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Investigation of the Antioxidant Potential of Kombucha Prepared Using *Salvia officinalis* L.

Cihan Düşgün[1,a,*]

[1]*Niğde Ömer Halisdemir University, Faculty of Science, Department of Biology, 51200, Niğde, Türkiye*
[*]*Corresponding author*

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Kombucha is a slightly acidic sugary drink made by fermenting sweetened tea. It is known for its numerous health advantages. The objective of this study is to explore the possible effects of *Salvia officinalis* on enhancing the biochemical characteristics of kombucha. The present investigation compared traditional kombucha, produced using green and black tea, with kombucha derived from *S. officinalis*, examining their antioxidant properties, total phenolic, and total flavonoid content. The fermentation process lasted for a duration of 14 days. The present study was performed to evaluate the antioxidant activity of fermented *S. officinalis*. The antioxidant potential was assessed using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity and copper (II) reducing antioxidant capacity (CUPRAC) techniques. The DPPH radical reduction percentages were determined to be 93.5±1.65% for kombucha of green tea, 90.6±1.51% for kombucha of black tea, and 88.5±1.68% for kombucha of *S. officinalis*. According to the CUPRAC results, green tea kombucha was found to have 321.58±2.12 mg TE/g, black tea kombucha 305.91±1.98 mg TE/g and *S. officinalis* kombucha 301.97±1.78 mg TE/g. Total phenolic content was 154.15±1.22 mg GAE/g for kombucha of green tea, 145.41±1.31 mg GAE/g for kombucha of black tea, and 124.52±1.25 mg GAE/g for kombucha of *S. officinalis*. The determined value for the total flavonoid content was 101.12±0.98 mg QE/g for kombucha of green tea, 99.41±0.97 mg QE/g for kombucha of black tea, and 92.73±0.78 mg QE/g for kombucha of *S. officinalis*. The findings indicate that *S. officinalis* can serve as a substitute medium for kombucha fermentation, resulting in the development of a novel kind of kombucha with similar chemical characteristics.

[a] *cihandusgun@ohu.edu.tr*   *https://orcid.org/0000-0003-2796-8356*

Introduction

Fermented products offer various health benefits. These products support intestinal health, strengthen the digestive system and positively affect the immune system. Fermented foods balance intestinal flora and improve overall health because they contain probiotic bacteria. In addition, the fermentation process increases the absorption of nutrients and supports the body's intake of vitamins and minerals. Some fermented products may help prevent cell damage and slow down the aging process thanks to their antioxidant properties (Gülhan, 2023a; Gülhan, 2023b; Gülhan, 2024; Gülhan et al., 2024). Kombucha is a beverage made from fermented tea that has a mildly sparkling, sweet and tangy flavor. It has originated in China, dating back 2000 years. Kombucha is traditionally made by fermenting green or black tea using a combination of acetic acid bacteria, lactic acid bacteria and yeast (Coelho et al., 2020). Kombucha's chemical structure is affected by the quantity of metabolites generated by microorganisms the nature of the substrate employed, and the fermentation conditions, including duration, temperature, and oxygen levels. A number of organic acids, including acetic, malonic, glucuronic, gluconic, pyruvic, tartaric, citric, L-lactic, oxalic, succinic, usnic, malic and D-saccharic acid-1,4-lactone, have been shown to be present in kombucha, according to studies that appeared in the literature. It also contains tea chemical components such as polyphenols (theaflavins, catechins, and flavonoids), vitamins (C, E, K, B1, B2, B3, B6 and B12), minerals (Fe, Cu, Ni, Zn and Mn), proteins and amino acids (Coelho et al., 2020; de Miranda et al., 2022; Tu et al., 2024). Kombucha beverage has been proposed to offer numerous health advantages because of its anti-inflammatory, antioxidant, antibacterial, antidiabetic, and antihypercholesterolemic properties. Furthermore, kombucha has been shown to possess liver-protective, brain-protective, and gastrointestinal therapeutic properties (Anantachoke et al., 2023; Diez-Ozaeta & Astiazaran, 2022; Mousavi et al., 2020).

