

Please note that this is an unedited version of the manuscript that has been accepted for publication. This version will undergo copyediting and typesetting before its final form for publication. We are providing this version as a service to our readers. The published version will differ from this one as a result of linguistic and technical corrections and layout editing.

<https://doi.org/10.17113/ftb.61.01.23.7789>

original scientific paper

Comprehensive Evaluation of Three Important Herbs for Kombucha Fermentation

Running head: The benefits of herbal flavoured kombucha

Burcu Emine Tefon Öztürk^{1*}, Berfin Eroğlu¹, Eda Delik¹, Mustafa Çiçek² and Esra Çiçek³

¹Biology Department, Faculty of Science, Akdeniz University, Dumlupınar Boulevard 07058, Antalya, Turkey

²Department of Biology, K.Ö. Science Faculty, Karamanoğlu Mehmetbey University, İbrahim Öktem Boulevard 70100, Karaman, Turkey

³Department of Biological Sciences, Middle East Technical University, Dumlupınar Boulevard 06800, Ankara, Turkey

Received: 18 May 2022

Accepted: 15 December 2022



SUMMARY

Research background. Kombucha is consumed worldwide for its beneficial health effects. Kombucha teas fermented with various herbal infusions have become very important nowadays. Although black tea is used for kombucha fermentation, kombucha teas fermented with different herbal infusions have gained great importance. In this study, three different traditional medicinal plants, namely hops (*Humulus lupulus L.*), madimak (*Polygonum cognatum*), and hawthorn (*Crataegus monogyna*) were used for the fermentation of kombucha beverages, and the bioactivity of these beverages was investigated extensively.

Experimental approach. The microbiological profile, bacterial cellulose formation, antibacterial and antiproliferative activity, antioxidant activities, sensory properties, total phenolic content, and flavonoid content of kombucha beverages were investigated. Liquid chromatography-coupled mass

*Corresponding author:

Phone: +902423102336

+902423103844

Fax: +02423102294

E-mail: burcutefon@akdeniz.edu.tr

Please note that this is an unedited version of the manuscript that has been accepted for publication. This version will undergo copyediting and typesetting before its final form for publication. We are providing this version as a service to our readers. The published version will differ from this one as a result of linguistic and technical corrections and layout editing.

spectrometry analysis (LC-MS / MS) was used to identify and quantify specific polyphenolic compounds in the samples.

Results and conclusions. According to the results, the hawthorn-flavoured kombucha, which has lower free radical scavenging activity than the other samples, came into prominence in terms of sensory properties. All kombucha beverages examined showed a strong cytotoxic effect on Mahlavu and HCT116 cell lines, but only the madimak-flavoured kombucha sample, which had a higher total phenolic/flavonoid content, demonstrated antibacterial activity against all microorganisms used in the study.

Novelty and scientific contribution. Considering the results of this study, madimak could be an effective herb for the development of new kombucha beverages, although its sensory properties still need to be improved. This study contributes to science in terms of producing new fermented beverages with improved beneficial health effects.

Keywords: Kombucha; bioactivity; antibacterial activity; cytotoxic activity; sensory evaluation; phenolic substance

INTRODUCTION

Kombucha is a slightly sweet and acidic beverage originating in Manchuria. It is traditionally prepared through fermentation of *Camellia sinensis* (black tea) and sugar by symbiotic culture of acetic acid bacteria (mainly *Komagataeibacter*, *Acetobacter* and *Gluconobacter*) and osmophilic yeasts (1). *Komagataeibacter xylinus*, in particular, is regarded as the most prominent microorganism of kombucha fermentation (2). In the production of kombucha, the medium is usually inoculated with a biofilm of bacteria and cellulose pellicles formed during a previous cultivation (SCOBY) and then incubated for 7 to 14 days under aerobic conditions. Sucrose, the primary carbon source for kombucha fermentation, is eventually oxidized to acetic acid by acetic acid bacteria during fermentation. The beverage is reported to be effective against metabolic diseases, psoriasis, constipation, indigestion, and hypertension (3). It is believed that the chemicals formed by metabolic activity such as lactic acid, acetic acid, glucuronic acid, and gluconic acid, free radical-binding vitamins such as C, B₂ and B₆, amino acids, antibiotics, catechins, and various micronutrients contained in the fermented tea are responsible for the beneficial properties of kombucha (4).

Since this fermented beverage is widely consumed due to its probiotic content, improving the antioxidant and antimicrobial properties of kombucha is an important issue. This situation prompted researchers to search for different aromatic plants and fermentation media rich in antioxidants, vitamins, nutrients, and antibacterial substances (5). Numerous other studies have previously

Please note that this is an unedited version of the manuscript that has been accepted for publication. This version will undergo copyediting and typesetting before its final form for publication. We are providing this version as a service to our readers. The published version will differ from this one as a result of linguistic and technical corrections and layout editing.

investigated the use of different herbs in kombucha preparation and evaluated their antioxidant and antimicrobial activities (6-8).

Hops (*Humulus lupulus L.*), madimak (*Polygonum cognatum*), and hawthorn (*Crataegus monogyna*) are herbs that have been used in traditional medicine for a long time. Hops has drawn a lot of attention in recent years for its sedative effects, digestive benefits, estrogenic properties, and potential cancer-preventive effects. The antibacterial effects of hops on mainly Gram-negative bacteria have been well documented by a variety of research groups (9-10). Madimak, a medicinal plant, is used in traditional Turkish medicine to treat diabetes mellitus and urinary tract diseases (11). Hawthorn, the third herb used in the study, is a member of genus *Crataegus*. Plants of this genus have been reported to have cardioprotective, anticarcinogenic, antioxidant, and anti-inflammatory effects on human health (12).

This study aims to investigate the antibacterial effect, microbiological profile, antioxidant and cytotoxic activities, total flavonoid and phenolic content, sensorial properties, and cellulose production of kombucha teas fermented with medicinal plant infusions. Some polyphenolic compounds of the samples were identified and quantified by liquid chromatography-coupled mass spectrometry analysis (LC-MS/MS). The traditional medicinal plants used in this study, namely hops, madimak, and hawthorn were selected for their therapeutic effects.

MATERIALS AND METHODS

Fermentation conditions of the kombucha beverages

In this study, black tea leaves (*Camellia sinensis*-Lipton, Unilever, Turkey), hops (*H. lupulus L.*-Antalya, Turkey), madimak (*P. cognatum*-Sivas, Turkey), and hawthorn (*C. monogyna*-Antalya, Turkey) were used to prepare kombucha beverages. The kombucha used for the experiments was a local isolate (Antalya, Turkey). Traditional kombucha fermented with medicinal herbs were named as hawthorn-flavoured kombucha (KH_a), hops-flavoured kombucha (KH), and madimak-flavoured kombucha (KM). Infusions prepared by only using black tea and herbs named as black tea (B) and herbal tea; hawthorn (H_a), hops (H), and madimak (M), and they were used as control groups.

The protocol defined by Marsh *et al.* (13) was adopted with minor modifications to prepare the traditional kombucha. Briefly, 5 % dry black tea leaves (*m/V*) were steeped in 1 L of boiling water for 3 min. Then, 9 % sucrose (Torku, Konya, Turkey) (*m/V*) was added, and the solution was boiled for another 1 min. Subsequently, the solution was cooled to room temperature (RT), and the dry leaves were removed by filtration. 100 mL of this black tea infusion with 2 % (*m/V*) SCOBY and 10 % (*V/V*) soup (starter culture) from the previous culture were inoculated into each glass jar for kombucha tea

Please note that this is an unedited version of the manuscript that has been accepted for publication. This version will undergo copyediting and typesetting before its final form for publication. We are providing this version as a service to our readers. The published version will differ from this one as a result of linguistic and technical corrections and layout editing.

fermentation. The sample flasks were incubated at RT. After 14 days of incubation, the liquid portion of the kombucha beverages was collected and centrifuged at 1370 g (Mistral 2000, MSE, UK) for 10 mins for further experiments.

To prepare herb-flavoured kombucha beverages 5 % dry black tea leaves (m/V) and 5 % dry herbal leaves (m/V) were added together to 1 L boiling water containing 9 % sucrose (m/V), and the same procedure described above was followed.

For herbal teas, 5 g of black tea, hops, madimak, and hawthorn leaves were added to 1 L of boiled water containing 9 % sucrose.

pH measurement

The pH values of the samples were measured on days 0 and 14 using a pH meter (Model 616.12.001, Isolab, Wertheim, Germany).

Microbiological profile

Microbiological profiling of the samples was performed on days 0 and 14 of fermentation. 1 mL samples were taken homogeneously from the fermentation vessels and serial dilutions of the samples were prepared with 0.05 M NaCl (Sigma Aldrich, St. Louis, MO, USA). Selective media were inoculated with 200 μ L of the 10^{-5} and 10^{-6} diluted samples, and this step was repeated 3 times for each sample. In order to count yeasts, Yeast Extract Glucose Chloramphenicol (YGC) (Merck, Darmstadt, Germany) was used as described by Coton *et al.* (14). Glucose Yeast Extract (GYC) was used for counting acetic acid bacteria (15). Plate Count Agar (PCA) (Merck, Darmstadt, Germany) was used to determine mesophilic bacterial colonies. De Man Rogosa Sharp Agar (MRS) (Merck, Darmstadt, Germany) was used for counting *Lactobacillus* and Plate Count Agar (PCA) (Merck, Darmstadt, Germany) was used to determine mesophilic bacterial colonies (14). Colonies were counted and colony forming units per millilitre (CFU/mL) were calculated according to Eqs 1 after incubation for five days at 30 °C for MRS, PCA and GYC plates and at 25 °C for YGC plates.

$$N = \frac{C}{[V \times (n_1 + (0.1 \times n_2))]} \times d \quad /1/$$

where d ; represent the most concentrated of the successive serial dilutions, n_1 ; number of replicates of samples prepared with the first of the serial dilutions, n_2 ; number of replicates of samples prepared with the second of the serial dilutions, N ; the total amount of microorganisms in one millilitre, V ; volume of serial dilution transferred to the samples (mL), C ; total number of colonies counted in samples (16).

Please note that this is an unedited version of the manuscript that has been accepted for publication. This version will undergo copyediting and typesetting before its final form for publication. We are providing this version as a service to our readers. The published version will differ from this one as a result of linguistic and technical corrections and layout editing.

Measurement of cellulose pellicle production

In order to measure the dry mass of the pellicles, the cellulosic biofilm of the kombucha samples was washed with dH₂O and incubated at 90 °C in 1 % NaOH (Sigma Aldrich, St. Louis, MO, USA) for 15 min. After incubation, the samples were washed with dH₂O and then treated with 1 % glacial acetic acid (Merck, Darmstadt, Germany) and air-dried (17).

