers cm^{-1} (21).

Analysis of volatile and bioactive compounds in roselle calyx extract blended *kombucha* by gas chromatography-mass spectrophotometry (GC-MS)

The fermented roselle calyx extract blended *kombucha* was analyzed for the presence of volatile and bioactive compounds using GC-MS (Model Shimadzu QP 2020). Hexane extract was prepared by dissolving 1 mL of *kombucha* sample overnight in 1 mL of n-hexane solvent. GC-MS instrumental set-up fitted with DB-5 fused-silica capillary column measuring 0.25 μm thick with 0.25 mm of internal diameter and length of 30 m was used. 1 μL of the sample with a split ratio of 20:1 was injected at the injection temperature of 260° C, whereas the interface and source temperature were set to 270°C and 230°C, respectively. Carrier gas helium was used with a total flow rate of 16.3 mL/min and the column flow rate was 1.21 mL/min. Mass spectra were recorded at 5 scans/sec with a scanning rate of 40-650 m/z. The mapping and spectral configurations were elucidated with the available mass spectral NIST MS search 2.2v database and compounds were identified. The relative abundance of the compounds was given as peak area (%) at different retention times. The compounds elucidated in the *kombucha* were characterized, validated and confirmed with NIST library matching by the procedure reported by Majumder *et al.* (22).

Statistical analyses

The sensory parameters and the various antioxidant activity data obtained were subjected to the student's t-test and differences between roselle blending treatments were discriminated at the 5% level of significance with the methods of Snedecor and Cochran (23).

Results

Sensory analyses of roselle blended *kombucha*

Roselle calyx extract blended *kombucha* at different levels in tea extract and *kombucha* obtained from tea extract alone was subjected to organoleptic hedonic evaluation (Fig. 2).

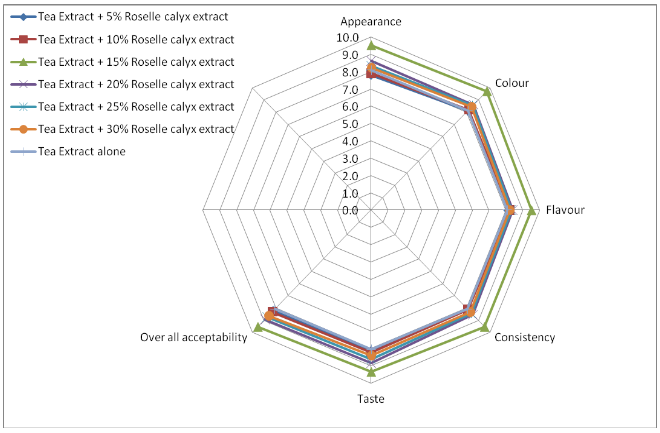

Fig. 2. Organoleptic scores of roselle blended *kombucha.*

The sensory scores of all the treatments scored above 8.0, which were acceptable to identify the volatile components. Filtered samples of freshly made *kombucha* were extracted and analyzed with hexane solvent. Among the different levels of roselle extract, an increase in concentration increased the overall acceptability of the fermented beverage, the highest being a 15% level of roselle extract. The response of the sensory panel observers registered a more appealing colour of black tea extract when fermented with 15% roselle calyx *kombucha*. The statistical analyses revealed that a significant difference ($p>0.05$) was observed between the control and roselle blended treatments.

SEM and FTIR spectrum of roselle calyx extract blended *kombucha*

The fermented *kombucha* was observed under a scanning electron microscope to assess the cellulosic microbial colonization. The results are given in Fig. 3, which shows the formation of cellulosic fibrils, the presence of budding yeasts and the cellulose-synthesizing bacteria *Komagataeibacter rhaeticus*. The present results of roselle blended *kombucha* of

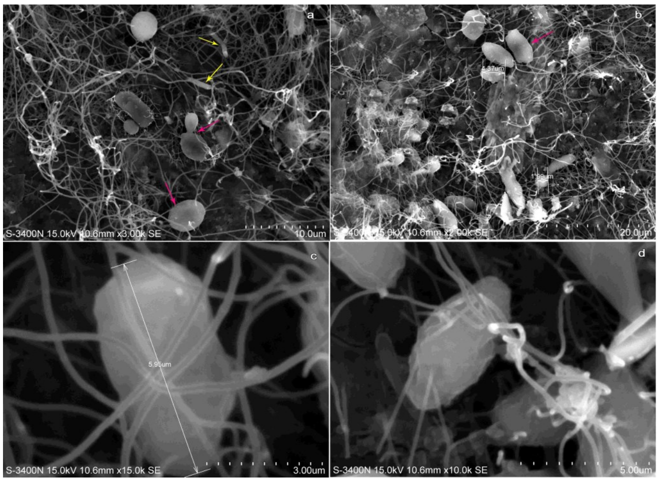

Fig. 3. SEM image showing yeast, bacterial cells and cellulose fibrils in roselle blended *kombucha.*

SEM clearly show the budding yeast cells and bacteria spinning the cellulose nanofibrils.

