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Physicochemical Characteristics of Bitter Leaf (*Vernonia amygdalina* Del.) Kombucha with Palm Sugar Addition

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Abstract

Functional drinks are processed drinks that contain food components that are beneficial for body health and have good sensory evaluation. Functional drinks can be used to prevent degenerative diseases such as diabetes mellitus. Kombucha is a functional drink fermented from tea and sugar using a kombucha starter or Symbiotic Culture of Bacteria and Yeast (SCOBY). Kombucha has functions as an antioxidant, antidiabetic, anticancer and antimicrobial. A variety of leaves that include phenols and bitter leaves with components that are beneficial to the body can be used to make kombucha. Palm sugar can be used as alternative sugar for kombucha fermentation because palm sugar has a low glycemic index of 35. This study aims to determine the effect of using palm sugar concentration on total dissolved solids, viscosity, pH value, total flavonoids, total phenols, and reducing sugar content. The research results show that the use of palm sugar can increase total dissolved solids, viscosity, pH value, total flavonoids, total phenols and reducing sugars. The best treatment is the use of 25% palm sugar with a total soluble solids value of 18.38˚Brix, viscosity of 1.37 cP, pH value of 3.43, total flavonoids of 0.275 mg QE/g sample, total phenols of 0.99 mg GAE/ml, and reducing sugar of 9.24%.

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Introduction

Functional food is processed food that contains one or more food components which, based on scientific studies, have certain physiological functions beyond their basic function, proven to be harmless and beneficial for health (BPOM, 2011). Functional food can be food or drink that serves to complete nutritional intake, provide sensory satisfaction, increase body resistance, source of prebiotics, fortify certain vitamins or minerals, and reduce the risk of certain diseases (Salsabila et al., 2021). Diabetes mellitus type 2 is a degenerative disease that can be prevented by consuming functional drinks. Research data shows that diabetes sufferers in Indonesia will reach 19.47 million people in 2021 and it is predicted that this will increase by 47%, namely around 28.57 million people in 2045 (IDF, 2021). Therefore, consuming functional drinks such as kombucha which has anti-diabetic potential can be done to prevent an increase in diabetes cases.

Kombucha is the result of fermenting a solution of tea and sugar using a starter culture of bacteria and yeast called Symbiotic Culture of Bacteria and Yeast (SCOBY). The yeast in SCOBY consists of *Saccharomyces* sp., *Zygosaccharomyces* sp., and *Pichia* sp., while the bacterium in SCOBY consists of acetic acid bacteria such as *Acetobacter xylinum*, *Acetobacter aceti*, *Gluconobacter xylinus*, *Gluconacetobacter xylinus*, lactic acid bacteria such as *Lactobacillus* sp., *Leuconostoc* sp., and *Lactococcus* sp. (Zubaidah et al., 2021). Kombucha fermentation lasts for 7 – 14 days in a glass bottle and stored at room temperature with range 23 – 35°C. The fermentation process by bacteria and yeast will produce a number of organic acids and vitamins which have various properties as antibacterial, antioxidant, antimicrobial, anticancer, anti-inflammatory and antidiabetic (Majidah et al., 2022).

Recent research has shown that kombucha made from snake fruit was effective as metformin in managing induced diabetes even better than the black tea kombucha (Zubaidah et al., 2019). In this study, kombucha will be made from bitter leaves that are known to have potential as antidiabetics. Bitter leaves (*Vernonia amygdalina* Del.) are an herbal plant containing bioactive compounds such as flavonoid alkaloids, phenolics, terpenes, tannins, terpenoids, triterpenoids, polyphenols, saponins and steroid glycosides which can reduce the activity of the enzymes α-amylase and α-glucosidase so it can be used as an alternative antidiabetic (Bestari, 2021). However, consumption levels of bitter leaves are still low because of their bitter taste. This can be reduced by processing it into a kombucha drink product using palm sugar.

