

The Effect of Fermentation Time on Phenolic Levels of Vanilla (*Vanilla planifolia*) Leaf Kombucha Tea

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Abstract : Phenolics are compounds that have hydroxyl groups and can be found in plant parts, such as vanilla leaves. Vanilla leaves can be used as a base for making kombucha tea. The length of time of fermentation can affect the resulting kombucha tea products. The purpose of this study was to determine the effect of fermentation time on the phenolic content of vanilla leaf kombucha tea. This study used experimental research which included titrated total acid level test, pH level test and phenolic content test with variations of 0, 4, 8 and 12 days. The results showed that the phenolic content of kombucha vanilla leaf tea on day 0 was 37.995 ppm; 42.188 ppm on the 4th day; 57.58 ppm on the 8th day; and 50.53 ppm on the 12th day. Therefore, the highest phenolic concentration was found on the 8th day.

INTRODUCTION

Nowadays, health is one of the things that needs to be maintained and cared for. Health can be maintained by eating nutritious food, exercising regularly, not sleeping late and getting enough rest. Recently, many diseases have arisen due to exposure to free radicals, such as pollution from industrial fumes, motor vehicle fumes and the consumption of unhealthy food or drinks. Examples of diseases caused by exposure to free radicals are cardiovascular disease, diabetes, stroke, cancer and Parkinson's disease (Simanjuntak, 2012).

Exposure to free radicals can be avoided by consuming healthy foods and drinks, especially foods or drinks that contain antioxidant compounds such as phenols, flavonoids and tannins which can fight free radicals. Antioxidants are compounds that have the ability to delay, slow down, or inhibit oxidative reactions and can fight free radicals formed from metabolism in the body (Agustina, 2017). Antioxidant potential can be obtained

from phenolic compounds. Sources of antioxidants can be found in polyphenolic compounds because polyphenols are available with donated hydrogen to combat damage caused by free radicals (Husniati et al., 2021).

Phenolics are compounds with groups (-OH) which can be found in various parts and types of plants. The structure of phenolic compounds varies, from simple phenols to complex phenols. Phenolic compounds include several aromatic compounds with one or more hydroxyl groups and can vary from simple molecules to complex polymers (Diniyah & Lee, 2020). Based on the number of phenolic hydroxyl groups, phenolic compounds are divided into phenolic acids, flavonoids, tannins and stilbenes (Singh et al., 2015).

In the food and beverage industry, phenolic compounds function as special flavoring agents, coloring agents and antioxidants in food and beverage products. In the pharmaceutical and health industries, this compound is used as an antioxidant, antibacterial

and anticancer (Christalina et al., 2018). Phenolic compounds that are rich in antioxidants can be obtained from natural ingredients such as vanilla leaves. Therefore, innovation is needed in food and beverage products that contain these antioxidant-rich ingredients. One of the most popular antioxidant-rich drink innovations is kombucha tea.

Kombucha tea is a type of tea made by fermenting sweet tea using a symbiotic culture of bacteria and yeast or commonly known as SCOBY (Leal et al., 2018). The bacteria that play a role in making kombucha tea from the acetic acid bacteria group are *Acetobacter aceti*, *Acetobacter pausterianus*, *Acetobacter xylinum*, *Bacterium gluconicum*, *Schizosaccharomyces pombe*, *Candida sp.*, *Kloeckera sp.*, *S. ludwigii*, *S. cerevisiae*, *Torulospora sp.*, *Zygosaccharomyces bailii* and *Pichia* species belonging to the yeast (Goh et al., 2012).

Kombucha tea can be made from plants that have high antioxidant content such as flavonoids and tannins. One of the plants that can be made for kombucha tea is vanilla leaves. Vanilla plant (*Vanilla planifolia*) is a plant that is widely used by the community. The plant has a stem with a smooth surface and knuckles. The diameter of the tree is 1-2 cm, the length of the trunk reaches 50 meters and the color is light or dark green (Ruhnayat, 2004). Vanilla is generally used as a food and beverage flavoring ingredient, as well as a raw material for perfume (Nurcahyani, 2013). The results of research by Menon and Nayeem (2013) on vanilla plants show that vanilla plants are able to treat and prevent toothaches, menstrual pain, stomach ulcers, fever, coughs and sore throats.