Determination of total phenolic content (TPC) by Folin-Ciocalteu assay

The colorimetric method described by Škerget *et al.* (18) was used to identify the total phenolic content (TPC) of kombucha samples. According to Singleton and Rossi (1965), the Folin-Ciocalteu reagent (FC) was prepared (19). The soup of kombucha samples was collected and centrifuged at 1370 g (Mistral 2000, MSE, UK) for 10 min. The supernatants were then filtered through a sterile filter with a pore diameter of 0.45 µm (GVS Filter Technology, Stanford, USA). 500 µL of the filtered kombucha samples and 2.5 mL FC (1:10) were mixed, vortexed and incubated for 2 min. Then 2 mL of 7.5 % Na₂CO₃ (Merck, Darmstadt, Germany) was added to the samples, vortexed for 30 s and kept in a water bath at 50 °C for 5 min. TPC was calculated as gallic acid equivalent (GAE)(µg/mL) after absorbance values were measured at 760 nm.

Determination of total flavonoid content (TFC) by aluminum chloride colorimetric method

The colorimetric method with aluminium chloride was used to identify the total flavonoid content (TFC) of kombucha samples (20). The soup of kombucha samples was collected and centrifuged at 1370 g (Mistral 2000, MSE, UK) for 10 min. Then, supernatants were filtered through a sterile filter with a pore diameter of 0.45 µm (GVS Filter Technology, Stanford, USA). To each of the 500 µL kombucha sample, 1.5 mL methanol (Isolab, Wertheim, Germany), 100 µL 10 % AlCl₃ (Merck, Darmstadt, Germany), 100 µL 1 M potassium acetate (Merck, Darmstadt, Germany), and 2.8 mL distilled water were added. Then, incubation was performed for 30 min at 25 °C in the dark. Absorbance values were measured at 415 nm, and TFC was calculated as quercetin equivalent (QE) in µg/mL.

Determination of phenolic compounds by LC-MS analysis

LC-MS analysis was performed with the Agilent HPLC 6430 system (Waldbronn, Germany) equipped with a C18 column (1.8 µm 2.1 × 150 mm) using the method developed by Fischer *et al.* (21). The resolution peaks were recorded on the HPLC chart according to the retention times of the standards prepared with methanol (Isolab, Wertheim, Germany). After dilution, each sample was homogenized with a vortex and centrifuged (Mistral 2000, MSE, UK) at 3350 g for 10 min. The clear

Please note that this is an unedited version of the manuscript that has been accepted for publication. This version will undergo copyediting and typesetting before its final form for publication. We are providing this version as a service to our readers. The published version will differ from this one as a result of linguistic and technical corrections and layout editing.

portion of the samples was collected and passed through 0.45 μm membrane filters (GVS Filter Technology, Stanford, USA) and injected into the LC-MS/MS instrument.

The study was performed in positive and negative ion modes. The mobile phase consisted of 0.01 % (V/V) formic acid (Merck Millipore, Darmstadt, Germany) and 5 μM format in methanol (Merck Millipore, Darmstadt, Germany): Water (5/95 V/V) (Merck Millipore, Darmstadt, Germany) (eluent A) and 0.01 % (V/V) formic acid and 5 μM format in methanol (eluent B). The flow rate was 0.3 mL/min, and the gradient program was optimized as follows: 5 % B isocratic (1 min), 30 % B (2 min), 60 % B (1 min), 60 % B isocratic (1 min), 70 % B (1 min), 80 % B isocratic (2 min), 100 % B (2 min), 5 % B isocratic (2 min), and 5 % B isocratic (5 min). The injection volume of all samples was 3 μL and the total run time was 15 min.

Measurement of free radical scavenging ability (RSA) by DPPH method

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) method described by Von Gadow *et al.* (22) was used to investigate free radical scavenging activity (RSA) of the beverages. Kombucha samples were centrifuged at 1370 g for 10 min (Mistral 2000, MSE, UK) and filtered through a filter with a pore diameter of 0.45 μm (GVS Filter Technology, Stanford, USA). 4 mL of methanolic DPPH solution (Sigma Aldrich, St. Louis, MO, USA) was added to 100 μL of the prepared samples and the reaction mixture was kept in the dark for 30 min. The absorbance of the samples was measured with a spectrophotometer (UV-5100, SOIF, Shanghai, China) at 516 nm and the results were expressed as ascorbic acid equivalents (AAE) in $\mu\text{M}/\text{mL}$.

Determination of antibacterial activity

The disk diffusion method was used to determine the antibacterial activities of the fermented beverages (23). *Bacillus cereus* (DSM 22648), *Escherichia coli* (ATCC 35218), *Klebsiella pneumoniae* (ATCC 13883), *Pseudomonas aeruginosa* (ATCC 27853), *Staphylococcus aureus* (ATCC 29213), and *S. epidermidis* (ATCC 12228) were the bacterial strains used in this study.

Fresh bacterial solutions were adjusted to 0.5 McFarland in NaCl (0.85 % (w/v)) (Merck, Darmstadt, Germany) and spread on Nutrient Agar Medium (NA) (Merck, Darmstadt, Germany). The soup of kombucha samples was collected and centrifuged at 1370 g (Mistral 2000, MSE, UK) for 10 min. The supernatant was then filtered through a sterile filter with a pore diameter of 0.45 μm (GVS Filter Technology, Stanford, USA). 20 μL of the filtered samples were then used to impregnate the empty disks (Bioanalyse, Ankara, Turkey). The disks were placed on inoculated plates. 20 μL ampicillin (30 $\mu\text{g}/\text{mL}$) (Sigma Aldrich, St. Louis, MO, USA) and 20 μL kanamycin (30 $\mu\text{g}/\text{mL}$) (Cayman Chemical, Ann Arbor, MI, USA) were used as positive controls in the experiments. Petri dishes were

Please note that this is an unedited version of the manuscript that has been accepted for publication. This version will undergo copyediting and typesetting before its final form for publication. We are providing this version as a service to our readers. The published version will differ from this one as a result of linguistic and technical corrections and layout editing.

incubated at 37 °C for 24 hours, and the diameters of the zones of inhibition were measured. The experiments were repeated 4 times.

Cell culture and viability assays

HCT116, a human colorectal carcinoma cell line, was cultured in RPMI 1640 medium without phenol red (Biological Industries, Beit-Haemek, Israel) supplemented with 10 % Fetal Bovine Serum (FBS) (Biowest, Nuaille, France), 1 % penicillin-streptomycin (Pen/Strep) (Biological Industries, Beit-Haemek, Israel) and 2mM L-glutamine (Biological Industries, Beit-Haemek, Israel). The human hepatocellular carcinoma cell line Mahlavu was cultured in Dulbecco's Modified Eagle Medium (DMEM) (Biological Industries, Beit-Haemek, Israel) containing 10 % FBS, 1 % Pen/Strep, and 1 % non-essential amino acids (Biological Industries, Beit-Haemek, Israel). Cells were grown as monolayers in an incubation chamber at 37 °C under a humidified atmosphere with 5 % CO₂. To investigate the effects of kombucha samples on cell viability and proliferation, the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) (Sigma-Aldrich, Taufkirchen, Germany) assay was performed as described previously (24). Briefly, in a final volume of 100 µl growth medium, Mahlavu and HCT116 cells were plated in microtiter plates at a density of 1x10³ and 1x10⁴ cell/well, respectively. The following day, the existing growth media was replaced with fresh media supplemented with kombucha beverages (0 - 4000 µg/mL), and the cells were incubated for 72 h. At the end of this incubation period, 10 µl of MTT solution, prepared at a concentration of 5 mg/mL in Dulbecco's Phosphate Buffered Saline (Biological Industries, Beit-Haemek, Israel) was added to the cells and the cells were incubated for an additional 4 h. Subsequently, the cells were incubated for another 24 h in the presence of 0.1 % SDS-HCL and absorbance was measured at 570 nm using a microplate spectrophotometer (Multiskan GO, Thermo Scientific, Waltham, USA). All experiments were performed at least in triplicate with five technical replicates and cell viability (%) was calculated using the following Eqs. 2:

$$\% \text{ Cell Viability} = \frac{A_{570} \text{ of treated cells} - A_{570} \text{ of blank sample}}{A_{570} \text{ of control cells} - A_{570} \text{ of blank sample}} \times 100 \quad /2/$$

Sensory evaluation

Twenty individuals between the ages of 19-40 years were selected from the Akdeniz University campus for sensory evaluation of the samples. The participants were not informed about the contents of the beverages, since the sensory analyses were blind tests. The analysis was performed according to the techniques described by Altuğ (25), and the beverages were rated from 1 (very poor) to 5 (very

Please note that this is an unedited version of the manuscript that has been accepted for publication. This version will undergo copyediting and typesetting before its final form for publication. We are providing this version as a service to our readers. The published version will differ from this one as a result of linguistic and technical corrections and layout editing.

good). Participants rated the visual and olfactory characteristics of the beverages. Sensory analysis experiments were carried out in accordance with the Declaration of Helsinki (26).

Statistical analysis

Results were presented as mean \pm standard deviation of three independent experiments. All statistical analyses were performed using Oneway ANOVA in IBM SPSS 22 software (SPSS, USA) and Tukey's HSD and Tamhane's T2 tests (27). IC₅₀ values were evaluated by analysis of variance followed by an appropriate posthoc test (Duncan test), and differences were considered statistically significant at $p < 0.05$. The relationship between the measured biological potentials (total phenolic content, flavonoid content, and antioxidant activity) and the relationship between these biological potentials and the identified compounds were investigated by Spearman's correlation analysis (28).

RESULTS AND DISCUSSION

pH variations

After 14 days of incubation, the pH values in the herbal infusions decreased; however, the changes in the pH values of the kombucha samples were considerably more remarkable (Table 1), most likely because of the conversion of sucrose into organic acid during fermentation (3). Marsh *et al.* (13) discovered that at the end of a 10-day fermentation the pH of kombucha cultures ranged between 3 to 3.50, which is also consistent with our results. According to Cardoso *et al.* (29) the pH discrepancies between fermented kombucha samples might be attributable to the predominance of distinct types of acetic acid and lactic acid bacteria. At the end of fermentation, the pH values of kombucha samples in our study varied from 2.7 to 3.6 (Table 1). According to Nummer (30), these values are considered safe for human consumption. pH values above 4.2 may question the microbiological safety of the fermented product and pH values below 2.5 may be harmful to consumers due to the high concentration of organic acid.

Profiling of microbiological composition

On day 0, the number of acetic acid bacteria, total mesophilic bacteria, yeast, and *Lactobacillus* ranged from 1.7×10^3 to 7.4×10^3 CFU/mL. After the 14-day fermentation period, the number of microorganisms increased to 10^7 in all samples, except for hawthorn-flavoured kombucha, in which the total number of mesophilic bacteria and yeasts was lower compared to the other samples (5.5×10^6 CFU/mL and 9.6×10^5 CFU/mL, respectively) (Table 2).