Kombucha is made using palm sugar because it has a low glycemic index. The glycemic index of palm sugar is 35, while granulated sugar is 58 (Aprilia and Suryana, 2022). Previous research showed that rats given palm sugar for 28 days in doses of 2.57 mg/kg BW, 5.14 mg/kg BW, and 10.28 mg/kg BW for 28 days were able to reduce blood glucose levels in rats (Swastini *et al*., 2018). Thus, using palm sugar will improve the taste of bitter leaf kombucha and is safe for consumption by diabetes mellitus sufferers. The purpose of this research is to determine the effect of using different concentrations of palm sugar on pH value, total acid, alcohol content, viscosity, total dissolved solids, vitamin C, total phenol, reducing sugar content, total flavonoids, antioxidant activity, organoleptic properties and hedonic quality.

Materials and Methods

Materials

The raw materials used in this research were SCOBY kombucha from Rumah Fermentasi Banten, bitter leaves from residential yards in Sumberpucung Subdistrict, Malang Regency, palm sugar merk “NEO”, mineral water, distilled water, folin-ciocalteu reagent, Na_2CO_3, gallic acid, buffer pH 4 and 7, $AlCl_3$ 0.1 M, ethanol, anhydrous glucose, reagent nelson A, nelson B reagent, and arsenomolybdate.

The tools used in this research were analytical scales, measuring cups, tea bags, glass jars, jar covers, adhesive ropes, dropper pipettes, aluminium foil, Erlenmeyer, beaker glass, test tubes, micropipettes, micropipette tips, hot plates, vortexes, cuvettes, UV-Vis spectrophotometry, Ostwald viscometer, and stopwatch.

Experimental Design

This research was carried out from September to November 2023, at the Food Chemistry and Nutrition Laboratory and the Food and Agricultural Products Engineering Laboratory, Faculty of Animal Husbandry and Agriculture, Diponegoro University for making and preparing samples, analyzing the total dissolved solids value, viscosity, pH value, and reducing sugar content. Chemistry and Biochemistry Laboratory, Sebelas Maret University for total flavonoid testing. Food and Agricultural Products Technology Testing Laboratory, Faculty of Agricultural Technology, Gadjah Mada University for total phenol analysis.

Preparation of Bitter Leaf Tea

The preparation of dry bitter leaf tea (*Vernonia amygdalina* Del.) was done by separating the stems and leaves from fresh bitter leaves. Fresh bitter leaves that have been separated from the stem were then dried in the sun for 3 days, 10 hours each day. Dried bitter leaves were crushed into smaller pieces and then placed in sterilized tea bags. The tea bag used measures 6 cm × 8 cm. The weight of dried bitter leaves per bag is 4 grams (Novitasari et al., 2018).

Kombucha Fermentation

The production of bitter leaf kombucha with the addition of palm sugar was carried out based on Winandari et al. (2022) with modifications. Briefly, started with boiling 2 liters of clean water, then brewing 16 grams per liter (w/v) of dried bitter leaves in a tea bag and letting it sit for 5 minutes until the color and juice from the bitter leaves come out. The bitter leaves in the bag were then removed, then palm sugar is added with different concentrations, i.e., 5% (T1), 10% (T2), 15% (T3), 20% (T4) and 25% (T5) (w/v). The palm sugar was stirred until it is all dissolved, then left to sit until the solution reaches a temperature that is the same as room temperature, which is around 25℃. The starter for kombucha fermentation, namely 10% (w/v) SCOBY, was placed in a prepared glass jar, then covered with a clean cloth and then tied so that the cloth does not come off the jar. Fermentation was carried out for 8 days. The kombucha fermentation process must be carried out in a clean, dry room and away from direct sunlight.

Determination of Total Dissolved Solids

Total dissolved solids were measured using a refractometer based on research by Nurhayati et al. (2020). Total dissolved solids from bitter leaf kombucha were determined using a digital handheld refractometer (Atago, Japan) at a temperature of 25˚C and calibrated with distilled water. One or two drops of sample were dropped onto the refractometer prism. The results of the dissolved solids test on the sample were expressed as ˚Brix.

