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Determination of Total Phenolic Content and Antioxidant Activity of Tepache Fermented Product as Probiotic Beverage

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Abstract

Tepache is a fermented beverage from Mexico that is made from pineapple peel. It can improve the balance of good bacteria and reduce the risk of gastrointestinal disease. Pineapple peel contains vitamin C, phenolic compounds, and flavonoid compounds, which have antioxidant function. There have been many studies on making tepache from pineapple peel. However, there is limited research about organoleptic tests with several influencing factors, such as the type of sugar and fermentation time. The research aimed to determine tepache beverages' total phenolic content and antioxidant activity. Tepache beverages are made with pineapple peel, brown sugar, and water, and then fermented for 1, 3, and 5 days. Gallic acid is used as a standard to determine total phenolic content. The DPPH test was carried out to determine its antioxidant activity. The result of the total phenolic contents in tepache beverages for 1, 3, and 5 days of fermentation was 0.0278 mg GAE/g sample, 0.0307 mg GAE/g sample, and 0.0436 mg GAE/g sample—antioxidant activity expressed by IC_{50} value. IC_{50} values in tepache beverages for 1, 3, and 5 days of fermentation were 160.198 ppm, 150.639 ppm, and 142.713 ppm. Tepache beverages have low total phenolic content levels and low to medium antioxidant activity.

Keywords: Antioxidants, DPPH, phenolic, pineapple skin, tepache

Introduction

Human life does not forget several important things, including health. The need for food is increasingly changing in the modern era because people's views on the importance of health and healthy lifestyles are changing. Besides being nutritious, food ingredients must also help the body physiologically. The body can benefit from functional foods by strengthening its immune system and improving its physical condition (Khoerunisa, 2020). Several drinks, including probiotic drinks, have a positive impact on health.

A process that causes chemical changes to the substrate by microorganisms is called fermentation (Suryani et al., 2017). Fermentation is a food processing process that utilizes microbes to produce new processed products with distinctive aroma and taste characteristics. Microbes' breakdown of the substrate in the fermentation process will produce alcohol, carbon dioxide, or organic acids (Azara & Saidi, 2021).

The beverage industry in Indonesia has developed rapidly. It produces products from various ingredients, such as fruit, vegetables, fruit juice, and other mixed ingredients. The fruit's flesh is the most often used, while other parts, such as the skin, are just thrown away and end up as waste. One fruit peel that can be used is pineapple peel (Sukriadi et al., 2022).

Pineapple peel has the potential to be used as a raw material for making functional food because it contains high levels of sugar and vitamin C. According to research by Hatam et al. (2013), the IC50 value of pineapple peel extract using maceration, Soxhlet extraction, and reflux techniques was 3.18 ppm, 2.78 ppm, and 2.95 ppm, respectively. Meanwhile, according to Widyanto et al. (2020), pineapple methanol extract has an IC_{50} of 1549.88 ppm. Apart from that, according to (2018), it contains 81.72 % water, 20.87 % crude fiber, 17.53 % carbohydrates, 4.41 % protein, and 13.65 % reducing sugar in pineapple skin, so it has the potential to be processed into a probiotic drink. Tepache is one of the products that can be made. This agrees with Sagita et al. (2023) in their research regarding the use of pineapple fruit waste in the production of the probiotic drink tepache.

The development of probiotic drink products using waste materials has been widely carried out. However, this research only covers physical quality and several influencing factors. Therefore, research on the effect of fermentation time on the antioxidant activity of tepache probiotic drink products is very necessary. The research

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aimed to determine tepache beverages' total phenolic content and antioxidant activity.

Methods

Materials

Pineapple peel obtained from Kalisapu Village, Slawi District, Tegal Regency, brown sugar, distilled water, methanol of pro analysis, gallic acid powder, DPPH powder, vitamin C powder, Na_2CO_3 powder, Folin-Ciocalteu reagent, potassium dichromate powder, iodoform, and $FeCl_3$.

Making tepache

After collection, the pineapple skin is sorted. Next, weigh 300 grams of pineapple skin and clean it with water. After cleaning, the pineapple skin is sliced into small pieces. Pineapple skin is placed in a glass fermentation container. 500 mL of distilled water dissolves 100 grams of brown sugar. Once dissolved, the brown sugar and pineapple skin are put into the fermentation container. Then, cloth covers the jar and is tied with a rubber band. The pineapple skin is fermented within 1, 3, and 5 days (Sukriadi et al., 2022).