The functional group of compounds present in black tea *kombucha* is depicted in Fig. 4a. FTIR fingerprint region for the black tea *kombucha* showed stretching vibration for -OH groups at 1418 cm^{-1}, 1263 cm^{-1} with $-CH_3$ stretching, symmetric CH_3 bending at 1134 cm^{-1}, strong stretching of C-O-C compounds at 1051 cm^{-1}, vinyl C-H group out of plane bend at 994 cm^{-1}, exhibiting ring deformation of phenyl and carbon compounds at 926 cm^{-1} and at 596 cm^{-1} C-alkyl groups were known to be present.

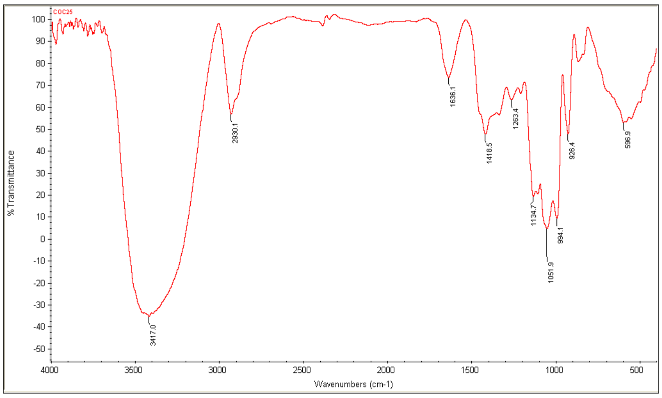

Fig. 4a. FTIR spectrum of the functional group of compounds in black tea *kombucha.*

In Fig. 4b, the peak transmittance % in the single bond region at the wavelengths of 3387 cm^{-1} and 2928 cm^{-1} represents the presence of hydrate molecules, hydroxyl group, variable stretching hydrogen bonds in O-H linking and C-H stretching bonds of lipids/fats, respectively. At 1734 cm^{-1}, carbonyl compounds C=C, aromatic compounds (olefinic compounds), ketones, aldehydes and esters were transmitted at 1622 cm^{-1}. In the fingerprint region at the wavelength of 1410 cm^{-1}, strong intensity of -OH groups, phenols or tertiary alcohol, organic sulphates/sulfur oxy compounds, at 1233 cm^{-1} strong intensity for aromatic compounds/proteins (amide III groups) with $-CH_3$stretching, 1076 cm^{-1} strong stretching of C-O-C compounds/C-F

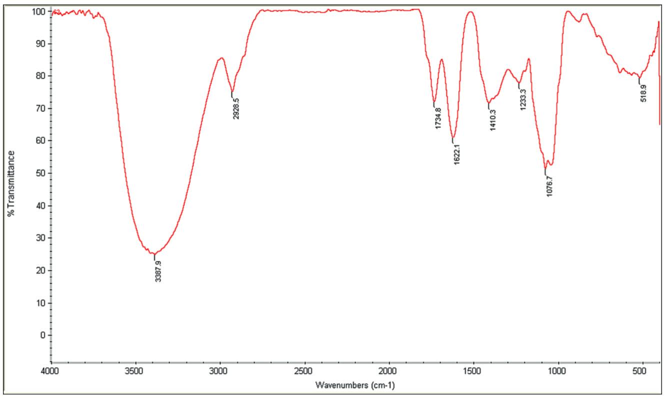

Fig. 4b. FTIR spectrum of the functional group of compounds in roselle blended *kombucha.*

stretching for aliphatic organo halogen compounds and at 518 cm^{-1} C-alkyl groups are transmitted in roselle blended *kombucha*.