Determination of Viscosity

Viscosity was measured using an Ostwald viscometer based on research Gustishio et al. (2023). A sample of 10 ml was put into an Ostwald viscometer then sucked up to the top mark and then allowed to fall to the bottom mark. The time for the distilled water to drop to the bottom was calculated using a stopwatch. This step was repeated using distilled water as a comparison fluid. The specific gravity of the sample is measured using a pycnometer. Sample viscosity was calculated by the Equation 1, where ρ sample: sample specific gravity (g/ml); t sample: sample flow time (s); ρ: specific gravity of water (1 g/ml); t water: water flow time (s); η water: water viscosity (1 cP).

$$\text{Viscocity (cP)} = \frac{(\rho\ \text{sample})\ \times (\text{t sample})}{(\rho\ \text{water}) \times (\text{t water})}\ \eta\ \text{water}$$

Equation 1

Determination of pH

The pH value of kombucha was measured based on research Puspaningrum et al. (2022) using a pH meter (Ohaus, USA) calibrated with pH 4 and 7 buffers.

Determination of Total Flavonoids

Testing for total flavonoids in bitter kombucha leaves was carried out based on research by Dwiputri and Feroniasanti (2019) using the aluminum chloride colorimetric method using a UV-Vis spectrophotometer. The test began with making a standard curve using quercetin 200 ppm. The quercetin solution was made by dissolving 10 mg of gallic acid in 50 ml of methanol, then diluting it to a concentration of 100, 80, 60, 40, 20, and 0 ppm. Two ml of each concentration of quercetin solution was taken in a test tube and 5 ml of 0.1 M $AlCl_3$ was added. The solution was vortexed for 15 seconds and left for 40 minutes.

The absorbance value was read using a UV-Vis spectrophotometer at a wavelength of 415 nm. The absorbance results were used to create a standard curve for quercetin as the y-axis, while the concentration was used as the y-axis. The test was carried out by diluting the sample, then taking 2 ml and mixing it with 5 ml of 0.1 M $AlCl_3$ in a test tube, then vortex for 15 seconds and incubating for 40 minutes. The absorbance value was read using a UV-Vis Spectrophotometer at a wavelength of 415 nm. Total flavonoids are calculated using below equation.

$$\text{Total Flavonoids} = \frac{\frac{\text{Absorbance}}{1000} \times \text{Dilution factor}}{\frac{Sample\ weight(\text{g})}{Extract\ volume\ (mL)}}$$

Equation 2

Determination of Total Phenols

The test used to test total phenols refers to Rosyada et al. (2023) using the folin-ciocalteu method. Standard solution preparation used to test total phenol with 5 variations in concentration, namely 10 ppm, 20 ppm, 30 ppm, 40 ppm, and 50 ppm. Preparation of a gallic acid calibration curve used to test total phenol with different concentration treatments was taken as 1 ml poured into a test tube. Subseqently, 0.5 ml of Folin-Ciocalteu was added to the solution and 4 ml of 7% Na_2CO_3 solution was added. The solution was homogenized using a vortex and incubated at room temperature for 8 minutes. After that, the absorbance of the solution was measured using UV-Vis spectrophotometry at a wavelength of 760 nm. The results obtained are a gallic acid curve with the linear equation y = ax + b.