Despite the fact that black and green tea are considered to be primary materials utilized in the manufacture of kombucha, there are several studies that have been conducted in which other raw materials have been utilized as alternative substrates. In recent studies, grape pomace (Barakat et al., 2024), corncob (Liu et al., 2024), soy whey (Feng et al., 2024) and *Sargassum fusiforme* (Harv.) (Tu et al., 2024) were used to produce kombucha by using different substrates. The findings indicate that kombucha made from alternate substrates exhibits superior advantages, chemical composition, and biological features compared to traditional kombucha.

Salvia officinalis, often known as Sage, is a member of the Lamiaceae family. Originally indigenous to the Middle East and Mediterranean areas, this plant has now become established in several parts of the world. *S. officinalis* has been utilized in traditional medicine to address various conditions including ulcers, rheumatism, gout, inflammation, tremors, diarrhea, paralysis and hyperglycemia. Over the past few years, there has been a significant focus on conducting thorough investigations on this plant to record its traditional usage and discover novel biological impacts. Studies have uncovered a diverse array of pharmacological actions associated with *S. officinalis*. The pharmacological findings that have been reported include the following effects: anti-inflammatory, anticancer, antioxidant, antibacterial, antimutagenic, hypoglycemic, antidementia and hypolipidemic (Ghorbani & Esmaeilizadeh, 2017).

This study examined the feasibility of integrating *S. officinalis* into kombucha to develop a novel beverage and enhance its chemical characteristics. This research compared normal kombucha, derived from green and black tea, with kombucha prepared by *S. officinalis*.

Materials and Methods

Kombucha Fermentation

To prepare kombucha, green and black tea leaves and *S. officinalis* leaves were provided in dry form. Plant samples were purchased from a local market in Niğde, Turkey. 70 g of sucrose was dissolved in 1 L of water and heated to the boiling point. After the heat was turned off, 7 g of dried plant materials were added and infused for 30 minutes. Then, the tea leaves were filtered and allowed to reach room temperature. 10% of the previous culture was inoculated into the infusion that reached room temperature and was left to ferment at 25±1°C. The same ratios and procedures were applied for green, black tea and *S. officinalis* (Jayabalan et al., 2008).

pH Determination

pH measurement was carried out with a pH meter (WTW series Multi 9620 IDS, Weilheim, Germany) in 3 repetitions before and at the end of fermentation.

Determination of DPPH Radical Scavenging Activity

The solutions' capacity to remove free radicals was assessed by measuring their free radical scavenging capabilities using 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical. The method proposed by Brand-Williams et al. (1995), was implemented with necessary modifications. Kombucha samples were filtered through a 0.22 microliter syringe filter to remove microorganisms. The supernatant was used after filtration. 20 µL of kombucha sample was put into the sample tubes, and 180 µL of 1mM DPPH was added onto it and incubated for 30 minutes in a light-proof place at room temperature. Experiments were performed in three parallels. Following incubation, absorbance was measured at 517 nm, using methanol as the reference solution. The decrease in absorbance indicated the remaining quantity of DPPH solution, which represents the activity of scavenging free radicals. The calculation of the DPPH radical scavenging activity percentage was performed using the following equation:

$$\text{Radical scavenging power (\%)} = \frac{A_0 - A_1}{A_0} \times 100$$

where A_0 is the absorbance of the control and A_1 is the absorbance of the extract sample/standard at 30 min. The analysis of all the samples was done in triplicate.