Please note that this is an unedited version of the manuscript that has been accepted for publication. This version will undergo copyediting and typesetting before its final form for publication. We are providing this version as a service to our readers. The published version will differ from this one as a result of linguistic and technical corrections and layout editing.

Neffe-Skocińska *et al.* (31) indicated that the amount of acetic acid bacteria and yeast was around 10^4 CFU/mL before fermentation and increased to 10^7 CFU/mL after 10 days of fermentation. However, Cardoso *et al.* (29) reached 10^5 - 10^6 CFU/mL of lactic acid bacteria, mesophilic bacteria, and yeast and acetic acid bacteria. It can be speculated that the increase in microbiological count depends on the sugar content and SCOBY composition used in the kombucha cultures.

Bacterial cellulose production

On day 0, the dry mass of SCOBY inoculated to all samples was 1.4 g/L. After 14 days of fermentation, it was found that cellulose production was highest in the traditional kombucha (12.8 ± 0.7 g/L). Among the herb flavoured kombucha samples, the highest cellulose production was detected in fermented hawthorn tea (10.23 ± 0.51 g/L). The lowest cellulose production was found in fermented hops flavoured tea (8.3 ± 0.36 g/L) and fermented madimak tea (7.9 ± 0.46 g/L). However, there was no statistically significant difference in the amount of cellulose produced by the samples on day 14 ($p > 0.05$). It is well known that cellulose production in kombucha fermentation depends on the carbon source. AL-Kalifawi and Hassan (32) showed that bacterial cellulose production was higher in kombucha samples prepared with 10 g/L black tea than with 5 g/L black tea. They also obtained the highest pellicle production in kombucha samples prepared with 100 g/L sucrose.

Total phenolic (TPC) and total flavonoid (TFC) content

On day 0, the highest total phenolic content among the herbal flavoured kombucha samples was observed in madimak-flavoured kombucha ($1469 \mu\text{g/mL GAE}$) (Table 3). The TPC of madimak tea ($633 \mu\text{g/mL GAE}$) was also higher compared to other infusions tested except B (Table 3). After 14 days of fermentation, total phenolic content was highest in madimak-flavoured kombucha samples ($3151 \mu\text{g/mL GAE}$) ($p < 0.05$). Velićanski *et al.* (33), Shahbazi *et al.* (7) and Vitas *et al.* (8) demonstrated that the phenolic compound content in flavoured kombucha samples increased with fermentation. Bhattacharya *et al.* (34) suggested that the enzymes released by bacteria and yeast degrade polyphenols and increase the number of phenolic compounds during fermentation. On the other hand, Amarasinghe *et al.* (35) showed that the total phenolic content of the fermented kombucha samples did not increase significantly with fermentation and hypothesised that the reduction in total phenolic content might be related to the phenolic compounds used by the kombucha microorganisms. According to Güldane *et al.* (4) many factors including cultivation area, climatic conditions, and quality of agricultural practises can affect the total phenolic content of tea used for fermentation. It should

Please note that this is an unedited version of the manuscript that has been accepted for publication. This version will undergo copyediting and typesetting before its final form for publication. We are providing this version as a service to our readers. The published version will differ from this one as a result of linguistic and technical corrections and layout editing.

also be considered that the antioxidant activities of kombucha are not always determined by the amount of total phenolic content, but the types of metabolites produced during fermentation may have a decisive effect (35, 36).

It was found that the total flavonoid content of traditional kombucha, hawthorn-flavoured kombucha and hops flavoured kombucha samples increased significantly with fermentation ($p < 0.05$) (Table 3). At the end of the fermentation period, the traditional kombucha had a total flavonoid content of 77 $\mu\text{g/mL}$ QE, the hawthorn-flavoured kombucha of 43 $\mu\text{g/mL}$ QE and the hops-flavoured kombucha of 46 $\mu\text{g/mL}$ QE. Although the highest level of total flavonoids was found in the madimak-flavoured kombucha beverage (109 $\mu\text{g/mL}$ QE) before fermentation, no significant difference was found between the unfermented and fermented madimak-flavoured kombucha samples ($p > 0.05$). Similarly, madimak had the highest total flavonoid content among teas (Table 3). There was also a moderate correlation between the total phenolic and flavonoid substance of the samples ($r = 0.58$, $p < 0.05$). In their study, Vitas *et al.* (8) prepared alternative kombucha drinks from six medicinal plants, namely winter savoury, peppermint, nettle, wild thyme, elderberry, and quince. They showed that alternative kombucha drinks prepared with winter savoury, peppermint and nettle had higher total flavonoid content than conventional products. In the same study, the researchers found that the total flavonoid content of kombucha samples prepared with quince decreased with fermentation. Shahbazi *et al.* (7) also demonstrated that the flavonoid content of kombucha samples fermented with medicinal herbs increased significantly. According to the researchers this increase could be explained by the production of catechin and epicatechin isomers.

LC-MS analysis of phenolic compounds

After fermentation, gallic acid was the major phenolic compound in all kombucha samples, except for the madimak-flavoured kombucha (Table 4). However, chlorogenic acid was the highest phenolic compound in madimak-flavoured kombucha. Caffeic acid and vitexin were higher in traditional kombucha in comparison to other samples. On the other hand, epicatechin was found to be higher in hawthorn-flavoured kombucha. Rutin and protocatechuic acid contents did not significantly differ between the beverages ($p > 0.05$). Some phenolic acids such as caffeic acid, rutin, protocatechuic acid, vitexin are known to be antioxidant, antibacterial, antimutagenic and anticarcinogenic agents (37). Gallic acid is important in terms of its anti-obesity properties (e.g. enhancement of insulin signalling, suppression of lipogenesis), as well as for anticancer, gastrointestinal mucosal protection, and antibacterial properties (37). The results showed that, caffeic acid and rutin are positively correlated with antioxidant capacity (Fig. S1), but negatively correlated with phenolic and flavonoid content (Fig. S2 and Fig. S3, respectively). On the contrary, chlorogenic

Please note that this is an unedited version of the manuscript that has been accepted for publication. This version will undergo copyediting and typesetting before its final form for publication. We are providing this version as a service to our readers. The published version will differ from this one as a result of linguistic and technical corrections and layout editing.

acid and protocatechuic acid correlated negatively with antioxidant capacity (Fig. S1) and positively with phenolic and flavonoid content (Fig. S2 and Fig. S3, respectively). Vitexin showed a negative correlation with phenolic content (Fig. S2), whereas it showed a positive correlation with the antioxidants and flavonoids (Fig. S1 and Fig. S3 respectively). Interestingly, epicatechin and gallic acid were found to be negatively correlated with all 3 biological potentials. As is well known, the phenolic compounds identified in this study by the LC analysis are only a few of the many substances found in kombucha. And it is quite possible that these substances work in concert with one another. As a result, the direct positive correlation of the mentioned phenolic compounds on the biological activities of kombucha may not always be clear. This study may not have identified phenolic compounds in kombucha that correlate strongly with other biological potentials.

In their study, Borges *et al.* (38) pointed out the relationship between gallic acid and antimicrobial activity, cell surface hydrophobicity, K⁺ leakage, charge and induced propidium iodide (PI) uptake. Chlorogenic acid, is one of the most available and naturally occurring phenolic acids in tea and green coffee (39). The pronounced radical scavenging activity of chlorogenic acid and its polyphenol degradation products was already demonstrated by Bøhn *et al.* (40) and was significantly high in madimak-flavoured kombucha beverages in our study. Chlorogenic acid is known to have antimicrobial activity, but is not effective against Gram-positive lactic acid bacteria, making it a food additive and preservative (41). The presence of chlorogenic acid in madimak-flavoured kombucha not only explains the high antibacterial activity of the beverage, but also increases the beverage's long shelf life potential due to its food preservative properties.

Free radical scavenging ability (RSA)

The traditional kombucha beverage fermented for 14 days showed the highest antioxidant activity (655 $\mu\text{M}/\text{mL}$) among all samples, followed by hops-flavoured kombucha (634 $\mu\text{M}/\text{mL}$) (Table 3). It was found that the antioxidant activity of traditional kombucha, hawthorn-flavoured kombucha, and madimak-flavoured kombucha increased significantly with fermentation ($p < 0.05$). There was a significant difference between the antioxidant activities of the unfermented hawthorn-flavoured kombucha and fermented hawthorn-flavoured kombucha (548 $\mu\text{M}/\text{mL}$ AAE and 610 $\mu\text{M}/\text{mL}$ AAE, respectively) ($p < 0.05$). Similarly, the madimak-flavoured kombucha and the traditional kombucha showed higher antioxidant activity (626 $\mu\text{M}/\text{mL}$ AAE and 655 $\mu\text{M}/\text{mL}$ AAE, respectively) compared to their non-fermented control. Moreover, the antioxidant activity of the madimak tea (568 $\mu\text{M}/\text{mL}$ AAE) and black tea (553 $\mu\text{M}/\text{mL}$ AAE) was significantly higher among all herbal infusions after 14 days (Table 3). In general, it can be concluded that the presence of black tea in kombucha cultures is associated with increased radical scavenging activity. In addition, it was found that there was a strong

Please note that this is an unedited version of the manuscript that has been accepted for publication. This version will undergo copyediting and typesetting before its final form for publication. We are providing this version as a service to our readers. The published version will differ from this one as a result of linguistic and technical corrections and layout editing.

correlation between the increase in total flavonoid content and the increase in antioxidant activity, and this correlation was statistically significant ($r=0.69$, $p<0.05$). However, the results of antioxidant capacity and total phenolic content showed that there was a weak correlation between these two tests, which was not statistically significant ($r=0.49$, $p>0.05$).

Chu and Chen (36) showed that the DPPH activity of various Taiwanese household kombucha samples increased with fermentation. However, on 10th day of fermentation, the increase was significant only for certain samples. The researchers concluded that the differences in the microbiota of the kombucha samples were responsible for the different radical scavenging activity. Tanticharakunsiri *et al.* (42) showed that radical scavenging activities of oolong tea and kitchen mint kombucha samples increased during the fermentation period and exhibited the highest antioxidant capacity on day 14 of fermentation. Shahbazi *et al.* (7) demonstrated that the radical scavenging activity, total phenolic and flavonoid content of kombucha samples flavoured with Shirazi thyme, cinnamon, and cardamom increased with 16 days fermentation. However, Vitas *et al.* (8) showed that the antioxidant activity of winter savory-, green tea-, and stinging nettle-flavoured kombuchas increased until the third day of fermentation and then decreased. In addition, the antioxidant activity of wild thyme-, elderberry-, quince-, and peppermint-flavoured kombuchas was high at the beginning of fermentation and decreased continuously with fermentation (8). It has already been shown that the microbial composition of the SCOBY has a great effect on the resulting beverage (35). It is known that the higher antioxidant activity of fermented kombucha compared to its non-fermented form is due to the bacteria and yeast enzymes causing structural changes in the polyphenols in the tea and the production of low molecular weight components. The antioxidant activities in kombucha cultures show time-dependent changes and a long fermentation time is not recommended due to the harmful accumulation of organic acids (43). Therefore, the fermentation duration was limited to 14 days in this study.