Determination of total phenol content was carried out by taking 1 ml of kombucha sample and pouring it into a test tube, then adding 0.5 ml of Folin-Ciocalteu and 4 ml of 7% Na_2CO_3 solution to the solution. The solution that was made was homogenized using a vortex and left for 8 minutes. After that, the absorbance of the solution was calculated at a wavelength of 760 nm. The total phenol content is calculated using Equation 3 where c is concentration (x value) (ppm), v is extract's volume (mL), D is dilution factor, and w is sample weight in g.

$$Total\ phenols\ (\frac{\text{mg}}{\text{g}}\text{GAE}) = \frac{c \times v\ \times D}{w}$$

Equation 3

Determination of Reducing Sugars

Testing for reducing sugar in bitter leaf kombucha is in accordance with research by Haryanti and Mustaufik (2020) using the Nelson-Somogyi method. Testing for reducing sugar begins by making a standard curve, namely by making a standard sugar solution by making an anhydrous glucose solution of 1 mg/ 10 ml of distilled water. Dilution of anhydrous glucose is carried out until a solution with a concentration of 0.02-0.08 mg/ml is obtained. The solution was added to a test tube to 1 ml, then 1 ml of Nelson's reagent was added. Based on research (Dewi et al., 2017), nelson solution is made by mixing nelson A and nelson B in a ratio of 25:1 (v/v). The reaction tube was heated in boiling water for 20 minutes, cooled, and 1 ml of Arsenomolybdate reagent was added, waiting until the Cu_2O precipitate dissolved again. 7 ml of distilled water was added to the test tube and then vortexed. The solution was then poured into a cuvette and read using a UV-Vis Spectrophotometer with a wavelength of 540 nm. A standard curve was created by showing the relationship between anhydrous glucose concentration and Optical Dencity (OD). Measurement of reducing sugars in kombucha samples using a UV-Vis spectrophotometer was carried out as in the standard curve preparation. The reducing sugar is then calculated using Equation 4 where A is absorbance and D is dilution factor.

$$Reducing\ sugar\ (\%) = \frac{A \times D}{Sample\ (mL)} 100\%$$

Equation 4

Data Analysis

Data on total dissolved solids, viscosity, pH and reducing sugars were analyzed using Analysis of Variance (ANOVA) at a significance level of 5% and if there was a real effect, it was continued with the Duncan Multiple Range Test (DMRT). Data on total flavonoid and total phenol values were analyzed descriptively.

Results and Discussion

Total Dissolved Solids

As can be seen in Table 1, the results showed that the use of palm sugar with different concentrations in bitter leaf kombucha made a significant difference ($p<0.05$) to the total soluble solids value. The total dissolved solids value in each treatment increased with an average value ranging from 4.50 – 18.38˚Brix. This value is close to the research of cascara kombucha fermented for 8 days with the use of 10% sugar at 11.75˚Brix (Nurhayati et al., 2020).

Table 1 Total Dissolved Solids, Viscosity and pH of Bitter Leaf Kombucha

Palm Sugar (%)	Total Dissolved Solids (˚Brix)	Viscosity (cP)	pH
5	$4.50±0.14^a$	$1.57±0.02^a$	$3.23±0.03^c$
10	$8.38±0.24^b$	$1.60±0.03^a$	$3.18±0.02^d$
15	$12.03±0.30^c$	$1.67±0.02^b$	$3.23±0.03^b$
20	$15.70±0.23^d$	$1.70±0.02^b$	$3.36±0.04^b$
25	$18.38±0.35^e$	$1.81±0.04^c$	$3.43±0.02^a$

*Different lowercase superscript indicates a significant difference ($p<0.05$) within the same column.

The total dissolved solids in bitter leaf kombucha are the ingredients dissolved in the solution. Sugar has the property of being easily soluble in water so that the more sugar added causes an increase in the total dissolved solids in the resulting solution (Breemer et al., 2021). Total dissolved solids consist of organic acid components, vitamins, pigments, total sugars and proteins contained in a material (Kamsina et al., 2015). The total sugar remaining from the breakdown of the fermentation process and the organic acids formed are counted as total dissolved solids (Pramesta and Sucahyo, 2022).

Viscosity

Based on the data in Table 1, variations in the use of palm sugar have a significant effect ($p<0.05$) on the viscosity of bitter leaf kombucha. Viscosity increases with the high concentration of palm sugar in kombucha. According to Tarihoran et al. (2022) adding sugar will increase viscosity because of sugar's ability to bind water, thereby reducing water content. Sugar can reduce viscosity because it is hygroscopic. The structure of the sugar molecule consists of hydroxyl groups which can bond with water molecules through hydrogen bonds so that the water content in the food will be reduced and reduce the viscosity of the product (Sumartini and Suliasih, 2021).