Copper (II) Reducing Antioxidant Capacity Method (CUPRAC)

For this study, 500 µL of Copper (II) Chloride ($CuCl_2$) solution and 500 µL of Ammonium Acetate (pH= 7.0, 1 M) solution were placed in 2.5 mL centrifuge tubes. 500 µL of Neocuproin ($C_{14}H_{12}N_2$) (7.5×10^{-3} M) solution was added to each tube. A volume of 100 µL of kombucha sample, with a concentration of 1 mg/mL, was added to the tubes and then diluted with distilled water to a final volume of 2 mL. In the case of empty samples, distilled water was used as a substitute for the extract. The specimen was subjected to incubation at both room temperature and in a water bath set at 50ºC for a duration of 30 minutes. The measurement of absorbance at a wavelength of 450 nm was taken using the blank sample as a reference (Selamoglu et al., 2017).

Analysis of Total Phenolic Content

The total phenol content was determined using the Folin–Ciocalteau reagent, following the method described by Düşgün et al. (2021). The Folin-Ciocalteau method relies on the generation of blue-hued molecules by the transfer of electrons from phenolic compounds to phosphomolybtic and phosphotungistic acids in an alkaline setting. These compounds are read spectrophotometrically at an average of 760 nm. 0.1 mL of the prepared kombucha samples was taken and added to 1 mL of Folin–Ciocalteau solution (diluted 10 times) and incubated for 5 minutes. 1 mL of 7.5% sodium bicarbonate solution was added. After 90 minutes of incubation, the absorbance of the samples was read at 765 nm. The outcomes were determined by applying gallic acid standards to the calculations (Ildız et al., 2022).

Analysis of Total Flavonoid Content

The total flavonoid content was determined as previously reported spectrophotometric method with minor modification (Zeynali et al., 2023). In summary, a 1 mL sample of kombucha was combined with an equal volume of $AlCl_3$ (2%) in methanol. In the same way, a blank was created by combining 1 mL of sample solution with 1 mL of methanol, excluding $AlCl_3$. Following a 10-minute period of incubation at ambient temperature, the absorbances of both the sample and the blank were measured at a wavelength of 415 nm. The blank absorbance was subtracted from the sample. Quercetin was used as a reference standard, and total flavonoid content was expressed as milligram quercetin equivalents (mg QE/g extract).

Statistical analysis

The study reported the findings of multiple analyses using the mean ± standard deviation format. The ANOVA was used to assess the means of the experimental results, and significance tests were conducted using Duncan's multiple range tests for statistical significance ($p < 0.05$).

Results and Discussion

Color analysis

This study examined the kombucha fermentation process on 3 substrates over a 14-day period. During the processes, *S. officinalis* kombucha as well as traditionally produced green and black tea kombucha were included. It was observed that the dark color turned into light during the fermentation period. In addition, the sour odor grew as the fermentation process progressed and a bacterial cellulose layer formed. The first three days of fermentation observed bacterial cellulose production on Kombucha's surface, which thickened as fermentation progressed. The fermentation conditions and yeast-acetic acid bacteria synergy increased cellulose yield (Tapias et al., 2022; Tapias et al., 2023). The difference of color change indicated that the fermentation process was carried out successfully. The similar indications were reported in other studies (Pure & Pure, 2016; Zou et al., 2021). Carbon is essential for the growth and metabolic processes of nearly all living microorganisms. Furthermore, it is a fundamental component of every substance that makes up protoplasm. The bacterial cellulose 'mother' (starter) culture requires a carbon source, primarily sucrose, as it cannot produce sufficient cellulose independently. Previously, (Gülhan, 2023c) have produced cellulose by kombucha fermentation using different carbon sources. In the present study, results of experiments conducted revealed that the using different carbon source present in the tea broth affects the synthesis of bacterial cellulose, and these findings are similar to the previous report by (Gülhan, 2023c). Therefore, the cellulose layer production can be attributed to use different carbon source including *S. officinalis*.