Antibacterial activity

In order to evaluate the antibacterial activity of the samples, the inhibition zones around the sample disks (in mm) were measured (Table 5). None of the kombucha beverages showed any antibacterial activity on day 0. After 14 days of fermentation, the traditional kombucha showed antibacterial activity against *K. pneumoniae*, *B. cereus*, *S. epidermidis*, and *E. coli*, and the madimak-flavoured kombucha showed antibacterial activity against all six bacterial strains. Furthermore, madimak-flavoured kombucha formed a larger zone of inhibition against *P. aeruginosa* than kanamycin ($p<0.05$). Hops flavoured kombucha showed antibacterial activity against *K. pneumoniae*,

Please note that this is an unedited version of the manuscript that has been accepted for publication. This version will undergo copyediting and typesetting before its final form for publication. We are providing this version as a service to our readers. The published version will differ from this one as a result of linguistic and technical corrections and layout editing.

B. cereus, *P. aeruginosa*, *S. epidermidis*, *E. coli*, and hawthorn-flavoured kombucha showed antibacterial activity against *K. pneumonia*, *S. aureus*, *B. cereus*, *S. epidermidis*, and *E. coli*. In contrast to ampicillin, which had no antibacterial activity against *E. coli*, all the herb-flavoured kombucha samples were effective against *E. coli* ($p < 0.05$). Besides, all flavoured kombucha samples formed a larger zone of inhibition than ampicillin against *K. pneumonia*. The zone size for hawthorn-flavoured kombucha and madimak-flavoured kombucha was statistically significant compared with ampicillin.

Sreeramulu *et al.* (3) observed that non-fermented black tea and traditional kombucha had no antibacterial activity on bacterial strains except *Campylobacter jejuni*. Previously, black tea kombucha fermented for 10 days was shown to inhibit the growth of *S. aureus* and *Listeria monocytogenes* (minimum inhibitory concentration: 250 $\mu\text{L/mL}$), whereas it had no antibacterial activity against *E. coli* and *Salmonella* (29). These differences could be due to the adoption of different parameters in kombucha fermentation, such as the origin of the SCOBY, the amount of soup, fermentation time, sugar concentration, and temperature, as these factors may lead to antibacterial compounds (bacteriocins, organic acids, enzymes, proteins *etc.*) being produced at different concentrations (29).

Cytotoxic effects of kombucha beverages on cancer cells

Anti-proliferative potential of traditional kombucha and kombucha beverages flavoured with different medicinal plants was investigated in two different cancer cell lines, HCT116 and Mahlavu. The efficacy of the kombuchas was assessed by measuring the percentage of surviving cells after 72 hours of incubation with different concentrations of the test compounds. The IC_{50} values of kombucha beverages are presented in Table 6. The results indicate that Mahlavu cells are generally more resistant to the cytotoxic effect of kombucha beverages compared to HCT116 cells. The only exception to this generalisation was hops-flavoured kombucha, which had a higher cytotoxic effect on Mahlavu cells. Fig. 1 also shows the effects of the tested kombucha beverages on cell viability in a dose-dependent manner. As it can be seen, extremely low concentrations of kombucha drinks (*i.e.* 31.25 $\mu\text{g/mL}$) slightly enhanced cell viability, while higher doses of kombucha beverages exhibited dose-dependent cytotoxic effects on Mahlavu and HCT116 cells. Cancer is known to be one of the leading causes of death worldwide (44), and this fact underscores the importance of research to develop novel molecules/drugs with anti-cancer properties. Much research in recent years has focused on the anti-cancer properties of kombucha as a functional beverage (45,46). It is known that the anticancer properties of kombucha vary depending on parameters such as the content of microorganisms in the symbiotic culture used for kombucha fermentation, fermentation time, sucrose

Please note that this is an unedited version of the manuscript that has been accepted for publication. This version will undergo copyediting and typesetting before its final form for publication. We are providing this version as a service to our readers. The published version will differ from this one as a result of linguistic and technical corrections and layout editing.

content, temperature, type of tea and the presence of herbal infusions (47). Herein, we investigated the growth inhibitory potential of traditional kombucha and kombucha beverages flavoured with hops, madimak, and hawthorn on the hepatocellular carcinoma cell line Mahlavu and the colorectal carcinoma cell line HCT116. The results revealed that each kombucha beverage had a strong cytotoxic effect on both cell lines. Madimak-flavoured kombucha proved to be more effective on HCT116 cells, while hops-flavoured kombucha had a stronger cytotoxic effect on Mahlavu cells. These antiproliferative effects of kombucha beverages could be due to polyphenols or secondary metabolites produced during the fermentation process (48). Nevertheless, further studies are needed on the toxicity of kombucha on various cancer cell lines and normal cell lines. In a recent study it was also revealed that the anti-cancer effect of doxorubicin on cancer cells increased in the presence of kombucha (49). In this context, it would be beneficial to investigate the combinatorial use of different chemotherapeutic agents together with kombucha fermented in the presence/absence of various herbal infusions.

Hedonic ratings of sensory evaluation

Participants rated kombucha beverages after 14 days of fermentation on a 5-point hedonic scale for appearance, odour, acidity, taste, and overall quality (Fig. 2). The test participants stated that the fermented beverages produced were generally blurry and had a pungent odour and taste. Traditional kombucha and hawthorn-flavoured kombucha were rated as the tastiest of all beverages (3.7). The odour of the kombucha samples was generally disliked by the participants. The odour (2.4) and appearance (2.9) of the hawthorn-flavoured kombucha received the best scoring among the samples. In general, the hawthorn-flavoured kombucha was perceived as the most pleasant drink according to the results of the sensory analysis (2.8). Ulusoy and Tamer (50) indicated that a shorter fermentation time leads to a slight decrease in the sugar content of the beverage, making subjects more likely to enjoy the taste of the beverage. The taste of the drink is likely to be appreciated by the participants because a short fermentation time prevents the formation of a vinegar taste caused by organic acids. Therefore, it is probable that the 14-day fermentation period was what led to the beverages in our study received a low taste rating.

CONCLUSIONS

The effects of three medicinal plants, namely hawthorn, hops, and madimak on the antimicrobial and antiproliferative properties, bioactivity, microbial profile, sensory properties, and cellulose production of kombucha beverage were examined in this study. Although the antioxidant activities and the total amount of phenolic substances in the three alternative kombucha beverages

Please note that this is an unedited version of the manuscript that has been accepted for publication. This version will undergo copyediting and typesetting before its final form for publication. We are providing this version as a service to our readers. The published version will differ from this one as a result of linguistic and technical corrections and layout editing.

were close, the hops-flavoured kombucha showed a strong cytotoxic effect on Mahlavu cells. Hawthorn-flavoured kombucha was rated as the most delicious drink by the participants. Madimak-flavoured kombucha also appears to be a promising product among kombucha beverages due to its strong antibacterial activity and high phenolic and flavonoid content. It also had a strong cytotoxic effect on HCT116 cell line. However, the results of the sensory analysis indicate that the sensory characteristics of all herb-flavoured kombucha beverages need to be improved to appeal to consumers. For instance, increasing the gluconic acid ratio of the beverages, determining the sugar, alcohol, and organic acid content, and determining the conditions of the fermentation process and their effects on sensory properties may be effective in improving the flavour quality and control of kombucha. In addition, the effects of these kombucha products on model organisms can be studied and their health-promoting effects commented on.

ACKNOWLEDGEMENTS

We thank M.Sc. Side Selin Su Yirmibeşoğlu and Asst. Prof. Dr. Gülgün Gündüz for their support.

FUNDING

This research received no specific grants from public, commercial, or nonprofit entities.

CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

SUPPLEMENTARY MATERIALS

Supplementary materials are available at: www.ftb.com.hr.

AUTHORS' CONTRIBUTION

Burcu Emine TEFON ÖZTÜRK, Berfin EROĞLU and Eda DELİK designed/performed the experiments, processed/interpreted the data, took part in the preparation, writing/revision of the article. Mustafa ÇİÇEK processed/interpreted the data, took part in the preparation, writing/revision of the article. Esra ÇİÇEK processed/interpreted the data, took part in the preparation of manuscript.

ORCID ID

B.E. Tefon Öztürk <https://orcid.org/0000-0003-1690-9879>

B. Eroğlu <https://orcid.org/0000-0002-9099-7603>

Please note that this is an unedited version of the manuscript that has been accepted for publication. This version will undergo copyediting and typesetting before its final form for publication. We are providing this version as a service to our readers. The published version will differ from this one as a result of linguistic and technical corrections and layout editing.

E. Delik <https://orcid.org/0000-0002-9047-2874>

M. Çiçek <https://orcid.org/0000-0002-7109-6500>

E. Çiçek <https://orcid.org/0000-0002-7364-4435>

REFERENCES

1. Battikh H, Bakhrouf A, Ammar E. Antimicrobial effect of kombucha analogues. *Lwt-Food Sci Technol.* 2012;47(1):71-7.
<https://doi.org/10.1016/j.lwt.2011.12.033>
2. Laureys D, Britton SJ and De Clippeleer J. Kombucha tea fermentation: A review. *J Am Soc Brew Chem.* 2020;78(3):165-174.
<https://doi.org/10.1080/03610470.2020.1734150>
3. Sreeramulu G, Zhu Y, Knol W. Kombucha fermentation and its antimicrobial activity. *J Agr Food Chem.* 2000;48(6):2589-94.
<https://doi.org/10.1021/jf991333m>.
4. Güldane M, Bayram M, Topuz S, Kaya C, Gök H, Bülbül M, *et al.* Determination of some properties in kombucha produced with white, black and green tea. *JAFAG* 2017;34(1):46-56.
5. Ayed L, Hamdi M. Manufacture of a beverage from cactus pear juice using “tea fungus” fermentation. *Ann Microbiol.* 2015;65(4):2293-9.
<https://doi.org/10.1007/s13213-015-1071-8>
6. Eroğlu B, Delik E, Tefon Öztürk BE. Investigation of biological activities and sensory properties of chasteberry flavoured kombucha. 12th International Scientific Research Congress; 2021 December 17–18; Ankara, Turkey; 2021. pp. 2-13.
7. Shahbazi H, Hashemi Gahrue H, Golmakani MT, Eskandari MH, Movahedi M. Effect of medicinal plant type and concentration on physicochemical, antioxidant, antimicrobial, and sensorial properties of kombucha. *Food Sci Nutr.* 2018;6(8):2568-77.
<https://doi.org/10.1002/fsn3.873>
8. Vitas J, Vukmanović S, Čakarević J, Popović L, Malbaša R. Kombucha fermentation of six medicinal herbs: Chemical profile and biological activity. *Chem Ind Chem Eng Q.* 2019(00):34-34.
<https://doi.org/10.2298/CICEQ190708034V>
9. Zanolli P, Zavatti M. Pharmacognostic and pharmacological profile of *Humulus lupulus* L *J Ethnopharmacol.* 2008;116(3):383-96.
<https://doi.org/10.1016/j.jep.2008.01.011>
10. Evrendilek GA. Empirical prediction and validation of antibacterial inhibitory effects of various plant essential oils on common pathogenic bacteria. *Int J Food Microbiol.* 2015;202: 35-41.