Volatile compounds in roselle blended *kombucha* characterized by GC-MS

The GC-MS analysis of 15% roselle blended *kombucha* resulted in the detection of 25 metabolites, which showed identities, retention times (Rts) and retention indices (RI values) of the identified compounds are shown in Table 1 and Fig. 5a–5c. The results of the present study showed different compounds formed within the retention time of 45 min in roselle blended *kombucha* were 13-hexyloxacyclotridec-10-en-2-one (37.64%), palmitin such as 1,3 dipalmitin (6.42%), glycidyl palmitate (3.3%), alkenes such as 1-heptadecene and heptadecatriene, alkanes namely

Table 1. Elucidation of volatile compounds in hexane extract of roselle blended *kombucha* using GC-MS

Sl. No.	Compounds elucidated	Molecular weight and formula	Rt (min)	RI	Area %
1.	13-hexyloxacyclotridec-10-en-2-one	280 $C_{18}H_{32}O_2$	33.4	2325	37.64
2.	1,3-dipalmitin, TMS derivative	640 $C_{38}H_{76}O_5$	42.6	4055	6.42
3.	2,3-diphenyl cyclopropyl)	332	42.1	2835	5.49
4.	Undecanedioic acid, monomethyl ester	230 $C_{12}H_{22}O_4$	30.4	1738	4.71
5.	Bis (2-ethyl hexyl) phthalate	390 $C_{24}H_{38}O_4$	44.0	2704	4.36
6.	Hexadecanoic acid Ethyl ester	330 $C_{19}H_{38}O_4$	43.4	2498	4.20
7.	Acetic acid	486 $C_{32}H_{54}O_3$	45.0	3203	3.88
8.	5,5-dimethyl-1,3-dioxane-2-ethanol	274 $C_{14}H_{30}O_3$	45.8	1490	3.88
9.	Stigmasterol	412 $C_{29}H_{48}O$	44.0	2739	3.71

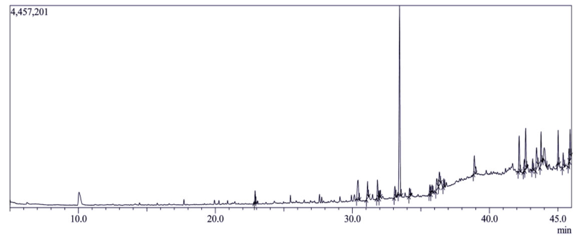

Fig. 5a. Volatile compounds in hexane extract of *kombucha* blended with roselle calyx elucidated using GC-MS.

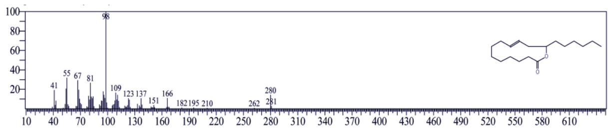

Fig. 5b. GC-MS peak elucidating 13-hexyloxacyclotridec-10-en-2-one in roselle blended *kombucha.*

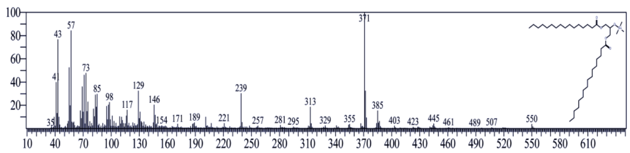

Fig. 5c. GC-MS peak showing 1,3-dipalmitin in roselle blended *kombucha.*

heneicosane, eicosane, 2-methylhexacosane, organic acids such as undecanedioic acid, acetic acid (3.88%), propenoic acid, fatty acid esters like hexadecanoic acid; 2-hydroxy-1-(hydroxymethyl)ethyl ester, monomethyl ester, linoleic acid; ethyl ester and ethyl oleates, phthalate esters like bis (2-ethylhexyl) phthalate and dibutyl phthalate, alcoholic compounds such as 5,5-dimethyl-1,3-dioxane-2-ethanol, sterol namely stigmasterol.

Total phenolic, flavonoids and DPPH, ABTS activity in roselle blended *kombucha*

The results are furnished in Table 2. The total phenolic and flavonoid content was significantly higher in 15% roselle blended *kombucha* at 218.34±0.20 and 89.54±0.21 mg mL^{-1}, respectively. The percentage of DPPH radical scavenging activity was also a maximum of 76.22% in 15% roselle blended *kombucha* than the black tea extract *kombucha* (72.50%).