Biological Activity Results

The pH values of green, black tea kombucha and *S. officinalis* kombucha at 25°C and on the 0th and 14th days of fermentation are given in Table 1. There was a decrease in pH levels in all infusion groups due to the increase in fermentation time (p <0.05). The reduction in pH levels in kombucha samples during fermentation is mostly attributed to the rise in organic acid concentration. The findings of Dusgun (2024), Kaewkod et al. (2019) and Velićanski et al. (2014) were similar with this study's data. The concentration of hydrogen ions in the surrounding environment is a key factor that can either promote or inhibit the growth of certain microorganisms in food. Bacteria that produce acetic acid can survive in media with pH levels ranging from 3.5 to 6.4. However, these bacteria thrive best when the pH is at least 5.4. The pH value of *S. officinalis* kombucha was 6.44±0.21 and 3.68±0.29 in before and end of the fermentation, respectively. Throughout the fermentation process, from day 0 to day 14, all groups experienced a decline in pH levels as a result of increased organic acid production. However, this value caused a slight increase in the values of black tea kombucha compared to the beginning on the 14th day of fermentation (Table 1). In previous research, it was discovered some molecules generated as a result of microbial activities can cause pH fluctuations by exhibiting a buffering effect, contingent upon the liquid concentration in the fermentation medium (Bressani et al., 2024; Ziemlewska et al., 2023). This gradual reduction in pH was attributed to the buffering capabilities inherent in the kombucha solutions. Bressani et al. (2024) reported that the pH of kombucha prepared using yam dropped rapidly within the first 5 days of fermentation, then dropped further by the 14th day. In another study, kombucha samples made from various teas (white, green, black, and red) initially had pH levels ranging from 5.34 to 6.53. The researchers observed that after 14 days of fermentation, the pH dropped significantly, reaching values between 2.32 and 2.53.

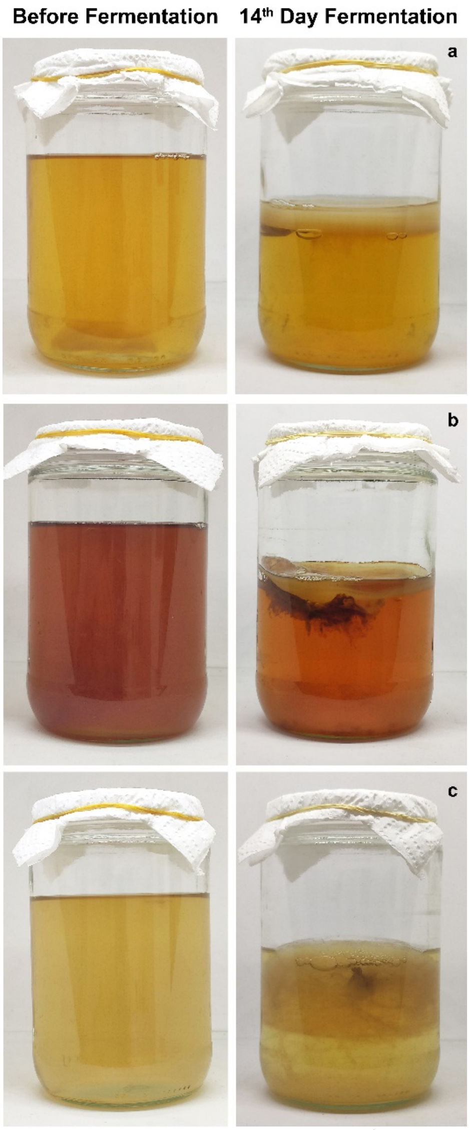

Figure 1 Kombucha fermentation for 14th day (a: Green tea kombucha, b: Black tea kombucha, c: *S. officinalis* kombucha)