Wet vanilla water content is 80% and will be 20% when dried. Vanilla beans weighing 100 grams contain 20 grams of water, 3-5 grams of protein, 11 grams of fat, 7-9 grams of sugar, 15-20 grams of fiber, 5-10 grams of ash, 1.5-3 grams of vanillin, 2 grams of resin. and vanillin acid. Compounds that play a very large role in the organoleptic properties are 4-hydroxy3-methoxy benzaldehyde (vanillin). Based on the isolation results, it can be seen that the components of fresh and dry vanilla glycosides include acids,

fatty aldehydes, alcohols, fatty esters, alkanes, alkanones, amines, benzene aldehydes, benzene esters, benzene alcohol and phenols, as well as other substances (Melawati, 2006).

In the manufacture of kombucha tea, one of the factors that can affect the resulting product is the fermentation time. Fermentation time will affect the levels of active compounds in kombucha tea. According to research by Puspaningrum et al., (2022) regarding kombucha cascara, the total phenol yield on day 0 of fermentation was $4.28a \pm 0.28$ mg GAE/100 ml, day 4 of $21.88e \pm 0.49$ mg GAE/100 ml, the 8th day was $22.37e \pm 0.61$ mg GAE/100 ml, the 10th day was $17.32c \pm 0.63$ mg GAE/100 ml, the 12th day was $13.31b \pm 0.09$ mg GAE/100 ml, and on the 14th day it was $18.62d \pm 0.51$ mg GAE/100 ml. Hapsari et al., (2021) research on red galangal kombucha obtained a total phenol yield on day 0 of fermentation of 759.36 ± 0.06 µgGAE/ml, day 2 of 780.69 ± 0.03 µgGAE/ml, the 4th day was 805.73 ± 0.16 µgGAE/ml, the 6th day was 824.79 ± 0.16 µgGAE/ml, the 8th day was 854.64 ± 0.07 µgGAE/ml, and the 10th day of 734.62 ± 1.15 µgGAE/ml. Research by Suhardini & Zubaidah (2016) showed that all fermented samples on day 0 amounted to 255,833 to 352,500 µg/ml GAE, day 8 amounted to 263,250 to 459,533 µg/ml GAE and day 14 amounted to 304,433 to 527,500 µg/ml GAE. The purpose of this study was to determine the effect of the length of fermentation time on the phenolic content of vanilla leaf kombucha tea.

METHOD

Research Design

This study used an experimental research type with a completely randomized design with variations in fermentation time of 0, 4, 8, 12 days. The treatment in this study are:

- P1 = 0 days fermentation time treatment
- P2 = 4 days fermentation time treatment
- P3 = 8 days fermentation time treatment
- P4 = 12 days fermentation time treatment

The research variables are:

Independent variable: length of fermentation time.

Dependent variable: antioxidant activity.

Control variables: concentration of kombucha culture, temperature, process of making kombucha tea.

Tools and Materials

Tool

Measuring cups, beaker glasses, measuring flasks, erlenmeyer, burettes and static, glass jars, stirrers, pipettes, volume pipettes, spoons, analytical scales, stoves, ovens, vortexes, aluminum foil, pH meters, UV-VIS spectrophotometer.

Material

Vanilla (*Vanilla planifolia*) leaves, water, tea, kombucha culture starter, aquades, granulated sugar, methanol p.a, Na₂CO₃, gallic acid, Folin-ciocalteu solution, PP indicator, NaOH.

Procedure

1. Making Vanilla Leaf Tea

Vanilla leaves are washed under running water until clean and cut into small pieces. Then dried in the sun until the leaves wilt for 30-60 minutes. The wilted vanilla leaves are dried in an oven at 55°C until the leaves are completely dry. The dried leaves are used as vanilla leaf tea.

2. Kombucha Starter Preparation

2000 ml of water is boiled until boiling and 200 grams of sugar (10% w/v) is added to the amount of water used and 10 grams of 0.5% (w/v) tea is added. Then filtered and the filtrate is covered with aluminum foil and let stand until the tea has room temperature. After that, 200 ml (10% b/v) of kombucha culture starter was added to the brewed tea and the container was tightly closed. The propagation of the kombucha starter culture was left for 14 days.