Discussion

The optimum ratio of roselle extract with black tea in *kombucha* fermentation was standardized by sensory analysis, which showed that the addition of roselle had a positive effect on the sensory quality of *kombucha*. Among various levels, 15% of the roselle extract received maximum acceptable scores of 95% in all the quality parameters. Similarly, Neffe-Skocinska *et al.* (24) illustrated a microbiologically safe and stable calyx extract beverage with a richness of vitamin C and increasing glucuronic acid levels combined with high overall sensory quality. Fermented milk

Table 2. Total phenolic and flavonoid content, DPPH and ABTS activity of roselle calyx extract blended *kombucha*

Substrates	Total phenolic content mg mL^{-1}	Total flavonoid content mg mL^{-1}	DPPH radical scavenging activity (%)	ABTS radical scavenging activity (%)
Black tea *kombucha*	208.87±0.73	85.42±0.05	72.50±0.02	97.68±0.53
Roselle calyx extract blended *kombucha*	218.34±0.20	89.54±0.21	76.22±0.05	97.26±0.26
CD (0.05%)	2.10	0.61	0.18	1.66

incorporated with roselle extract and fermented using probiotic bacteria was organoleptically accepted with higher sensory scores and was found to promote hypolipidemic and hypoglycemic effects in experimental mice (25).

The starter culture used for *kombucha* fermentation and the formation of its typical cellulosic fibrils were confirmed by SEM analysis in the present study. The change in functional group after fermentation is also studied by FTIR analysis. Dima *et al*. (26) illustrated that boiled green tea infusion sweetened with high fructose corn syrup and fermented using a symbiotic culture of bacteria and yeast, such as *Komagataeibacter*, *Gluconobacter*, *Lactobacteria* and yeasts like *Dekkera*, *Pichia* and *Zygosaccharomyces.* In the same study, researchers showed the characteristic feature for the occurrence of C-H bond in $-CH_2$ and $-CH_3$ groups with FTIR spectroscopic absorption bands at 2941 and 2843 cm^{-1}, respectively. In the present study, similar types of bands were also formed, representing the C-H bonds. In experimental evidence elaborated by Sigiro *et al*. (27) using FTIR and SEM to study the white floating polysaccharide synthesized by *kombucha* cultures, through FTIR spectroscopy, O-H, CH_2, H-O -H and C-O groups were identified and evidentially proved that the molecules were pure compact layers of cellulose purely synthesized from glucose, offering crystalline cellulosic fibrils with nanopores. Further, in the same study, the SEM analysis showed the surface morphology of irregularly woven cellulosic fibrils with micropore sizes that are less than 2 mm. Earlier studies by Brza *et al*. (28) have elucidated and concluded that tea extract solution showed the transmittance peak for polyphenols at 3388 cm^{-1}, exhibiting the abundance of carboxylic acid with O-H/N-H bonds, amino acids showing C=C bonds at 1636 cm^{-1} and C-O-C stretching at 1039 cm^{-1}. The present findings conform with the peaks identified in the above research.

Pure and Pure (29) experimentally evaluated the usage of nettle leaf and banana peel wastes as an alternative substrate instead of black tea for the preparation of antioxidant-rich fermented beverage *kombucha.* Similarly, Juhari *et al*. (30) elucidated aroma and odorants such as terpenes, aldehydes, esters, furans and ketones in dried roselle calyx gathered from eight different nations. Huang *et al*. (31) experimentally proved that microbial biotransformation through fermentation of *H. sabdariffa* calyx using lactic acid bacteria and yeasts improved the nitric oxide scavenging activity, DPPH, aglycones, flavonoids and total polyphenolic compounds. The pharmacological effects as antimicrobial, antioxidant, antihyperlipidemic, immunomodulatory, anticancer, antidiabetic, anti-inflammatory, and antihypertensive properties of fermented *kombucha* beverages produced from different kinds of raw materials and mixtures such as tea, coffee, herbs, milk and fruits had been witnessed in different countries using co-cultures of acetic acid bacteria and yeasts that live in symbiotic association with each other in sweetened substrates (32).

In the present study, the volatile fermentative compound formed in roselle calyx extract blended *kombucha* with a major peak area of 37.64% was 13-hexyloxacyclotridec-10-en-2-one, followed by 1,3-dipalmitin. Suffys *et al*. (33) explored the volatile organic and odour-active compounds such as phenethyl alcohol and isoamyl alcohol (Δ-3-carene, α-phellandrene, γ-terpinene, m- and p-cymene), carboxylic acids, alcohols and terpenes. The aromatic compound, namely 2-phenyl ethanol, provided sweet floral bready and honey flavour for *kombucha* and citrus floral sweet notes due to the dominant compounds, geraniol and linalool. Due to fermentation and formation of α-farnesene, intense herbal citrus lavender bergamot notes were produced. Khan *et al.* (34) conducted preliminary photochemical screening in Kleinex Willd (*Ischaemum pilosum*) using different solvents and reported the presence of alkaloids, anthraquinone, glycosides, coumarins, flavonoids, phenols, reducing sugars, saponins, steroids, tannin and triterpenes. They further elucidated the presence of bioactive compounds by soxhlet extraction method using acetone as an extractant by GC-MS and confirmed the compounds like phenol, 4-bis (1,1-dimethyl ethyl), eicosane, n-hexadecanoic acid, 2, 6-bis (1,1-dimethyl ethyl) phenol and heptadecane that were known to possess bioactive and therapeutic abilities.