Screening for phytochemical compounds

Secondary metabolites of Tepache beverages were identified using Reiza et al.'s methods.

a. Alkaloids

The sample solution was put into 3 different test tubes of 2 mL each, then 1 mL of 2 N HCl was added. Mayer's reagent was added to the first tube, Wagner's reagent to the second tube, and Dragendorff's reagent to the third tube.

b. Flavonoids

A total of 2 mL of sample was dissolved in 2 mL of 70 % ethanol, then heated for approximately 2 minutes. After heating, 4 - 5 drops of concentrated HCl and 0.1 gram of Mg powder are added. Positive results are indicated by the appearance of a yellow-orange to dark red color within 3 minutes.

c. Tannins

A total of 2 mL of sample was dropped into 1 % $FeCl_3$ solution. A positive tannin test result is if the solution changes color to blackish green or blackish blue.

d. Saponins

A total of 5 mL of sample was put into a test tube. Next, the solution is shaken for 1 minute; add 1 N HCl if foam appears. The foam formed can last 5 minutes, then the extract is positive for containing saponin.

e. Steroids or terpenoids

A total of 5 mL of sample was put into a test tube, then dissolved with 0.5 mL of chloroform, and 0.5 mL of anhydrous acetic acid was added, followed by 2 mL of concentrated sulfuric acid through the tube wall. The formation of a brown or violet ring at the border of the solution indicates the presence of triterpenoids, whereas if a greenish blue ring appears, it indicates the presence of steroids

Alcohol test

Samples were tested for alcohol content qualitatively using color reagents, namely:

a. Potassium Dichromate 3.5 %

A total of 0.5 mL of sample was diluted with 15 mL of distilled water. After that, it was reacted with 12.5 mL of $K_2Cr_2O_7$ until the color changed to orange. Then, the solution is heated and cooled. If the sample is positive for containing alcohol, the solution will change color to blackish green after being heated (Rakhmatullah et al., 2022).

b. Iodoform

A total of 5 mL of sample was carefully reacted with 1 mL of 1 N NaOH and 2 mL of 0.1 N iodine. If alcohol is present, the solution will give off a characteristic iodoform aroma and form a yellow precipitate (Oktaviani et al., 2011).

Phenolic preliminary test

a. Test with Folin-Ciocalteu

A 0.5 mL sample was reacted with 2.5 mL of Folin-Ciocalteu. After 10 minutes, it was reacted with 7.5 mL of Na_2CO_3 solution. Look at the color changes that occur (Dalming et al., 2023).

b. Test with $FeCl_3$

A total of 5 mL sample was reacted with 1 mL of $FeCl_3$. The appearance of red, green, blue, purple, or black indicates the presence of phenolics in it (Bayani, 2016).

Determination of total phenolic content

a. Preparation of Na_2CO_3 20%

To make a 20 % Na_2CO_3 solution, put 20 grams of Na_2CO_3 in 80 mL of distilled water, boil until the Na2CO3 powder dissolves, leave it for 24 hours, filter it, and add distilled water up to 100 mL (Pramiastuti et al., 2018).

b. Measurement of Gallic Acid Standard Solution

Gallic acid solutions with 100, 200, 300, 400, 500, and 600 ppm concentrations were pipetted in 0.1 mL each. Then, 7.9 mL of distilled water and 0.5 mL of Folin-Ciocalteu were added. Then the solution was homogenized using a vortex within 1 minute. The solution was transferred into a 10 mL measuring flask, and 20% Na_2CO_3 solution was added to the mark. Then the solution was incubated for 27.5 minutes. Absorption was measured at a wavelength of 657.5 nm, and a linear regression equation $y = a + bx$ was created (Hapsari et al., 2018).

c. Determination of Total Phenolic Content of Tepache

A test tube is filled with 0.1 mL of sample. The sample was added with 7.9 mL of distilled water and 0.5 mL of Folin-Ciocalteu reagent, then homogenized using a vortex within 1 minute. The solution was transferred into a 10 mL measuring flask, and 20 % Na_2CO_3 solution was added to the mark. Then the solution was incubated for 27.5

minutes. Absorption was measured at a wavelength of 657.5 nm (Hapsari et al., 2018).