Please note that this is an unedited version of the manuscript that has been accepted for publication. This version will undergo copyediting and typesetting before its final form for publication. We are providing this version as a service to our readers. The published version will differ from this one as a result of linguistic and technical corrections and layout editing.

<https://doi.org/10.1016/j.ijfoodmicro.2015.02.030>

11. Yeşilyaprak GN. (2017). Presentation of the biological characteristics of some plant species offered for sale. Master's thesis, Kütahya Dumlupınar Üniversitesi/Fen Bilimleri Enstitüsü (in Turkish).

12. Keskin N, Mammadov R, İli P. Investigation of *Crataegus aronia* variety *dentata* Browicz extract on spleen: Histochemical study. Pamukkale Medicine Journal 2012(2):68-74 (in Turkish).

13. Marsh AJ, O'Sullivan O, Hill C, Ross RP, Cotter PD. Sequence-based analysis of the bacterial and fungal compositions of multiple kombucha (tea fungus) samples. Food Microbiol. 2014;38:171-8.

<https://doi.org/10.1016/j.fm.2013.09.003>

14. Coton M, Pawtowski A, Taminiau B, Burgaud G, Deniel F, Coulloume-Labarthe L, *et al.* Unraveling microbial ecology of industrial-scale kombucha fermentations by metabarcoding and culture-based methods. FEMS Microbiol. Ecol. 2017;93(5).

<https://doi.org/10.1093/femsec/fix048>

15. El-Salam SSA. 16S rRNA gene sequence detection of acetic acid bacteria isolated from tea kombucha. New York Sci J. 2012;5:55-61.

16. Halkman KA. Food microbiology and its applications. 2nd Ed. 6. Ankara, Turkey: Ankara University Faculty of Agriculture Food Engineering Department Publishing; 2000 (in Turkish).

17. Keshk SM, Sameshima K. Evaluation of different carbon sources for bacterial cellulose production. Afr J Biotechnol. 2005;4(6):478-82.

18. Škerget M, Kotnik P, Hadolin M, Hraš AR, Simonič M, Knez Ž. Phenols, proanthocyanidins, flavones and flavonols in some plant materials and their antioxidant activities. Food Chem. 2005;89(2):191-8.

<https://doi.org/10.1016/j.foodchem.2004.02.025>

19. Singleton VL, Rossi JA. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. AJEV. 1965;16(3):144-58.

20. Dwiputri MC, Feroniasanti YL, editors. Effect of fermentation to total titrable acids, flavonoid and antioxidant activity of butterfly pea kombucha. JPCS, IOP Publishing; 2019: p 012014.

<https://doi.org/10.1088/1742-6596/1241/1/012014>

21. Fischer UA, Carle R, Kammerer DR. Identification and quantification of phenolic compounds from pomegranate (*Punica granatum* L.) peel, mesocarp, aril and differently produced juices by HPLC-DAD-ESI/MSⁿ. Food Chem. 2011;127(2): 807-821.

<https://doi.org/10.1016/j.foodchem.2010.12.156>

22. Von Gadow A, Joubert E, Hansmann C. Comparison of the antioxidant activity of rooibos tea (*Aspalathus linearis*) with green, oolong and black tea. Food Chem. 1997;60(1):73-7.

[https://doi.org/10.1016/S0308-8146\(96\)00312-3](https://doi.org/10.1016/S0308-8146(96)00312-3)

Please note that this is an unedited version of the manuscript that has been accepted for publication. This version will undergo copyediting and typesetting before its final form for publication. We are providing this version as a service to our readers. The published version will differ from this one as a result of linguistic and technical corrections and layout editing.

23. Bauer AW. Antibiotic susceptibility testing by single disc method. *Am J Clin Pathol.* 1996;45(4): 149-158.
24. Akhavantabasi S, Akman, HB, Sapmaz A, Keller J, Petty EM, Erson AE. USP32 is an active, membrane-bound ubiquitin protease overexpressed in breast cancers. *Mamm Genome.* 2010;21(7–8):388–397.
<https://doi.org/10.1007/s00335-010-9268-4>
25. Altuğ T. Sensory test techniques. Ege University; 1993 (in Turkish).
26. Anon. WMA Declaration of Helsinki – Ethical Principles for Medical Research Involving Human Subjects. 2017. Available from: <https://www.wma.net/policies-post/wma-declaration-of-helsinki-ethical-principles-for-medical-research-involving-human-subjects/>
27. IBM. IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: International Business Machines Corporation; 2013. Available from: <https://www.ibm.com/tr-tr/products/spss-statistics>
28. Yang J, Lagishetty V, Kurnia P, Henning SM, Ahdoot AI, Jacobs JP. Microbial and chemical profiles of commercial kombucha products. *Nutrients.* 2022;14(3):670.
<https://doi.org/10.3390/nu14030670>
29. Cardoso RR, Neto RO, dos Santos D'Almeida CT, do Nascimento TP, Pressete CG, Azevedo L, *et al.* Kombuchas from green and black teas have different phenolic profile, which impacts their antioxidant capacities, antibacterial and antiproliferative activities. *Food Res Int.* 2020;128:108782.
<https://doi.org/10.1016/j.foodres.2019.108782>
30. Nummer BA. Special report: Kombucha brewing under the food and drug administration model food code: Risk analysis and processing guidance. *J Environ Health.* 2013;76:8-11.
31. Neffe-Skocinska K, Sionek B, Scibisz I, Kolozyn-Krajewska D. Acid contents and the effect of fermentation condition of kombucha tea beverages on physicochemical, microbiological and sensory properties. *Cyta-J Food.* 2017;15(4):601-7.
<https://doi.org/10.1080/19476337.2017.1321588>
32. AL-Kalifawi EJ, Hassan IA. Factors influence on the yield of bacterial cellulose of kombucha (Khubdat Humza). *Baghdad Sci J.* 2014;11:1420-1428.
33. Velićanski AS, Cvetković DD, Markov SL, Tumbas Šaponjac VT, Vulić JJ. Antioxidant and antibacterial activity of the beverage obtained by fermentation of sweetened lemon balm (*Melissa officinalis* L.) tea with symbiotic consortium of bacteria and yeasts. *Food Technol Biotechnol.* 2014;52(4):420-9.
<https://doi.org/10.17113/ftb.52.04.14.3611>

Please note that this is an unedited version of the manuscript that has been accepted for publication. This version will undergo copyediting and typesetting before its final form for publication. We are providing this version as a service to our readers. The published version will differ from this one as a result of linguistic and technical corrections and layout editing.

34. Bhattacharya S, Gachhui R, Sil PC. Hepatoprotective properties of kombucha tea against TBHP-induced oxidative stress via suppression of mitochondria dependent apoptosis. *Pathophysiology*. 2011;18(3):221-34.

<https://doi.org/10.1016/j.pathophys.2011.02.001>

35. Amarasinghe H, Weerakkody NS, Waisundara VY. Evaluation of physicochemical properties and antioxidant activities of kombucha “tea fungus” during extended periods of fermentation. *Food Sci Nutr*. 2018;6:659-665.

<https://doi.org/10.1002/fsn3.605>

36. Chu S-C, Chen C. Effects of origins and fermentation time on the antioxidant activities of kombucha. *Food Chem*. 2006;98(3):502-507.

<https://doi.org/10.1016/j.foodchem.2005.05.080>

37. Bortolomedi BM, Paglarin CS, Brod, FCA. Bioactive compounds in kombucha: A review of substrate effect and fermentation conditions. *Food Chem*. 2022;385:132719.

<https://doi.org/10.1016/j.foodchem.2022.132719>

38. Borges A, Ferreira C, Saavedra MJ, Simoes M. Antibacterial activity and mode of action of ferulic and gallic acids against pathogenic bacteria. *Microb Drug Resist*. 2013;19(4):256-265.

<https://doi.org/10.1089/mdr.2012.0244>

39. Naveed M, Hejazi V, Abbas M, Kamboh AA, Khan GJ, Shumzaid M, WenHua L. Chlorogenic acid (CGA): A pharmacological review and call for further research. *Biomed Pharmacother*. 2018;97:67-74.

<https://doi.org/10.1016/j.biopha.2017.10.064>

40. Bøhn SK, Blomhoff R, Paur I. Coffee and cancer risk, epidemiological evidence, and molecular mechanisms. *Mol Nutr Food Res*. 2014;58(5): 915-930.

<https://doi.org/10.1002/mnfr.201300526>

41. Puupponen-Pimiä R, Nohynek L, Meier C, Kähkönen M, Heinonen M, Hopia A, Oksman-Caldentey KM. Antimicrobial properties of phenolic compounds from berries. *J Appl Microbiol*. 2001;90(4):494-507.

<https://doi.org/10.1046/j.1365-2672.2001.01271.x>

42. Tanticharakunsiri W, Mangmool S, Wongsariya K, Ochaikul D. Characteristics and upregulation of antioxidant enzymes of kitchen mint and oolong tea kombucha beverages. *J of Food Biochem*. 2021;45(1):e13574.

<https://doi.org/10.1111/jfbc.13574>

Please note that this is an unedited version of the manuscript that has been accepted for publication. This version will undergo copyediting and typesetting before its final form for publication. We are providing this version as a service to our readers. The published version will differ from this one as a result of linguistic and technical corrections and layout editing.

43. Jayabalan R, Malbaša RV, Lončar ES, Vitas JS, Sathishkumar M. A review on kombucha tea—microbiology, composition, fermentation, beneficial effects, toxicity, and tea fungus. *Compr Rev Food Sci Food Saf.* 2014;13(4):538-550.

<https://doi.org/10.1111/1541-4337.12073>

44. Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A, Bray F. Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *Ca-Cancer J Clin.* 2021;71(3):209–249.

<https://doi.org/10.3322/caac.21660>

45. Jayabalan R, Chen P-N, Hsieh YS, Prabhakaran K, Pitchai P, Marimuthu S, *et al.* Effect of solvent fractions of kombucha tea on viability and invasiveness of cancer cells-characterization of dimethyl 2-(2-hydroxy-2-methoxypropylidene) malonate and vitexin. *Indian J Biotechnol.* 2011;10:75–82.