The lowest viscosity was in treatment T1 (5%) 0.86 cP, while the highest viscosity was in treatment T5 (25%), namely 1.37 cP. This value is higher than the research results of Nurhayati et al. (2020) which states that kombucha fermented for 8 days using 10% granulated sugar has a viscosity of 0.90 – 0.98 cP. A lower viscosity value of kombucha will be preferred by consumers because it is lighter and fresher to drink. The difference in value is caused by high sugar concentration, which component sucrose and fructose dissolve easily in water so they can increase the amount of dissolved substances which affect product viscosity (Arziyah et al., 2022).

pH value

As shown in Table 1, variations in the use of palm sugar have a significant effect ($p<0.05$) on the pH value of bitter leaf kombucha. These data show that there was a decrease in pH at using palm sugar 10%, then an increase at using palm sugar 15% to 25% The decrease in pH is caused by the production of organic acids by microorganisms during the fermentation process.

According to Ayuratri and Kusnadi (2017), the low pH value in kombucha is caused by the accumulation of acids which decompose and release free protons. The data listed in Table 3 shows that the use of palm sugar exceeding T2 (10%) causes an increase in pH. Thus, the optimum amount of palm sugar used in bitter leaf kombucha is 10%, because excess sucrose availability will disrupt the balance of the fermentation medium. According to Nur et al. (2021) bacteria easily experience lysis in fermentation media that are too concentrated due to increased osmotic pressure. The average pH in this study ranged from 3.18 – 3.43. This is in accordance with statement Mclntyre et al. (2023) which states that the pH of tea before fermentation starts from 5 and the pH of kombucha after fermentation must be in the range 2.5 – 4.2 to prevent the growth of *B. cereus* or *C. perfringens* spores which can grow on tea leaves.

Total Flavonoids

Based on the data in Table 2, the flavonoid content in kombucha of bitter leaves increases significantly along with the addition of palm sugar concentration, it affects microbial activity in hydrolyzing sugar to form simpler compounds by lactic acid bacteria. This is in accordance with research by Putri et al. (2023) that when the fermentation process takes place, the flavonoid content will increase due to an increase in the phenol content. This is due to an enzymatic reaction that stimulates the formation of flavonoids during fermentation. The enzymatic reaction is influenced by microbial activity in the SCOBY which degrades polyphenolic compounds into simpler ones.

Table 2 Total Flavonoids, Total Phenols, and Reducing Sugar of Bitter Leaf Kombucha

Palm Sugar (%)	Total Flavonoids (mg QE/g)	Total Phenol (mg GAE/ml)	Reducing Sugar (%)
5	0.135	0.45	1.90±0.36[a]
10	0.166	0.61	2.91±0.87[a]
15	0.167	0.96	6.41±0.56[b]
20	0.238	1.06	7.43±0.90[b]
25	0.275	0.99	9.24±0.80[c]

*Different lowercase superscript indicates a significant difference ($p<0.05$) within the same column.

According to Sintyadewi and Widnyani (2021), the increase in total flavonoids during the fermentation process is due to the activity of lactic acid bacteria which form enzymes to break down sugars and degrade complex phenolic compounds, as well as releasing phenolic compounds from the substrate to form flavonoids. According to research by Tu et al. (2024), it was stated that the total flavonoids in black tea kombucha were 0.72 mgQE/g. Black tea at 10 grams per liter is fermented for 8 days, producing higher phenolic compounds than bitter leaf kombucha with only 8 grams of leaves per liter. This value proves that the number of leaves used affects the total flavonoids. This is in accordance with the research of Yuningtyas et al. (2021) who reported that the concentration of leaves used affects the value of flavonoids in kombucha, as evidenced by the amount of African leaves used which is less than black tea leaves producing a smaller amount of flavonoids. In addition, the low total flavonoids in bitter leaf kombucha are due to the age of the leaves used. Young bitter leaves do not contain flavonoids, this is because of the synthesis phase that occurs during the leaf aging process (Putri, 2019).