Antioxidant assay

Antioxidant assay was carried out using the DPPH method (Nadia et al., 2016). The samples' concentrations were 100, 120, 140, 160, and 180 ppm. Vitamin C was used as a positive control. The vitamin C solution was prepared according to Ibrahim et al. (2024). The concentrations of the vitamin C used were 0.5, 1, 2, 4, 6, and 8 ppm. A total of 0.2 mL of the test solution was taken with a measuring pipette, and then 3.8 mL of DPPH 0,05 mM solution was added. The solution was homogenized and left in a place with minimal light for 35 minutes. Absorption was measured at a wavelength of 515.5 nm. The percentage value of antioxidant inhibition was calculated using the equation:

$$\% \text{ inhibition} = \frac{\text{Absorption control} - \text{Sample absorbance}}{\text{Absorption control}} \times 100\% \quad (1)$$

Results and Discussion

Tepache beverage

The tepache beverage has a diversity of microbes. The microbes in tepache beverages are dominated by lactic acid bacteria (Lactobacillus, Acetobacter, and Lactococcus) and fungi (Saccharomyces, Gibberella, Candida, and Kabatiella). When the fermentation occurs, the sugar is transformed into ethanol and carbon dioxide to decrease the sugar content. The sugar contained inside tepache beverages is converted into ethanol with the help of Saccharomyces cerevisiae. The fermentation reaction of the tepache beverages' seen in **Figure 1** (Najini et al., 2024).

$$C_6H_{12}O_6 \rightarrow 2C_2H_5OH + 2CO_2$$

Carbohydrate Ethanol Carbon dioxide

Figure 1. Tepache Beverage Fermentation Reaction

Phytochemical results

The purpose of conducting phytochemical screening tests is to determine the secondary metabolite content in tepache beverages with varying fermentation times. Tepache beverages were continued with qualitative phytochemical tests to identify these secondary metabolites. Based on the results, the content of secondary metabolites is shown in **Table 1.**

Table 1. The secondary metabolite of tepache beverages

Secondary Metabolite	Results		
	1 day	3 days	5 days
Alkaloid	+	+	+
Flavonoid	+	+	+
Tanin	+	+	+
Saponin	+	+	+
Triterpenoid	+	+	+

This result is similar to the secondary metabolites in the tepache beverage, which also contains alkaloids, flavonoids, tannins, and saponins (Rezaldi et al., 2022). Although obtained from different locations, the dominant metabolite was the same.

Alcohol test

The tepache fermentation process involves converting sugar (glucose, fructose, or sucrose) into ethanol (alcohol), carbon dioxide, and adenosine triphosphate (ATP) as an energy source, similar to the fermentation mechanism in other alcoholic beverages (Devi et al., 2024). Therefore, it is necessary to test for the presence of alcohol in tepache beverage using potassium dichromate and iodoform tests with different fermentation times.

a. Potassium Dichromate 3.5 %

The principle of this reaction is an oxidation-reduction reaction. Potassium dichromate can be used to identify the presence of alcohol due to the specific color change of potassium dichromate as it reacts with ethanol. Potassium dichromate, which is initially orange, will change to chromium (III) sulfate, which is blue because it is reduced by ethanol. There is a color change to green in tepache beverages after the sample is reacted with potassium dichromate. This happens because the alcohol level in tepache beverages is small, so there will also be much less reducing agent (Kartika, 2022). The reaction is in **Figure 2.**

$$3CH_3CH_2OH + 2K_2Cr_2O_7 + 8H_2SO_4 \rightarrow 3CH_3COOH + 2Cr_2(SO_4)_3 + 2K_2SO_4 + 11H_2O$$

Figure 2. Alcohol Test Reaction with Potassium Dichromate

b. Iodoform

In this test, an iodine solution in KI solution is used as the main sample to identify alcohol, while NaOH is used as a sample to influence the changes that occur in iodine (Antonius et al., 2021). The test results showed the formation of a yellow precipitate, so it could be concluded that the tepache beverages positively contained alcohol. The reaction in the iodoform test is in **Figure 3.**

$$C_2H_5OH + 6KI + 6NaOH \rightarrow 2CHI_3 + 6NaI + 6KOH$$

Figure 3. Alcohol test reaction with Iodoform

Phenolic preliminary test

The preliminary phenolic test aims to determine the presence of phenolic content qualitatively.