Table 1. Kombucha analyzes and results

Analyses	Initial pH	Final pH	Total phenol (mg GAE/g)	Total flavonoid (mg QE/g)	DPPH (%)	CUPRAC (mg TE/g)
Green tea kombucha	6.58±0.19[a]	3.65±0.26[a]	154.15±1.22[a]	101.12±0.98[a]	93.5±1.65[a]	321.58±2.12[a]
Black tea kombucha	6.67±0.27[a]	3.51±0.23[a]	145.41±1.31[a]	99.41±0.97[a]	90.6±1.51[a]	305.91±1.98[b]
S. officinalis kombucha	6.44±0.21[a]	3.68±0.29[a]	124.52±1.25[b]	92.73±0.78[b]	88.5±1.68[b]	301.97±1.78[c]

*The values shown are the average (mean) values with the standard deviation (S.D.) calculated from three separate measurements conducted in parallel. Distinct letters in the same column indicate statistically significant differences between the examined samples ($p<0.05$).

The research found that on the 14th day, pH levels ranged from 3.51 to 3.68, which falls within the acceptable acidity range for drinkable beverages. However, it's important to note that consuming drinks with extremely low pH levels may negatively impact the digestive system. Therefore, when it comes to kombucha, both the duration of fermentation and the quantity consumed are equally significant factors to consider.

Total phenolic substance content is shown in Table 1. The highest total phenolic substance content was observed in kombucha prepared with green tea (154.15±1.22 mg GAE/g). In addition, 145.41±1.31 mg GAE/g was found in black tea kombucha, while 124.52±1.25 mg GAE/g was found in kombucha prepared with *S. officinalis*. Total phenolic substance content was determined in all samples as a result of kombucha fermentation.

Total flavonoid substance content results are shown in Table 1. According to the results, green tea showed the highest flavonoid substance content with 101.12±0.98 mg QE/g, while kombucha prepared with black tea was found to be 99.41±0.97 mg QE/g. In kombucha prepared with *S. officinalis*, 92.73±0.78 mg QE/g total flavonoid substance content was determined.

There were similar patterns observed in the relationship between the overall amount of phenolic compounds and flavonoid compounds, with the highest levels occurring after 14 days of fermentation. On the 14th day of fermentation, the total phenolic and flavonoid substance content of kombucha containing *S. officinalis* increased slightly, while traditional kombucha remained consistent during the whole fermentation process. Zhang et al. (2020) produced kombucha from rose and jujube kernel. Additionally, it was discovered that the overall quantity of phenolic minerals reached its highest point three days after the commencement of fermentation, then declined, and then stayed stable until the 10th day of fermentation (Zhang et al., 2020).

The results of the DPPH antioxidant activity test were found to be at the highest level with 93.5±1.65% for green tea kombucha, while it was found to be 90.6±1.51% for black tea kombucha, as shown in Table 1. In *S. officinalis* kombucha, it was measured as 88.5±1.68%. It was determined that it showed antioxidant properties as a result of the fermentation process.

Following the DPPH test results, the Copper reduction test results are shown in table 1. As a consequence of the findings, green tea kombucha was found to be 321.58±2.12 mg TE/g, while black tea kombucha was found to be 305.91±1.98 mg TE/g. *S. officinalis* kombucha was found to be as effective as traditionally prepared green tea and black kombucha, with a very high level of 301.97±1.78 mg TE/g.