3. Making Vanilla Leaf Kombucha Tea

36 grams (0.5% w/v) of vanilla leaf tea is brewed using 7200 ml of boiling water. Then added sugar with a concentration of 20 grams

(10% w/v) stirred. The infusion of vanilla leaf tea is tightly closed with aluminum foil and let stand until room temperature. 20 ml of liquid kombucha starter was added and fermented for 0, 4, 8 and 12 days in a closed container and filtered after the fermentation time was complete.

4. Test Levels of Total Titrated Acid

The titrated total acid level test was carried out using the principle of acid-base titration. The test was carried out by placing 10 ml of the sample in a 100 ml volumetric flask and then adding distilled water up to the mark. After that, 10 ml of the filtrate was taken and put into the Erlenmeyer and 3 drops of PP indicator were added. The solution was titrated with 0.1 N NaOH solution until the color of the sample solution changed from clear to pink. Total acid is calculated using the formula:

$$\text{Total Acid (\%)} = \frac{V_{\text{NaOH}} \times N_{\text{NaOH}} \times \text{BM} \times 100\%}{V_{\text{sample}} \times 1000} \quad (1)$$

Information :

V_{NaOH} : Volume NaOH for titration

N_{NaOH} : Standard concentration of NaOH

V_{samples} : The volume of the sample used for the titrate

BM : Molecular weight of acetic Acid

5. pH level Test

pH measurement was measured using a pH meter. Vanilla leaf kombucha tea is put in a beaker glass and immersed in a pH meter.

6. Phenolic Level Test

Gallic Acid Standard Solution Standard Curve

Standard gallic acid solutions were made in various concentrations of 10, 20, 30, 40, 50 ppm. A standard solution of gallic acid for each concentration was taken 1 ml, put into a test tube and added 0.5 ml of Folin-ciocalteu then allowed to stand for 8 minutes while shaking. Into the solution was added 4 ml of 7% Na₂CO₃ solution and vortexed for 1 minute. Measurements were made at a wavelength of 760 nm.

Sample Absorption Measurement

A sample of vanilla leaf kombucha tea was taken as much as 1 ml and added 0.5 ml of Folin-ciocalteu, allowed to stand for 8 minutes while shaking. 4 ml of 7% Na₂CO₃ solution was added and vortexed for 1 minute. The absorbance was calculated at a wavelength of 760 nm. Measurements were made at a wavelength of 760 nm. The total phenol content can be calculated using the following formula:

$$\text{TPC} = c \cdot v \cdot \text{fp}$$

Description:

TPC : total phenolic content (mg/L GAE)
 c : concentration (x value) (ppm)
 v : sample volume (ml)
 fp : dilution factor

RESULTS AND DISCUSSION

Tea is one of the most consumed drinks by Indonesian people. Tea has the ability to detoxify, improve blood and urine circulation, reduce joint pain and increase the body's resistance to disease (Sa'diyah & Lestari, 2020). Over time, tea continues to develop, including kombucha. Kombucha tea in some regions has other names, as in Russia it is called "tea kvass". Kombucha is a traditional Chinese drink made from fermented green tea or black tea by SCOBY (Symbiotic Culture of Bacteria and Yeast) biofilm (Cardoso et al., 2020). SCOBYs are colonies of fermenting bacteria that act as starters in making kombucha. SCOBY is made from the bacteria *Acetobacter xylinum*, *Gluconobacter*, *Ascomycetous*, and *Saccharomyces ludwigii* (Mousavi et al., 2020). These microorganisms play a role in changing the texture, aroma, color and quality of fermented products (Riadi et al., 2020).

The main ingredients for making kombucha tea are green tea and sugar which are then fermented for 7 to 10 days with the help of microorganisms. The presence of added sugar stimulates the growth of microorganisms and becomes a natural biocontrol during

fermentation (Negara & Meilani, 2023). During fermentation, sugar is converted by bacteria and yeast into main compounds such as acetic acid, ethanol and glucuronic acid, as well as additional compounds such as lactic acid, phenolic compounds, vitamins B and enzymes. Fermentation increases the concentration of active ingredients in kombucha tea (Purnami et al, 2018). This is what makes kombucha tea healthier than regular tea. Kombucha contains epicatechin gallate, epigallocatechin, catechin, epicatechin, and epigallocatechin gallate which are useful as anti-carcinogenic and antioxidants (Mousavi et al., 2020).