a. Test with Folin-Ciocalteu

This test is carried out to ensure that the sample used contains phenolic compounds. This method relies on colorimetric reduction and oxidation reactions. The result of the reaction between the phenolic-hydroxyl group and Folin-Ciocalteu will form a blue phosphotungstate-phosphomolybdate complex, which can be detected by a UV-Visible spectrophotometer (Dalming et al., 2023). Based on the test results, the solution

changed color to blue, indicating that the tepache beverage positively contained phenolics. The reaction that occurs is shown in **Figure 4.**

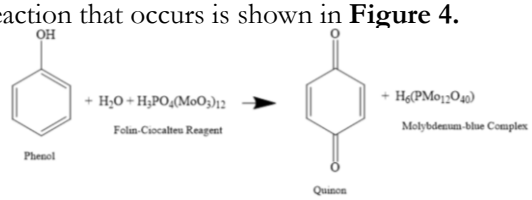

Figure 4. Reaction of Phenolic Compounds with Folin-Ciocalteu

b. Test with $FeCl_3$

Phenolics were identified using $FeCl_3$ solution. The result of the reaction between the Fe^{3+} ion and the phenolic group will form a green, blue, or black color in the solution (Putri et al., 2018). Based on the test results, the solution changed color to green, indicating that the tepache beverage positively contained phenol. This reaction is shown in **Figure 5.**

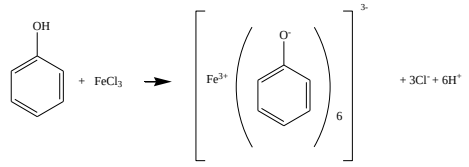

Figure 5. Reaction between Phenol and $FeCl_3$

Determination of total phenolic content

The aim of determining total phenolic content is to determine the phenolic content quantitatively in tepache drinks with varying fermentation times. Gallic acid (3,4,5-trihydroxy benzoic acid) is a hydrobenzoic acid classified as a simple phenol. Gallic acid is used as a standard because it is a natural phenolic compound with a strong antioxidant effect (Selviyana, 2019). Determination of total phenolic content was carried out using the Folin-Ciocalteu reagent. The hydroxyl group in phenolic compounds can react with the Folin-Ciocalteu reagent to form a blue molybdenum-tungsten complex, which can be detected using a UV-Vis spectrophotometer.

In determining phenolic content, the initial step taken is determining the maximum wavelength by measuring the absorbance of standard solutions at a wavelength of 400-800 nm. Moreover, in this research, the maximum wavelength for the standard solution was 657.5 nm. Standard solutions with concentrations of 100, 200, 300, 500, and 600 ppm were then measured for their absorbance at 657.5 nm, which was obtained from measuring the highest absorbance of gallic acid as measured using spectrophotometry. The results of gallic acid absorbance measurements can be seen in **Table 2.**

The total phenolic content in tepache beverages was determined by measuring the sample absorption at a wavelength of 657.5 nm, which was repeated three times to produce accurate data. The results of determining the total phenolic content of tepache beverages are in **Table 3**.