The amount of total phenolic and flavonoid content in kombucha made from *S. officinalis* was comparable to that of typical kombucha made from green and black tea. The quantity of total phenolic and flavonoid content that was discovered to be present in kombucha that was made with green tea was the greatest. Additionally, high phenolic content levels were determined in kombucha samples prepared with black tea kombucha and *S. officinalis* kombucha. Because *S. officinalis* is a rich source of polyphenols and other bioactive compounds (Belcadi et al., 2023), the rise in total phenolic and flavonoid content may be attributed to the fact that green and black tea are similarly high in polyphenols but poor in vitamins, organic acids, and minerals (Khaleil et al., 2020; Zhou et al., 2022). A number of studies have shown that the fermentation process has the potential to raise both total phenolic and flavonoid content. Additionally, the mixing of alternate substrates has the potential to raise total phenolic and flavonoid content in comparison to traditional kombucha. For example, Khaleil et al. (2020) *Psidium guajava* L. examined the fermentation of kombucha with (hibiscus) and found that the total phenolic content of this sample increased after fermentation. In a separate study conducted by Xiong et al. (2023), kombucha was made from bamboo leaf. It was shown that the levels of total phenolic and flavonoid content increased following the fermenting process (Zubaidah et al., 2018). In accordance with Pure & Pure (2016), the polyphenols that can be found in things like fruits, vegetables, and tea leaves undergo degradation during the kombucha fermentation process, leading to an increase in total phenolic and flavonoid content. Polyphenols are bioactive substances characterized by many phenol chemicals within every molecule. In the fermentation process of kombucha, bacteria secrete an enzyme that breaks down polyphenols into smaller molecules, hence leading to an increase in total phenolic and flavonoid content (Xiong et al., 2023; Zubaidah et al., 2018).

In addition, this investigation revealed a correlation between the antioxidant activity, as observed by the DPPH test and CUPRAC test, and the total phenolic content. The rise in antioxidant activity can be attributed to the augmentation of chemical components, including ascorbic acid, phenolic substances, flavonoids and other organic acids, that are generated fermentation process (Ojo & de Smidt, 2023). In this investigation was revealed that the antioxidant activity of kombucha was notably higher when *S. officinalis* were dried prior to the preparation procedure, in comparison to other methods. Multiple studies have demonstrated that *S. officinalis* exhibits antioxidant properties as a result of its content of polyphenolic chemicals and flavonoids. Furthermore, the antioxidant capabilities differ according on the specific forms and extraction methods employed. Ben Akacha et al. (2023) reported that dried *S. officinalis* exhibited a 96% reduction in the activity of scavenging free radicals, surpassing the

57% inhibition observed in fresh plant leaves. According to the study by Belcadi et al. (2023), they determined antioxidant properties in extracts of the *S. officinalis* plant prepared using various solvents. In addition, in their study using the CUPRAC method, they revealed that *S. officinalis* extracts have antioxidant properties resulting from the bioactive compounds contained in them (Zeynali et al., 2023).

The total phenolic content, total flavonoid content, antioxidant activity, and organic acid content of kombucha are all affected by a variety of factors, including microorganisms the kind of substrate, the fermentation duration, temperature, and pH (Jayabalan et al., 2008; Khaleil et al., 2020). For this reason, it is essential to do more research on a pilot scale and determine the optimal ratio of dried *S. officinalis* in order to enhance the chemical composition of kombucha products.

Conclusion

Sage, also known as *Salvia officinalis*, has the ability to improve the fermentation process of traditional kombucha. This research marks the first attempt to create kombucha using *S. officinalis*. The study aimed to evaluate the potential of *S. officinalis* as a substitute for the traditional black and green tea substrates in kombucha production. To assess its viability, the researchers compared the physicochemical attributes, total phenolic and flavonoid content, and antioxidant properties of kombucha made with *S. officinalis* against those made with green and black tea. As a result of the analyses made on kombucha prepared using *S. officinalis*, high total phenolic, total flavonoid substance and high antioxidant activity were observed in samples taken on the 14th day of fermentation, similar to traditional kombucha prepared with green and black. This research supported the concept of creating a functional beverage by utilizing medicinal plants with tea properties to produce kombucha. The newly developed functional beverage products demonstrated high compatibility and exhibited enhanced bioactive properties and health advantages compared to traditional kombucha tea, showing promise for industrial production. Further in vivo studies are necessary to validate the health effects of kombucha made with *S. officinalis* on humans.

Declarations

Conflict of Interest
The author declares no conflict of interest.

Fund Statement
There is no financial support.

Data Availability
All the data generated or analyzed during this study are included in this published article.

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