One of the factors in the process of brewing kombucha tea that can affect the final result is the fermentation time. The fermentation time for kombucha tea ranges from 8-12 days at a temperature of 18-28°C (Wistiana & Zubaidah, 2015). Fermentation time will affect the alcohol content and active compounds in kombucha tea. According to research conducted by Goh et al., (2012) with kombucha tea using black tea, it can be seen that the best product is fermented for 8 days because it produces the highest cellulose precipitate, namely 66.9%.

Kombucha tea has a sour taste due to an increase in organic acid compounds during fermentation so that the pH of kombucha tea decreases. According to research by Wistiana & Zubaidah (2015), the longer the fermentation time, the total acid increases. This happens because the metabolism of yeast and bacteria to sucrose produces a number of organic acids such as acetic acid, gluconic acid, and glucuronic acid. Thus, the higher the organic acids in kombucha tea, the more sour it will taste.

The acidity level of a material is usually expressed by the pH value. The pH of the fermented product is closely related to the amount of acid produced. The pH value has an inverse relationship with the total acid titrated. The lower the pH value, the higher the total amount of acid being titrated (Prastujati et al., 2018). The pH value and total titrated acid of vanilla leaf kombucha tea can be seen in table 1. According to Sulistiawaty & Solihat (2022), the pH value produced during the fermentation

process is said to be safe for consumption if it tastes between 4.28 to 3.36. A pH value below 2.5 poses a health risk because the acetic acid content is too high.

Table 1: pH value and total titrated acid

Treatment	pH Value	Total Titrated Acid (%)
P1	5.56	0.003
P2	3.67	0.012
P3	3.06	0.024
P4	2.37	0.048

Description: P1 (0 day fermentation time), P2 (4 days fermentation time), P3 (8 days fermentation time), P4 (12 days fermentation time)

According to research by Puspitasari et al., (2017), on the 1st day of fermentation (pH 5.93) it gradually decreased on the 3rd day of fermentation (pH 5.31), on the 5th day (pH 5.12), the 9th day (pH 4.06) and the 11th day (pH 3.65). The results of research by Pratiwi et al., (2011) concerning the effect of fermentation time on the physical and chemical properties of kombucha from seaweed substrates yielded data, that is, the pH value decreased from day 0 to day 16, from pH 4.89 decreased to pH 3.09.

Nurhayati et al., (2020) conducted research on kombucha cascara tea (ripe coffee cherries) and could conclude that the fermentation time affected the physical, chemical and organoleptic properties. The best results after 8 days of fermentation because the total phenol is high, the pH and total acidity are still within safe limits for consumption. According to research by Yanti et al., (2020) on sour sop leaf preparations, kombucha for 12 days had the highest antibacterial activity with the ability to inhibit *Escherichia coli* of 16.28 mm and *Staphylococcus aureus* of 17.08 mm.

The decrease in pH occurs due to the formation of acetic acid. Yeast cells will hydrolyze sucrose into glucose and fructose to produce ethanol, while bacteria will metabolize glucose into gluconic acid and fructose into acetic acid. *Acetobacter* sp. in kombucha culture oxidizes ethanol to acetaldehyde and then to acetic acid (Puspitasari et al., 2017). The

decrease in the pH of kombucha tea also occurs because during fermentation, yeast synthesizes sugar into ethanol and is broken down by acetic bacteria into organic acids, such as acetic acid and gluconic acid and several concentrations of organic acids result in a decrease in the pH of the fermentation medium.

According to Devita et al., (2019) the longer the fermentation time, the more lactic acid produced by these lactic acid bacteria. These bacteria break down lactose into lactic acid, causing an increase in free hydrogen ions. Lactic acid bacteria are a non-pathogenic probiotic group, belonging to the Gram-positive bacteria group, in the form of coccus (round) or bacillus (rod), non-spore forming, catalase negative and oxidase positive, and lactic acid production. The rapid production of lactic acid by lactic acid bacteria inhibits the growth of other unwanted bacteria. In general, lactic acid bacteria belong to the Lactobacillaceae family, namely *Lactobacillus* sp. and Streptococcaceae, especially *Leuconostoc*, *Streptococcus* and *Pediococcus* (Putri et al., 2018).