46. Srihari T, Arunkumar R, Arunakaran J, Satyanarayana U. Downregulation of signalling molecules involved in angiogenesis of prostate cancer cell line (pc-3) by kombucha (lyophilized). *Biomed Prev Nutr.* 2013;3(1):53–58.

<https://doi.org/10.1016/j.bionut.2012.08.001>

47. Martínez Leal J, Valenzuela Suárez L, Jayabalan R, Huerta Oros J, Escalante-Aburto A. A review on health benefits of kombucha nutritional compounds and metabolites. *CYTA J Food.* 2018;16(1):390–399.

<https://doi.org/10.1080/19476337.2017.1410499>

48. Mousavi SM, Hashemi SA, Zarei M, Gholami A, Lai CW, Chiang WH, *et al.* Recent progress in chemical composition, production, and pharmaceutical effects of kombucha beverage: A complementary and alternative medicine. *Evid.-based Complement Altern Med.* 2020;2020.8

<https://doi.org/10.1155/2020/4397543>

49. Rasouli L, Aryaeian N, Gorjian M, Nourbakhsh M, Amiri F. Evaluation of cytotoxicity and anticancer activity of kombucha and doxorubicin combination therapy on colorectal cancer cell line HCT-116. *J Educ Health Promot.* 2021;10:376.

https://doi.org/10.4103/jehp.jehp_1456_20

50. Ulusoy A, Tamer CE. Determination of suitability of black carrot (*Daucus carota* L. spp. *sativus* var. *atrorubens* Alef.) juice concentrate, cherry laurel (*Prunus laurocerasus*), blackthorn (*Prunus spinosa*) and red raspberry (*Rubus idaeus*) for kombucha beverage production. *J Food Meas Charact.* 2019;13(2):1524-1536.

<https://doi.org/10.1007/s11694-019-00068-w>

Please note that this is an unedited version of the manuscript that has been accepted for publication. This version will undergo copyediting and typesetting before its final form for publication. We are providing this version as a service to our readers. The published version will differ from this one as a result of linguistic and technical corrections and layout editing.

Table 1. pH values of 0 and 14 days of kombucha cultures and tea infusions

Samples	Length of fermentation (days)	
	0	14
Traditional kombucha	(3.8±0)	(2.8 ±0) ^{*a}
Hawthorn-flavoured kombucha	(4.1±0)	(3.1±0.1) ^{*b}
Hops-flavoured kombucha	(4.5±0)	(3.6±0.3) ^{*c}
Madimak-flavoured kombucha	(4.3±0)	(2.7±0) ^{*a}
Hawthorn tea	(6.4±0.2)	(6.2±0.1) ^{ns, d}
Hops tea	(7.3±0.1)	(7.1±0.1) ^{ns, e}
Madimak tea	(6.4±0.1)	(6.3±0.1) ^{ns, d}

*: Defines significant difference between day 0 and day 14 ($p < 0.05$), ns: Defines that there is no significant difference between day 0 and day 14 ($p > 0.05$), different superscript lowercase letters indicate the statistical significance of samples on the 14th day ($p < 0.05$). Results are presented as mean \pm standard deviation

Table 2. Microbiological characteristics of kombucha cultures (CFU/mL) on fermentation day 0 and 14

	Day 0	Day 14			
	K, KH, KH, KM	K	KHa	KH	KM
AAC	(2.3±0.4 × 10 ³)	(3±0.3 × 10 ⁷) ^{*a}	(1.3±0.1 × 10 ⁷) ^{*c}	(1.9±0 × 10 ⁷) ^{*b}	(1.3±0 × 10 ⁷) ^{*c}
TMB	(6.7±0.2 × 10 ³)	(3.2±0.4 × 10 ⁷) ^{*a}	(5.5±0.6 × 10 ⁶) ^{*c}	(1.3±0.4 × 10 ⁷) ^{*b}	(1.3±0.2 × 10 ⁷) ^{*b}
Yeast	(1.7±0.1 × 10 ³)	(2.7±0.7 × 10 ⁷) ^{*a}	(9.6±0.6 × 10 ⁵) ^{*d}	(1.7±0.2 × 10 ⁷) ^{*b}	(1.3±0.1 × 10 ⁷) ^{*c}
<i>Lactobacillus</i>	(7.4±0.3 × 10 ³)	(2.8±0.1 × 10 ⁷) ^{*a}	(1.6±0.1 × 10 ⁷) ^{*d}	(2.1±0.1 × 10 ⁷) ^{*b}	(1.9±0.1 × 10 ⁷) ^{*c}

AAC: Acetic acid bacteria, TMB: Total mesophilic bacteria, K: Traditional kombucha, KHa: Hawthorn-flavoured kombucha, KH: Hops-flavoured kombucha, KM: Madimak-flavoured kombucha, *: Defines significant difference between day 0 and day 14 ($p < 0.05$), ns: Defines that there is no significant difference between day 0 and day 14 ($p > 0.05$), different superscript lowercase letters indicate the statistical significance of samples on the 14th day ($p < 0.05$). Results are presented as mean \pm standard deviation.

Please note that this is an unedited version of the manuscript that has been accepted for publication. This version will undergo copyediting and typesetting before its final form for publication. We are providing this version as a service to our readers. The published version will differ from this one as a result of linguistic and technical corrections and layout editing.

Table 3. Antioxidant activity ($\mu\text{M}/\text{mL}$ AAE), total phenolic content ($\mu\text{g}/\text{mL}$ GAE) and total flavonoids ($\mu\text{g}/\text{mL}$ QE) of fermented beverages on day 0 and 14

	Ascorbic Acid Equivalent ($\mu\text{M}/\text{mL}$ AAE)		Gallic Acid Equivalent ($\mu\text{g}/\text{mL}$ GAE)		Quercetin Equivalent ($\mu\text{g}/\text{mL}$ QE)	
	Day 0	Day 14	Day 0	Day 14	Day 0	Day 14
K	(625 \pm 1.2)	(655 \pm 5) ^{*a}	(1262 \pm 2)	(2601 \pm 3) ^{*b}	(34 \pm 0.4)	(77 \pm 0.8) ^{*b}
KHA	(548 \pm 3.8)	(610 \pm 2.7) ^{*b}	(1354 \pm 3)	(3013 \pm 2) ^{*a}	(38 \pm 0.1)	(43 \pm 0.2) ^{*c}
KH	(633 \pm 3)	(634 \pm 1.6) ^{ns, a}	(1245 \pm 1.2)	(2974 \pm 3) ^{*a}	(39 \pm 0.7)	(46 \pm 0.3) ^{*c}
KM	(559 \pm 3)	(626 \pm 2.6) ^{*a}	(1469 \pm 1.8)	(3151 \pm 3) ^{*a}	(109 \pm 1.3)	(112 \pm 0.2) ^{*a}
B	(565 \pm 3.2)	(553 \pm 3.4) ^{*c}	(1254 \pm 1)	(796 \pm 1) ^{*c}	(16 \pm 0.1)	(13 \pm 0.2) ^{*e}
HA	(209 \pm 1.8)	(206 \pm 3) ^{ns, d}	(273 \pm 3)	(419 \pm 1.8) ^{*d}	(11 \pm 0.1)	(10 \pm 0.1) ^{ns, e}
H	(234 \pm 2.5)	(223 \pm 3.2) ^{*e}	(440 \pm 0.2)	(526 \pm 3) ^{*d}	(21 \pm 0.2)	(14 \pm 0.2) ^{*e}
M	(554 \pm 3.5)	(568 \pm 1.4) ^{*c}	(633 \pm 1.7)	(771 \pm 4.5) ^{*c}	(62 \pm 0.5)	(65 \pm 0.7) ^{*d}

K: Traditional kombucha, KHA: Hawthorn-flavoured kombucha, KH: Hops-flavoured kombucha, KM: Madimak-flavoured kombucha, B: Black tea, Ha: Hawthorn, H: Hops, M: Madimak, "*" indicated statistical significance ($p < 0.05$) and "ns" indicated statistically non-significant difference ($p > 0.05$) between non-fermented and 14 days fermented samples. Results are presented as mean \pm standard deviation. Different superscript lowercase letters indicate the statistical significance of samples on the 14th day ($p < 0.05$)

Table 4. The content of polyphenols (mg/L) of kombucha samples after fermentation

	K	KHa	KH	KM
Caffeic acid	(84.9 \pm 0.9) ^a	(26.5 \pm 1.2) ^b	(1.8 \pm 0.1) ^c	(15.0 \pm 2.4) ^{bc}
Chlorogenic acid	(26.0 \pm 0.4) ^c	(103.0 \pm 2.3) ^b	(15.5 \pm 0.7) ^c	(868.4 \pm 6.8) ^a
Epicatechin	(32.9 \pm 0.5) ^b	(51.1 \pm 4.6) ^a	(32.9 \pm 1.5) ^b	(14.3 \pm 0.2) ^c
Gallic acid	(666.2 \pm 19.5) ^b	(879.1 \pm 5.8) ^a	(432.5 \pm 10.7) ^d	(572.1 \pm 14.7) ^c
Protocatechuic acid	Nd ^a	(0.6 \pm 0) ^a	(0.2 \pm 0) ^a	(1.1 \pm 0) ^a
Rutin	(56.0 \pm 0.8) ^a	(42.1 \pm 1.2) ^a	(41.7 \pm 0.8) ^a	(40.2 \pm 0.7) ^a
Vitexin	(80.3 \pm 3.3) ^a	(68.3 \pm 1.7) ^{ab}	(55.3 \pm 1.4) ^b	(74.5 \pm 0.8) ^a

K: Traditional kombucha, KHa: Hawthorn-flavoured kombucha, KH: Hops-flavoured kombucha, KM: Madimak-flavoured kombucha, nd: not detected; results are presented as mean \pm standard deviation. Different superscript lowercase letters indicate the statistical significance of samples on the 14th day ($p < 0.05$)

Please note that this is an unedited version of the manuscript that has been accepted for publication. This version will undergo copyediting and typesetting before its final form for publication. We are providing this version as a service to our readers. The published version will differ from this one as a result of linguistic and technical corrections and layout editing.