Table 2. Results of absorbance measurements of standard gallic acid solutions

Concentration (ppm)	Absorbance
100	0.199
200	0.238
300	0.375
500	0.518
600	0.644

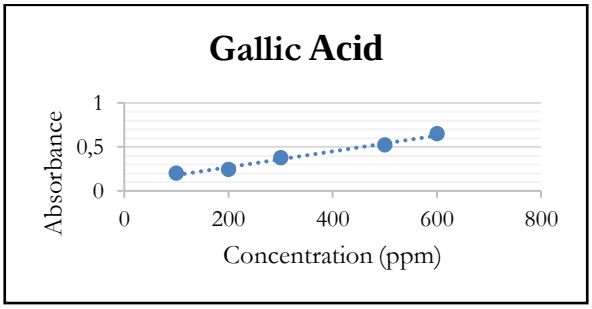

Figure 6. Gallic Acid Calibration Curve

Table 3. Results of Total Phenolic Content of Tepache Beverages

Fermentation Time	Replication	Absorbance	Total Phenolic Content (mg GAE/g)	Average (mg GAE/g)
1 Day	R1	0.463	0.0276	0.0278
	R2	0.463	0.0276	
	R3	0.469	0.0281	
2 Days	R1	0.506	0.0308	0.0307
	R2	0.505	0.0307	
	R3	0.505	0.0307	
3 Days	R1	0.655	0.0419	0.0436
	R2	0.653	0.0417	
	R3	0.724	0.0470	

Antioxidant activity

This test was carried out to see the antioxidant activity in tepache beverage, which was treated with different fermentation times. Determination of antioxidant activity was carried out at a wavelength of 515.5 nm with an incubation time of 35 minutes. The samples tested for their antioxidant activity were tepache drinks with varying fermentation times of 1, 3, and 5 days, while vitamin C was used as a comparison. Concentration variations of 0.5, 1, 2, 4, 6, and 8 ppm were used to assess vitamin C. Tepache drink samples were tested for antioxidant activity at 100, 120, 140, 160, and 180 ppm concentrations. The concentration of the sample solutions is made at the same concentration so that their antioxidant activities can be compared with each other.

Table 4. IC_{50} Value of Vitamin C and Tepache

Replication	Vitamin C	Fermentation 1 Day	Fermentation 3 Days	Fermentation 5 Days
1	3.780	161.076	148.454	142.994
2	3.533	160.678	151.746	142.710
3	3.646	158.839	151.716	142.434
Average	3.660	160.198	150.639	142.713
Category	Very strong	Weak	Weak	Moderate

Bacterial growth activity that is less than optimal can cause antioxidant activity to be moderate. Lag, log, stationary, and death phases are the four phases of BAL development. According to research conducted by Sabira & Suryani (2023) in tepache, lactic acid bacteria begin their initial growth phase (lag phase) and adaptation phase on the third day. Apart from that, research by Yanti et al. (2023) shows that BAL increased significantly during the 24-hour incubation period since entering the log phase. This significant growth occurred up to 72 hours. The log phase covers growth from 24 to 72 hours. During the 72 to 96-hour incubation period, overall BAL values continued to increase. These results indicate that the stationary phase occurs within 72 to 96 hours of incubation, leading to a stable LAB growth trend.

When fermentation occurs, yeast and bacteria metabolize sucrose into several organic acids, increasing the amount of organic acids. In addition, the invertase enzyme hydrolyzes sucrose into glucose, decreasing the total sugar value (Puspaningrum et al., 2021).

The longer the fermentation time, the higher the organic acids in tepache. This is because the longer the fermentation, the higher the acetic acid produced from the metabolism of the Acetobacter xylinum bacteria. The fermentation results will become more acidic as the fermentation progresses. Higher organic acids in tepache drinks can influence antioxidant activity, so the IC_{50} results on day 5 are stronger than on days 1 and 3 (Puspaningrum et al., 2022).

The results of the phenolic content determination test showed low phenolic content. This impacts the tepache's weak antioxidant activity. This aligns with research by Jemima et al. (2023) regarding the antioxidant activity of tepache using the DPPH and FRAP methods. The results show that the IC_{50} value of tepache drinks is 198.51 ppm in the weak category.

Apart from the content of natural bioactive compounds, the length of fermentation of a drink also influences its antioxidant level. According to Shintawati (2022), kombucha reaches its antioxidant peak on the 7th day of fermentation and begins to decrease on the 10th day. After the seventh day of fermentation, the acidic conditions made the phenolic compounds more stable, making it difficult to release protons, resulting in decreased antioxidant concentrations. This condition of phenol degradation reached an optimum state compared to the fermentation of less than 7 days.

Conclusions

Based on the research, the optimum fermentation time for tepache's antioxidant activity is on the fifth day. Fermentation time has a significant influence on tepache's antioxidant activity. The longer the fermentation time, the stronger the antioxidant activity will be. This is indicated by the IC50 values for 1, 3, and 5 days of fermentation, namely 160.198 ppm, 150.639 ppm, and 142.713 ppm.

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