Kombucha tea is trusted to contain phenolic compounds. These phenolic compounds have the potential as antioxidants. Sources of antioxidants can be found in polyphenolic compounds because polyphenols have the availability of hydrogen which is donated to counteract damage caused by free radicals (Husniati et al., 2021). Phenolic compounds are compounds that have groups (-OH) and can be found in various parts and types of plants. In the food and beverage industry, phenolic compounds function to provide a distinctive aroma, as coloring agents, and as antioxidants in food and beverage products. In the pharmaceutical and health industries, these compounds are used as antioxidants, antimicrobials, and anticancer (Christalina et al., 2018). The way phenolic compounds work is to denature proteins in bacteria. These compounds can be adsorbed into bacterial cells because they contain hydrogen bonds. If the phenolic level is low, phenolic forms protein complexes with weak bonds which will soon be decomposed and followed by phenolic penetration into the

bacterial cell, causing precipitation and protein denaturation (Wibisono et al., 2020).

The first step in carrying out a quantitative test of phenolic compounds is to prepare a standard phenolic solution. The standard solution consists of 100 ppm stock solution and solutions with various concentrations (10 ppm, 20 ppm, 30 ppm, 40 ppm, 50 ppm). The phenolic standard used in this lab is gallic acid or 3,4,5-trihydroxybenzoic acid ($C_6H_2(OH)_3CO_2H$). Gallic acid is a derivative of hydroxy benzoic acid which belongs to the simple phenolic acid group (Ansory et al., 2023).

In the phenolic content test process, the extract was added Folin Ciocalteu reagent and 7% Na_2CO_3 . The oxidation reaction of Folin by phenolic (alkaline salt) or hydroxyl-phenolic groups will reduce the phosphomolybdic-phosphotungstic acid present in Folin-Ciocalteu (Ansory et al., 2023). The Folin-Ciocalteu reagent will react with the hydroxyl group and form a blue molybdenum-tungsten complex which can be detected with a spectrophotometer (Andriani & Murtisiwi, 2018). The addition of Na_2CO_3 serves to achieve an alkaline state in the Folin reaction to cause proton dissociation of phenolic compounds into phenolic ions (Ansory et al., 2023). Na_2CO_3 can form an alkaline environment so that it can be observed that the greater the phenolic content present in the extract, the greater the intensity of the blue color produced (Toripah et al., 2014).

Solutions of various concentrations (10 ppm, 20 ppm, 30 ppm, 40 ppm, 50 ppm) were searched for absorbance using a UV-Visible Spectrophotometer instrument. The absorbance of each concentration can be seen in table 2.

Table 2: Data for determining gallic acid standard curves

Concentration (ppm)	Absorbance
10	0.38
20	0.585
30	0.705
40	1
50	1.255

After obtaining the absorbance of each concentration, the next step is to make a standard gallic acid standard curve that is used to find equations and linear regression. Gallic acid standard curve can be seen in Figure 1.

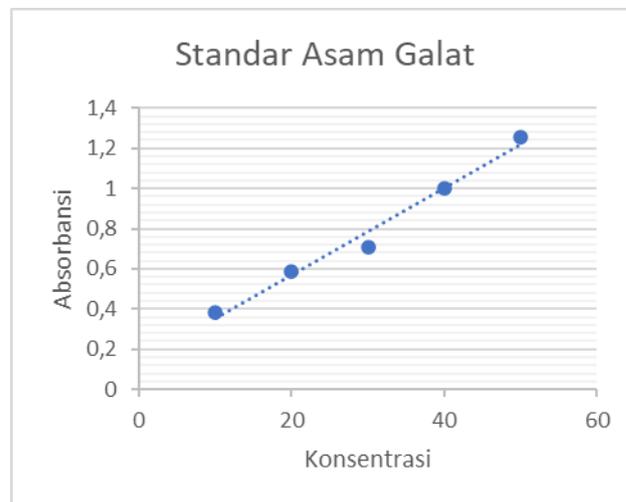


Figure 1: Standard Gallic Acid Standard Curve

The standard gallic acid standard curve has a regression equation y (absorbance) = $0.0217x$ (concentration) + 0.1355 with a coefficient of determination $R^2 = 0.9815$. The curve can be said to be linear because the coefficient of determination (R^2) is 0.9815 or close to one. The phenolic concentration can be determined by substituting the absorbance of the sample into Y in the equation. The magnitude of X indicates the concentration of phenolic in the sample.