The antimicrobial activity against pathogenic microorganisms of *kombucha* is largely attributable to acetic acid. Acetic acid is known to inhibit and destroy many gram-positive and gram-negative microorganisms (35). The roselle blended *kombucha* produced acetic acid that may be attributed to antimicrobial activity. Khalid (36) found two chemicals, 13-hexyloxacyclotridec-10-en-2-one and di-(2-ethylhexyl) phthalate (DEHP), that act against tumour cells in the extract of *Ambrosia maritima* utilizing spectroscopy and *in silico* molecular docking and prediction methods. Thus, the current study identified the development of an anti-cancerous compound, 13-hexyloxacyclotridec-10-en-2-one, in roselle blended *kombucha*. A similar study conducted by Kumari *et al*. (37) in the *Acacia nilotica* extract elucidated 13-hexyloxacyclotridec-10-en-2-one, which was shown to have antibacterial activity and also polyunsaturated fatty acid, namely 9, 12-octadecadienoic acid, which was found to inhibit growth of pathogenic yeast, *Candida albicans* and spoilage bacteria, namely *Streptococcus* sp. The current GC-MS elucidation of roselle blended *kombucha* also identified the same type of chemicals and the results are consistent with the previous findings.

Key odorant compounds and aroma-rich and aroma-active molecules such as furans, furfural and 5-methyl-2-furfural expressing bready and caramel notes, organic acids, alcohols, aldehydes, esters, ketones, lactones, pyranone, pyrrole, volatile phenols and terpenes were obtained by scoby fermentation of hot and cold brewed Beninese roselle calyx infusions (38). Sutthiphatkul *et al.* (15) found that fermenting black tea and roselle calyx extract-based *kombucha* for 10 days with co-cultures of *Acetobacter pasteurianus* AJ605 and *Zygosaccharomyces bailii* YN403 in 8:2 (v/v) ratio resulted in high antioxidant activity, DPPH and ABTS scavenging ability and high sensory acceptability. Similarly, the addition of *Komagataeibacter rhaeticus* (NAIMCC TB-3976) and yeast, specifically *Brettanomyces bruxellensis* (CAP 9), increased the acceptability of the roselle blended *kombucha*. However, Jayabalan *et al.* (39) revealed that *kombucha* tea ethyl acetate fractions contained two cytotoxic and anti-invasive chemicals, dimethyl 2-(2-hydroxyl -2 methoxypropyllidene malonate and vitexin. While experimenting on the prolonged fermentation studies of *kombucha* by Chen and Liu (40) using black tea as a fermentative substrate, organic acids such as gluconic acid were produced after 6th day of fermentation and the concentration of acetic acid increased very slowly, deciding the fruity taste of the final product. Using the endemic medicinal herb, *Curcuma sahuynhensis*, Tran *et al.* (41) conducted GC-MS studies to elucidate the secondary metabolites such as 6,10,14-trimethylpentadecan-2-one, phytol, 1-ethyl butyl hydroperoxide, isoborneol, 1-methylpentyl hydroperoxide and neophytadiene, etc., in the hexane extract of *C. sahuynhensis* that were found to exhibit anti-cancer principles, cytotoxic effect on human cancer cell lines. Hence, the present research findings prove that entirely different types of compounds exhibiting various properties were elucidated. Shanmuganathan *et al.* (42) identified the volatile compounds in fresh juice, a sugary-rich beverage from chewing sugarcane, using a headspace analyzer in GC-MS and reported hexadecanal, eicosenoic acid, hexadecanoic acid, octadecadienoic acid, methyl esters, 2,3-dihydroxy propyl ester, glycidyl oleate, fumaric acid, glycidyl oleate, glycidyl palmitate, silane, octadec-9-enolide and hexasiloxane that were present in a relatively abundant quantity, contributing to the typical grassy, nutty and waxy flavor of the chewing cane. However, the present findings of GC-MS mapping of roselle blended *kombucha*, entirely different types of compounds, except the hexadecanoic acid, were elucidated.