Table 5. Average diameter (in mm) of inhibition zones of 14 day fermented samples

Samples	Inhibition zones					
	KP	SA	BC	PA	SE	EC
Ampicillin	(7±0) ^{hi}	(24.5±1.9) ^a	(11±1.4) ^{cde}	(10.5±1) ^{cdef}	(17.5±1) ^b	(6±0) ⁱ
Kanamycin	(17±1.2) ^b	(16±0.7) ^b	(16±1.1) ^b	(8±0) ^{ghi}	(17±1.4) ^b	(16.5±1) ^b
K	(8±0.2) ^{ghi}	(6±0) ⁱ	(8±0.8) ^{ghi}	(6±0) ⁱ	(8±0.6) ^{ghi}	(8±0) ^{ghi}
KHa	(9±1.4) ^{efgh}	(9±1.4) ^{efgh}	(8±0.4) ^{ghi}	(6±0) ⁱ	(8±1.1) ^{ghi}	(9±1.2) ^{efgh}
KH	(8±0.2) ^{ghi}	(6±0) ⁱ	(10±0.4) ^{defg}	(10±0.2) ^{defg}	(8±0.1) ^{ghi}	(10±0) ^{defg}
KM	(8.5±1) ^{fgh}	(9±1.4) ^{efgh}	(12±0) ^{cd}	(12.5±1) ^c	(10.5±1) ^{cdef}	(12±0) ^{cd}
B	(6±0) ⁱ	(6±0) ⁱ	(6±0) ⁱ	(6±0) ⁱ	(6±0) ⁱ	(6±0) ⁱ
Ha	(6±0) ⁱ	(6±0) ⁱ	(6±0) ⁱ	(6±0) ⁱ	(6±0) ⁱ	(6±0) ⁱ
H	(6±0) ⁱ	(6±0) ⁱ	(6±0) ⁱ	(6±0) ⁱ	(6±0) ⁱ	(6±0) ⁱ
M	(6±0) ⁱ	(6±0) ⁱ	(6±0) ⁱ	(6±0) ⁱ	(6±0) ⁱ	(6±0) ⁱ

The discs used in the study have a diameter of 6 millimetres. A value of 6 in the table indicates that no inhibition zone is formed. Results are presented as mean ± standard deviation. BC: *B. cereus*, SA: *S. aureus*, SE: *S. epidermidis*, EC: *E. coli*, KP: *K. pneumonia*, PA: *P. aeruginosa*. K: Traditional kombucha, KHa: Hawthorn-flavoured kombucha, KH: Hops-flavoured kombucha, KM: Madimak-flavoured kombucha, B: Black tea, Ha: Hawthorn tea, H: Hops tea, M: Madimak tea, different superscript lowercase letters indicate the statistical significance of samples on the 14th day ($p < 0.05$)

Table 6. *In vitro* cytotoxic effects of kombucha beverages against two: HCT116 (human colorectal carcinoma) and Mahlavu (human hepatocellular carcinoma)

Sample	¥,∅ IC ₅₀ (mg/ml)	
	HCT116	Mahlavu
Traditional kombucha	(0.81±0.07) ^{de}	(1.47±0.29) ^a
Madimak-flavoured kombucha	(0.63±0.03) ^e	(1.14±0.12) ^{bc}
Hops-flavoured kombucha	(0.79±0.03) ^{de}	(0.67±0.16) ^{de}
Hawthorn-flavoured kombucha	(0.90±0.09) ^{cd}	(1.34±0.12) ^{ab}

¥ IC₅₀, half-maximal inhibitory concentration

∅ Results are presented as mean ± standard deviation.

(Values indicated with different superscript lowercase letters differ from each other at the level of $p < 0.05$.)

Please note that this is an unedited version of the manuscript that has been accepted for publication. This version will undergo copyediting and typesetting before its final form for publication. We are providing this version as a service to our readers. The published version will differ from this one as a result of linguistic and technical corrections and layout editing.

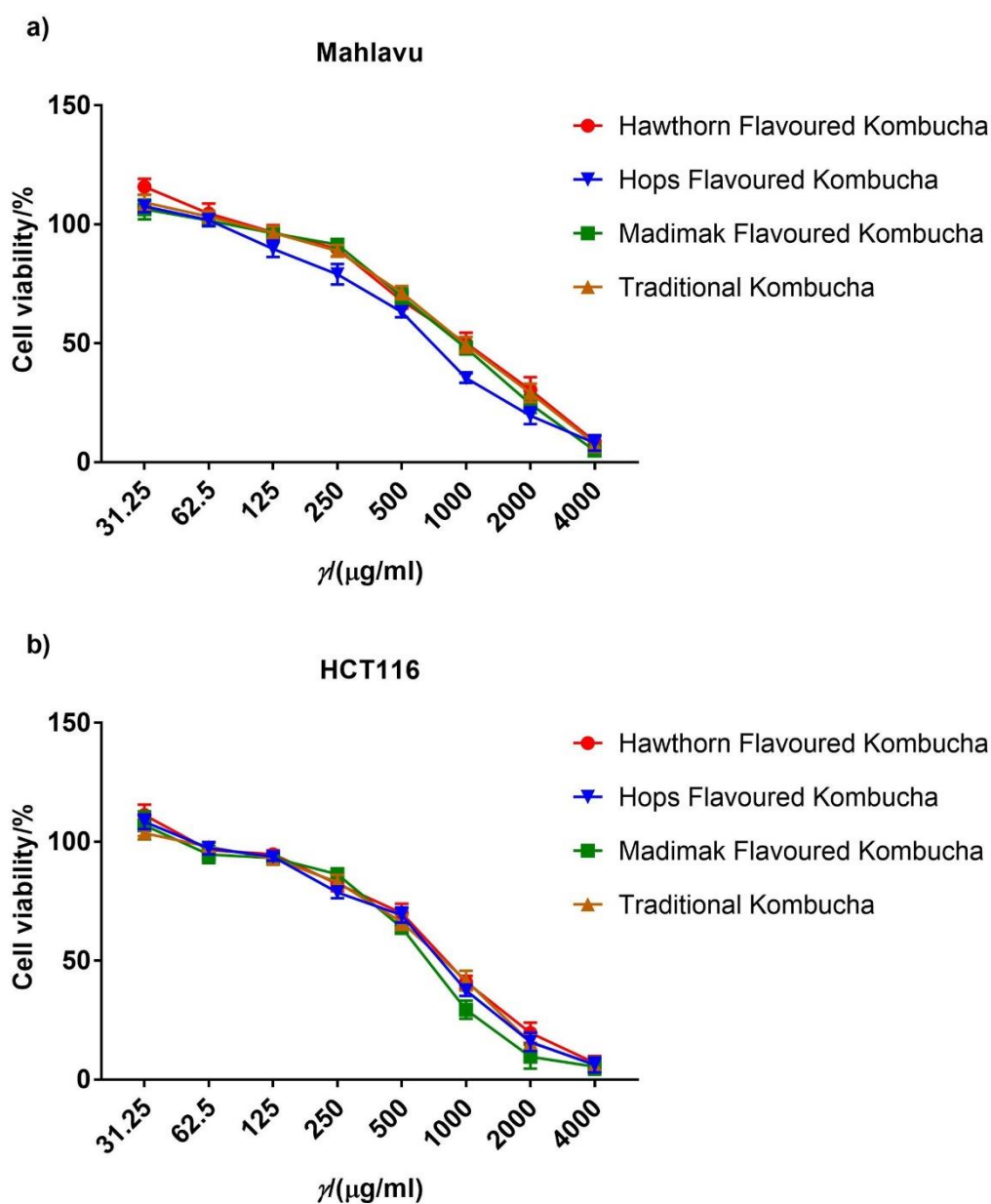


Fig. 1. Effects of varying concentrations of different kombucha beverages on viabilities of a) Mahlavu and b) HCT116 cells

Please note that this is an unedited version of the manuscript that has been accepted for publication. This version will undergo copyediting and typesetting before its final form for publication. We are providing this version as a service to our readers. The published version will differ from this one as a result of linguistic and technical corrections and layout editing.

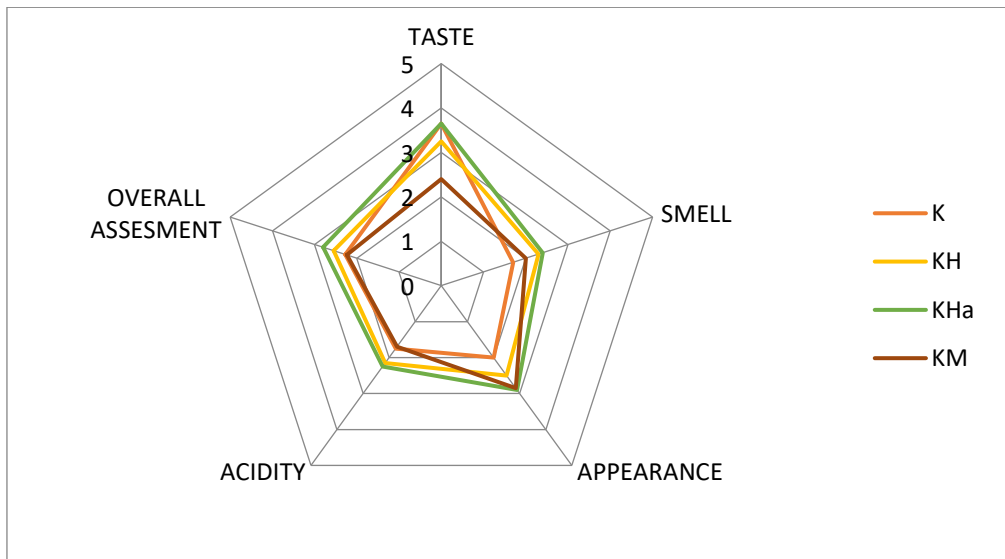


Fig. 2. Sensory analysis results of 14-day-old kombucha samples

Please note that this is an unedited version of the manuscript that has been accepted for publication. This version will undergo copyediting and typesetting before its final form for publication. We are providing this version as a service to our readers. The published version will differ from this one as a result of linguistic and technical corrections and layout editing.