Once the phenolic concentration is known, then TPC is calculated. Determination of TPC is part of the analysis that has to do with phenolic content and antioxidant activity. Samples containing relatively high levels of phenolic secondary metabolites usually have high antioxidant activity (Handayani et al., 2022). The total phenolic content of each extract is expressed as gallic acid equivalents (GAE). GAE is a general reference for measuring how many phenolic compounds are present in a material (Samin et al., 2013).

Based on the phenolic concentration present in each sample, the TPC value of vanilla

leaf kombucha tea was obtained in each treatment. The TPC value of each treatment can be seen in table 3 and figure 2.

Table 3: Phenolic Content

Treatment	Absorbance	TPC (mg/L GAE)
1	0.96	37.99
2	1.051	42.18
3	1.385	57.58
4	1.232	50.52

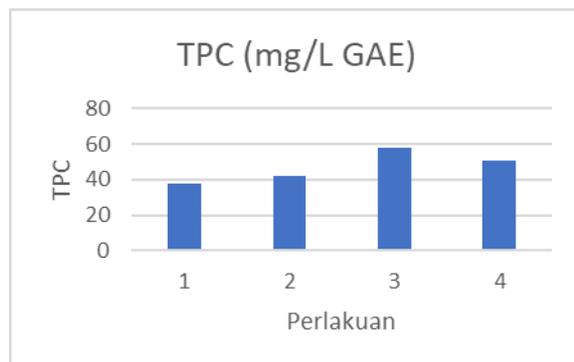


Figure 2. TPC value diagram for Vanilla Leaf Kombucha Tea

The TPC content of vanilla leaf kombucha tea at P1 (fermentation day 0) was 37.99 mg/L GAE, at P2 (fermentation day 4) was 42.188 mg/L GAE, at P3 (fermentation day 8) was 57.58 mg/L GAE and at P4 (12th day of fermentation) 50.52 mg/L GAE. Based on the data obtained, it can be seen that phenolic compounds increased at P2 and P3. According to Pebiningrum & Kusnadi (2018), the increase in phenolic levels is caused by enzymes released by bacteria and yeast in kombucha which will degrade complex compounds into simple compounds during fermentation. The increase in total phenolics is closely related to the metabolic activity of microbes during fermentation, where these microbes are able to modify bioactive components such as polyphenol groups, tannins and flavonoids. The presence of lactic acid bacteria in fermentation contributes to the conversion of phenolic complexes into simple ones and the depolymerization of phenolics which have high molecular weights (Rahmi et al., 2020).

Lactic acid bacteria present in kombucha can produce phenolic compounds. These bacteria carry out metabolism that can produce phenolic compounds by degrading ferulic acid and cinnamic acid into 4-vinyl phenol and 4-vinyl guaiacol (Winarsi et al., 2019). The fermentation process will increase the amount of phenolic compounds due to the decarboxylation process of cinnamic acid components such as trans-4-hydroxy-methoxycinnamic acid (ferulic acid (FA) and trans-4-hydroxycinnamic acid (p-coumaric acid (PCA)) to form phenolic compounds, namely 4-vinylguaiacol (4-VG) and 4-vinylphenol (4-VP) by microorganisms (Beek & Priest, 2000; Pebiningrum & Kusnadi, 2018).

Total phenolic decreased at P4 (12th day of fermentation) with a TPC value of 50.52 mg/L. The decrease in phenolic content can be caused by the sugar content which decreases with the duration of fermentation. The longer the fermentation lasts, the more the sugar content is depleted, causing the phenolic content to decrease after getting the optimum point. This is because microorganisms have run out of sugar as a food source (Puspitasari et al., 2017). The reduction of phenolic compounds can also be caused by the breakdown of these compounds, the dissolution of water-soluble phenolic compounds into water, and chemical rearrangements such as the binding of phenolic compounds with other organic materials (Diniyah & Lee, 2020).

CONCLUSION

Based on the research that has been done, the TPC value of vanilla leaf kombucha tea was obtained at 37.99 mg/L GAE on day 0, 42.188 mg/L GAE on day 4, 57.58 mg/L GAE on day 3. 8, and 50.53 mg/L GAE on the 12th day. The highest phenolic concentration was found on the 8th day. Thus it can be concluded that the length of time of fermentation affects the phenolic concentration in vanilla leaf kombucha tea.

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