Zhao *et al.* (43) discovered 7 different types of volatile flavour components that were in a state of dynamic change throughout the fermentation process in *kombucha*, such as alcohols, aldehydes, ketones, acids, esters and benzenoids. The top 5 acids were acetic acid, butanoic acid, citric acid, L-lactate, and succinic acid and they shared the largest peak area of all volatile flavour components. Roselle calyx fermented for 10 days with the yeast isolated from fresh palm wine, namely *Saccharomyces cerevisiae* in Nigeria by Ejuama *et al.* (44), resulted in yielding a high antioxidant-rich beverage posing maximum content of ascorbic acid, flavonoids, and phenol. Hibiscus-based *kombucha* fermented scoby showed volatile compounds analyzed constituted by 9 classes of a complex mixture of compounds, namely, esters, ketones, carboxylic acids, alcohols, aldehydes, amines, hydrocarbons, lactones and terpenes. Among them, the esters were the majority, ranging from 20.0 to 28.0%; it was also possible to observe that 2-octenal was the most abundant compound in all samples, ranging from 52.7 to 63%. The most important are ethyl butanoate, 2,6-dimethyl-4 -heptanol, linalool, 3-octenyl acetate, ethyl caproate, 3-octanol, hexadecanoic acid and 2-octen-1-ol, with emphasis still on the 2-octenal that was more abundant in the evaluated samples, representing more than 50.0% of the volatile fraction. It was also seen that the substances identified in the volatile profile of the different *kombucha* samples studied are mainly related to fruity and floral odor notes (45). Exploration of bioactive metabolites using the methanol extract of *kombucha* tea using GCMS by Jothilakshmi *et al.* (46) presented 18 different compounds such as dodecane, heptadecane, octadecane, hexadecane, octacosane, heneicosane, tricosane and nonane, organic acids namely acetic acid and thiophene-2-carboxylic acid, and flavour compounds such as neopentyl-2-oxobutanoate, benzaldehyde dioctyl acetal and santolin diacetylene. Bishop *et al.* (47) elaborately discussed the chemical composition of *kombucha* tea and indicated the presence of organic acids, amino acids, catechin, ethanol, polyphenols, vitamins, D-saccharic acid-1,4-lactones and biogenic amines.

Mendonca *et al.* (48) fermented leaves and stem portions of *Hibiscus sabdariffa* using scoby cultures for *kombucha* flavoured with grape juice and found that phenolic and flavonoid contents can be improved than the conventional *kombucha*. When the bioactive compounds of *kombucha* were analyzed using GC-MS, it was found to contain 17 types of molecules such as catechin, chlorogenic acid, coumaroyl quinic acid, crypto chlorogenic acid, epicatechin, gallocatechin, kaempferol rutinoside, kaempferol 3-O-rhamnoside neochlorogenic acid and quercetin (49). Apart from natural colouration, yogurt coloured and fermented with carrot juice and roselle calyx extract has been shown to boost antioxidant activity (50). Yogurt mixed with natural pigments from roselle calyx extract enhanced the redness, total polyphenol, ABTS, DPPH radical scavenging activity and flavonoid content (51). Similar results were obtained in the roselle blended *kombucha* used in the present experiment.

Conclusion

The study on the roselle calyx extract blended *kombucha* demonstrates that blending black tea extract with 15% roselle calyx extract significantly enhanced the functional and sensory properties. Furthermore, the response of the sensory panel observers registered a more appealing colour of black tea extract when fermented with 15% roselle calyx *kombucha*. The findings reveal a higher content of bioactive compounds, particularly phenolics and flavonoids and an increase in antioxidant activities (DPPH and ABTS) compared to black tea *kombucha* alone. The fermentation process, involving *Komagataeibacter rhaeticus* and *Brettanomyces bruxellensis*, also produced unique volatile compounds like 13-hexyloxacyclotridec-10-en-2-one and organic acids, contributing to antimicrobial properties and potential anticancer principle compounds. The improved organoleptic qualities and appealing colour of the roselle blended *kombucha* indicate its market potential as a naturally colored, functional beverage. These results open new avenues for innovation in the *kombucha* market, especially in integrating underutilized natural ingredients like roselle calyx to enhance product quality and the appealing appearance of fermented beverages.

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Authors' contributions

JK, GG worked on conceptualization; UT, GG, JK on formal analysis; GG on funding acquisition; JK, GG, UT on software; GG, JK, UT on writing original draft; JK, GG, AS, UT on writing, review and editing. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interest to declare.

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