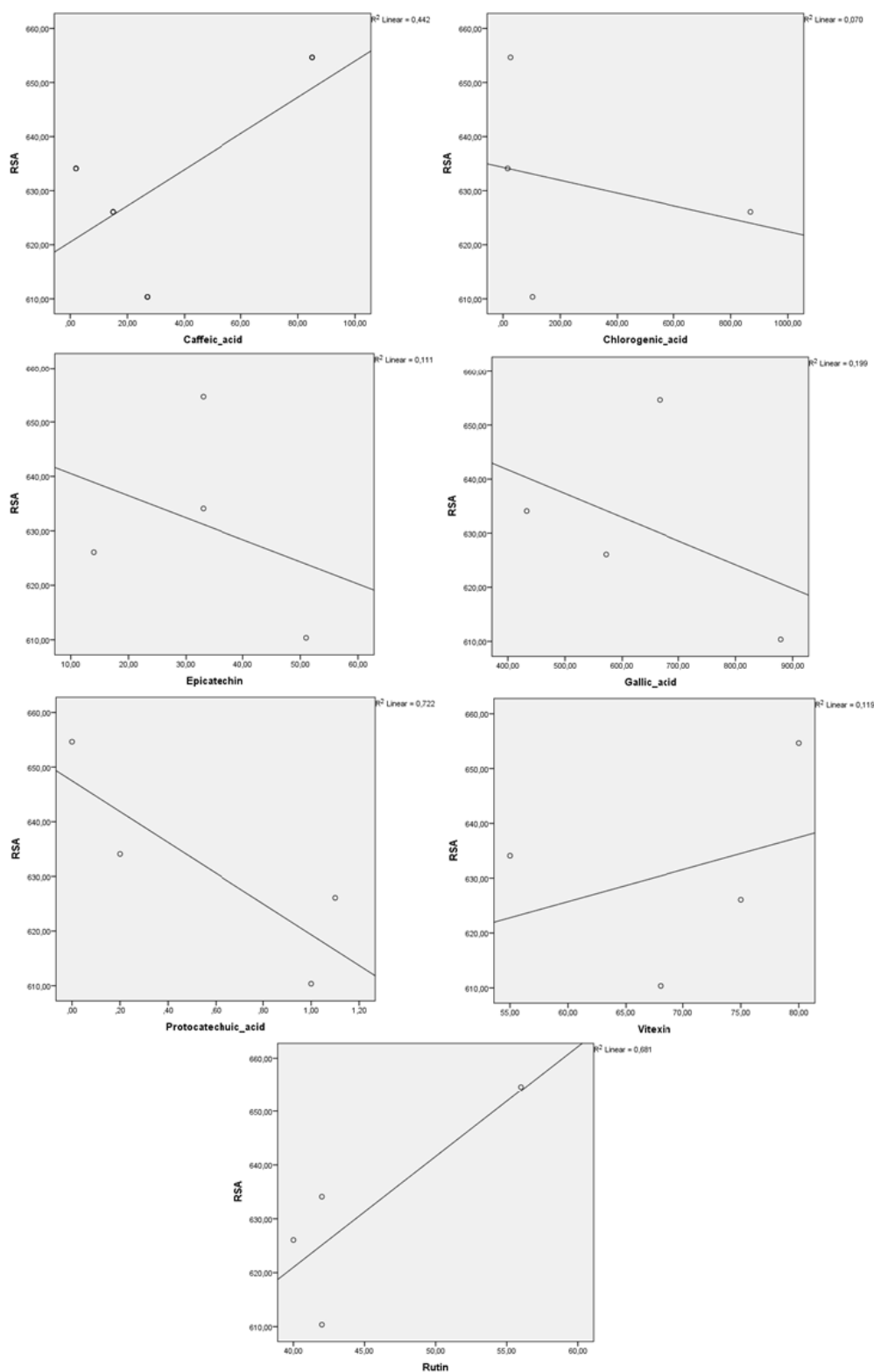


Fig. S1. Spearman correlation between identified compounds and measured free radical scavenging ability (RSA)

Please note that this is an unedited version of the manuscript that has been accepted for publication. This version will undergo copyediting and typesetting before its final form for publication. We are providing this version as a service to our readers. The published version will differ from this one as a result of linguistic and technical corrections and layout editing.

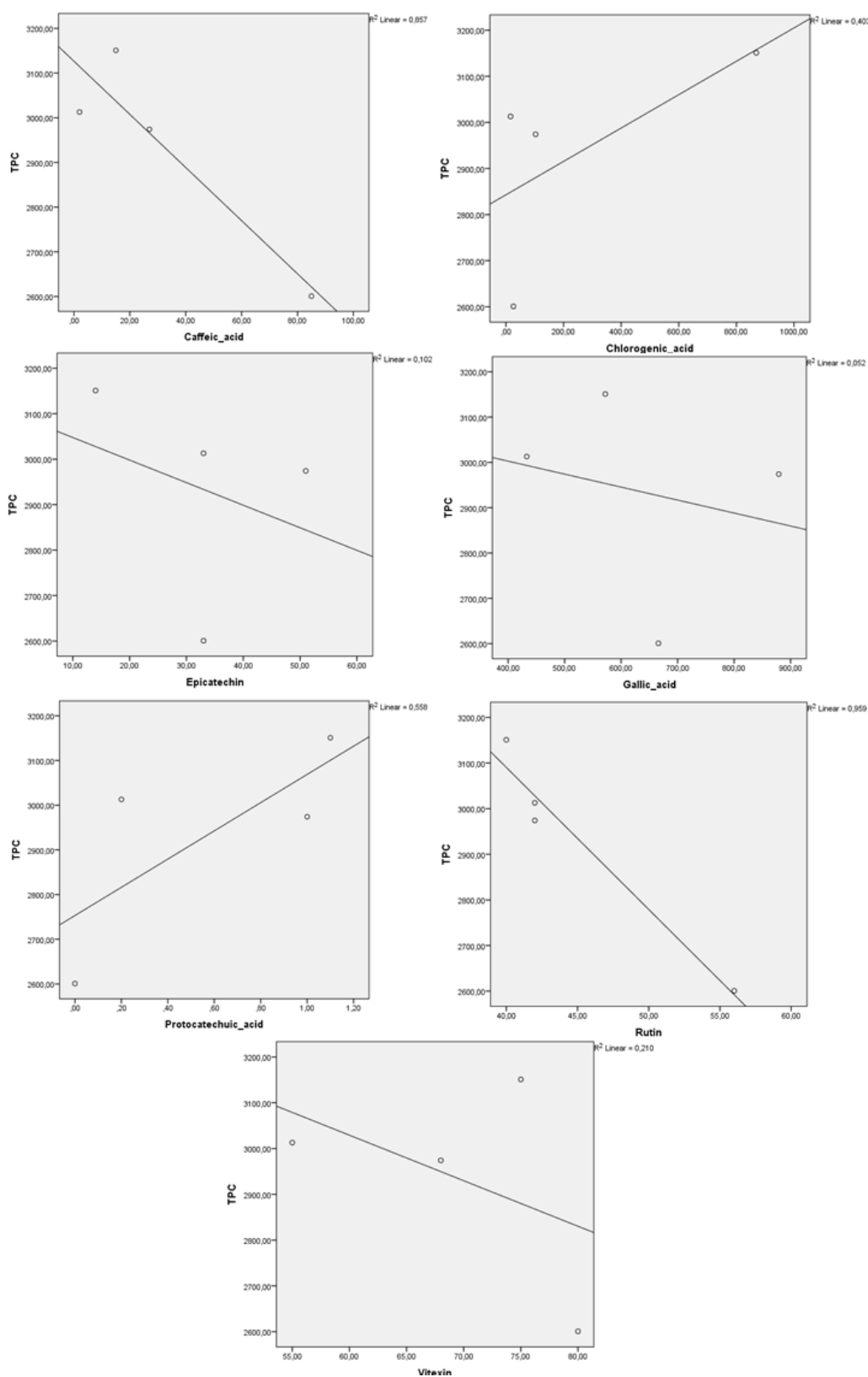


Fig. S2. Spearman correlation between identified compounds and measured total phenolic content (TPC)

Please note that this is an unedited version of the manuscript that has been accepted for publication. This version will undergo copyediting and typesetting before its final form for publication. We are providing this version as a service to our readers. The published version will differ from this one as a result of linguistic and technical corrections and layout editing.

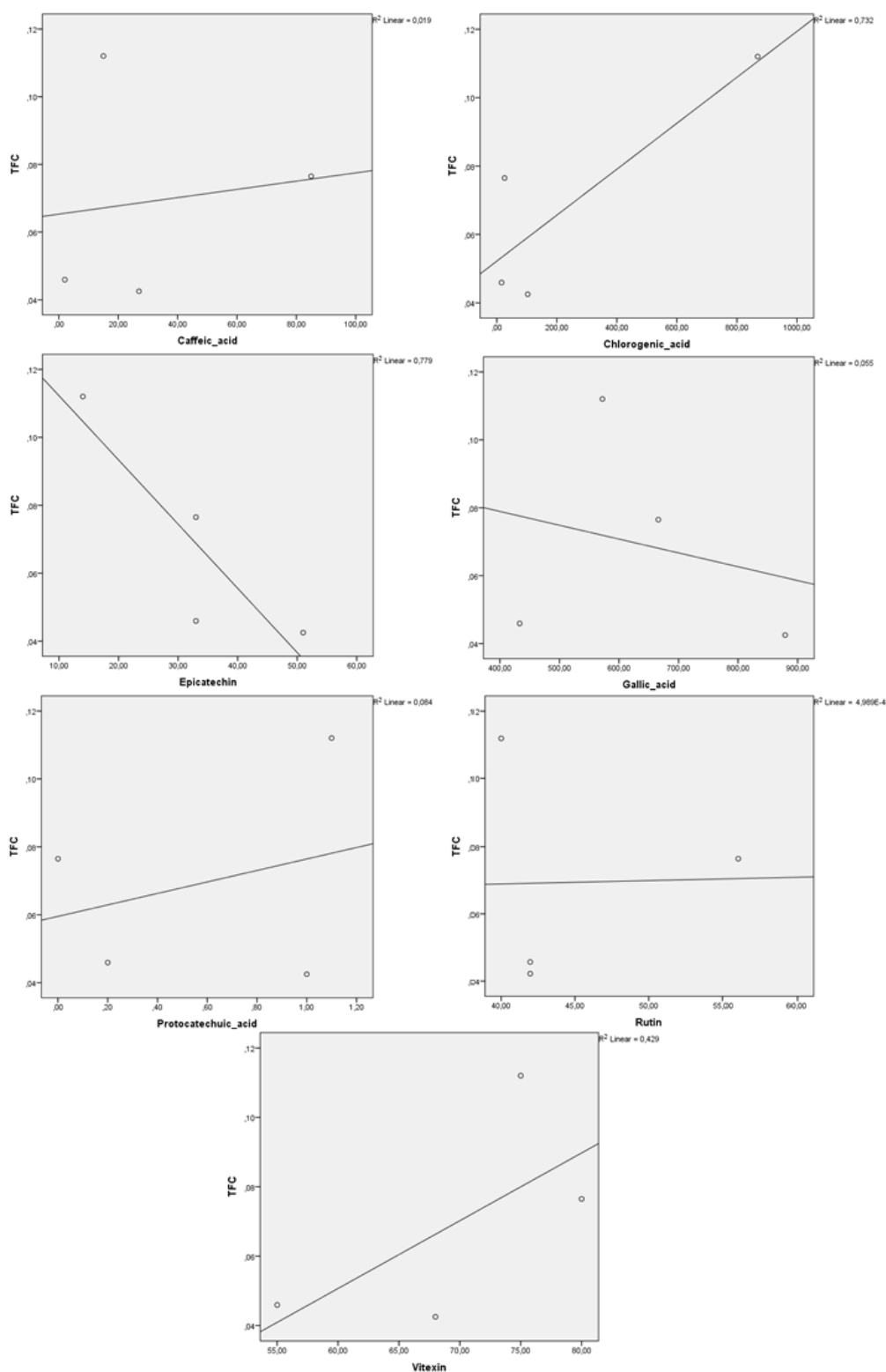


Fig. S3. Spearman correlation between identified compounds and measured total flavonoid content (TFC)