Total Phenol

Based on Table 2, the use of palm sugar with different concentrations in bitter leaf kombucha gives a total phenol value that tends to increase significantly. The lowest value was obtained at a palm sugar concentration of 5%, while the highest value was obtained at a palm sugar concentration of 20%. The total phenolic value of bitter leaf kombucha using palm sugar ranged from 0.45 – 1.06 mg GAE/ml. Total phenol tends to increase due to the addition of palm sugar which contains phenol. According to Badmus et al. (2016) powdered palm sugar has a phenol content of 5.82 mg GAE/100 g. Apart from that, the raw material used in making kombucha in the form of bitter leaves also contains phenol so that it can enrich the total phenol of the kombucha produced. Extraction of bitter leaves using methanol has a phenol content of 3.471 g GAE/ 100 g (Suhendy et al., 2022).

According to Ayuratri and Kusnadi (2017), kombucha phenolic compounds are increasing because the metabolism of microorganisms can convert complex polyphenolic compounds into simple compounds. A decrease in total phenols occurred in the treatment of bitter leaf kombucha made with a palm sugar concentration of 25%. This is due to the phenol compounds contained in African leaf kombucha are easily damaged by the brewing process with high temperatures. This is supported by the opinion (Dewata et al., 2017) which states that bioactive ingredients such as phenols can be damaged at high temperatures and have an optimal temperature range between 0 - 90˚C. Phenolic compounds can experience damage at high temperatures, oxygen, light, and enzymes affecting the stability of bioactive compounds such as polyphenols (Mahardani and Yuanita, 2021).

Reducing Sugar Content

Based on Table 2, the greater the added sugar concentration, the greater the reducing sugar content significantly. This is in accordance with research by Habibah et al. (2017) that the greater the sugar concentration added to the kombucha solution, the greater the reducing sugar content. Based on research by Saputra et al. (2017) stated that the reducing sugar content in bay leaf kombucha using 10% sugar was 8.56%, while using 20% sugar had a reducing sugar content of 25.57%. This result is higher when compared to bitter leaf kombucha using 10% palm sugar, namely 2.91% and using 20% palm sugar, namely 7.43%. This is because palm sugar contains less sucrose, so it is degraded more quickly by microbes.

The yeast *(Saccharomyces cereviseae*) can decompose sucrose in palm sugar into alcohol and other compounds, followed by oxidation of alcohol into acetic acid by acetic acid bacteria. The difference in the amount of sugar concentration added will affect the growth of microbes in kombucha, so it will also affect the chemical compounds (Yanti et al., 2020). The increase in reducing sugars according to the concentration used in kombucha can occur due to hydrolysis activity by the invertase enzyme. Yeast (*Saccharomyces cerevisiae*) can break down the sucrose in palm sugar into alcohol and other compounds, then continue with the oxidation of the alcohol into acetic acid by acetic acid bacteria. As the fermentation process continues, the reducing sugar content will decrease (Putri et al., 2023). The microorganisms in the solution will utilize the sugar in the media as a source of nutrition which is then converted into alcohol and CO_2. CO_2 gas will react with water vapor which then forms carbonic acid (Wulandari, 2017).

Conclusion

Based on the results, it can be concluded that the overall concentration of palm sugar used can increase total dissolved solids, viscosity, pH value, total flavonoids, total phenols and reducing sugars. The best treatment is the use of 25% palm sugar. Further research is needed on the duration of fermentation, SCOBY concentration, and type of sugar in making bitter leaf kombucha. This is expected to produce bitter leaf kombucha with total dissolved solids, viscosity, pH value, total flavonoids, total phenols and reducing sugars that meet the standards.

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