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Antioxidant Assay of Kefir Peanut (*Arachis hypogaea* L.) with Variations in Concentration and Fermentation Time

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ABSTRACT

Kefir is a processed beverage produced through pasteurized milk fermentation using a starter from kefir grains. These grains contain groups of lactic acid bacteria and yeast. Peanuts can be used as a raw material for kefir products and *Arachis hypogaea* L. has antioxidant activity due to a high nutritional content namely vegetable oil, protein, minerals, and essential fatty acids. This study aimed to determine the highest antioxidant activity with variations in concentration and time fermentation of peanut kefir (*Arachis hypogaea* L.). This research on the antioxidant activity test method used the DPPH method with UV-Vis spectrophotometry. The results of testing the antioxidant activity of peanut kefir with variations in the concentration of kefir grains of 0%, 1%, 2%, and 3% had IC50 values of 64.929 ppm, 57.675 ppm, 54.742 ppm, and 51.870 ppm, respectively. The one-way ANOVA test showed a significant difference between the IC50 value and the concentration of kefir grains. The highest antioxidant in 3% concentration determined the fermentation time with 0, 12, 24, 36, 48, and 60 hours variations. The results of testing the antioxidant activity of kefir peanut with variations in the fermentation time it was had IC50 values of 64.319 ppm respectively, 62.609 ppm, 59.376 ppm, 56.321 ppm, 51.870 ppm, and 51.384 ppm. The one-way ANOVA test results showed no significance in the 0 with 12 hours completion time and 48 with 60 hours. The research results on peanut kefir show that kefir grains of 3% and the duration of fermentation for 48 hours have strong antioxidant activity.

Keywords: Antioxidants; *Arachis hypogaea* L.; Fermentation; IC50; Kefir; Kefir grains

INTRODUCTION

Free radicals are atoms, molecules, or ions. They contain one or more unpaired electrons in their outermost orbitals and have a high reactivity (Di Meo and Venditti, 2020). Free radicals are harmful to the human body because they can damage the components of the cells due to an imbalance between the amount of free radicals and the antioxidant activity produced by the body. So we need antioxidants that can help protect the body from the effects of free radicals (Nurmazela et al., 2022). Antioxidants are compounds that can neutralize free radicals to prevent degenerative reactions by donating electrons to stop the chain reaction of free radicals (Souhoka et al., 2019).

One source of antioxidants is fermented kefir products. Kefir is traditionally processed using raw materials in the form of animal milk. But in its development, kefir can be made from vegetable raw materials (Hidayah, 2019). Kefir is a processed beverage produced through pasteurized milk fermentation using a starter form of kefir grains (Sulmiyati et al., 2018). Kefir grains contain groups of lactic acid bacteria and yeast. Peanuts can be used as a raw material for making kefir (Viogenta et al., 2021). Peanut kefir (*Arachis hypogaea* L.) has the potential to have strong antioxidants because it has a high nutritional content and is the main source of vegetable oil, protein, minerals, and essential fatty acids (Stella, 2019). A peel-off gel mask made from peanut kefir was proven to have strong antioxidant activity (Khairunnisa et al., 2022).

One of the factors that can affect the process of making kefir is fermentation time and concentration. Excessive fermentation time and higher concentrations of kefir grains in the manufacture of kefir will result in higher densities of lactic acid bacteria and yeast. Based on previous research, the 48-hour fermentation time increased the number of lactic acid bacteria and decreased again at 72 hours. The bacteria entered the death phase because the available nutrients had been completely broken down by the activity of lactic acid bacteria (Kinteki et al., 2019; Viogenta et al., 2021; Yusriyah and Agustini, 2014).

The types of starter microbes, temperature, fermentation time, and raw materials used can affect kefir components and the chemical composition contained in kefir. The process of standardizing the quality of kefir is needed to produce kefir with optimal chemical, microbiological, and organoleptic quality

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(Yusriyah and Agustini, 2014). This study aims to determine the best antioxidant activity of kefir grains and fermentation time in peanut kefir production.

MATERIALS AND METHODS

Materials

The materials used in this research included peanuts (*A. hypogaea* L.), kefir grain (kefiree), D-glucose (MERCK 108337), aquadest (*Water One*), methanol p.a (MERCK), 2,2-difenil-1-pikrilhidrazil (DPPH) (sigma), and quercetin (sigma).

Methods

Kefir Peanut Extract

Peanuts were sorted, washed, and then mashed using a blender and added aquadest (1:8). The mixture of aquadest and peanuts was filtered to get peanut filtrate. Peanut extract added 4% D-glucose (b/v). The peanut extract was sterilized using an autoclave for 5 minutes at 121°C. Furthermore, variations in the concentration of kefir grains 0%, 1%, 2%, and 3% were added with a fermentation time of 48 hours. Peanut kefir with the concentration of kefir grains with the highest antioxidant value was then carried out variations of fermentation time of 0, 12, 24, 36, 48, and 60 hours to know the best kefir fermentation time to produce antioxidant activity.

Determination of Maximum Wavelength (λ max)

1 mL of 0.4 mM DPPH solution was added to 1 mL of methanol p.a in a 5 mL volumetric flask. Then the absorbance of the solution was measured using a UV-Vis spectrophotometer at a wavelength of 450-550 nm (Udayani et al., 2022).

Determination of Operating Time

1 mL of 0.4 mM DPPH standard solution was added to 4 mL of 100 ppm quercetin standard solution. The mixture was then read with a UV-Vis spectrophotometric instrument at the previously obtained wavelength every 2 minutes for 60 minutes (Khairunnisa *et al.*, 2022).

Quercetin Antioxidant Activity Assay as Control Comparison

Quercetin standard solution (100 ppm) was prepared with 2.5 mg of quercetin dissolved in methanol. 100 ppm quercetin was used as a standard solution by making a series of 2, 4, 6, 8, and 10 ppm levels. 1 mL of 0.4 mM DPPH solution was pipetted and added to each assay series. The solution was then read for its absorbance at the maximum wavelength. Measurement of antioxidant activity was expressed in the percentage of inhibition and IC_{50} value. The equation can calculate the percent inhibitor:

$$\%\ inhibition = \frac{Ab - As}{Ab}\ x\ 100\%$$

Ab: Absorbance of blank (methanol); As: Absorbance of quarcetin

Antioxidant Activity Assay of Peanuts Kefir with the DPPH Method

A standard solution of 1000 ppm peanut kefir, then a series of 10, 15, 20, 25, and 30 ppm solutions were made in a 10 mL volumetric flask. Then 1 mL of 0.4 mM DPPH was added to the solution, incubated at room temperature, and protected from sunlight. The solution is then read for its absorbance at the maximum wavelength (Khairunnisa *et al.*, 2022). Measurement of antioxidant activity was expressed in the percentage of inhibition and IC50 value. The equation can calculate the percent inhibitor:

$$\%\ inhibition = \frac{Ab - As}{Ab}\ x\ 100\%$$

Ab: Absorbance of blank (methanol); As: Sample absorbance.

Data Analysis

The inhibition percentage obtained was then entered into the regression equation with ppm concentration as the x-axis and the percent inhibition value as the y-axis. The linear regression equation y=a+bx was used to determine the IC50 value obtained from the x value after replacing y=50. Data analysis carried out in this study was in the form of quantitative analysis. Measurement of antioxidant activity is expressed in percentage of inhibition and IC50 value and statistical analysis using IBM SPSS® version 26 with analysis of variances.

RESULTS

Determination of the maximum wavelength was carried out to obtain the wavelength of the DPPH compound with the maximum absorbance value in a certain absorption area, where light absorption will be transmitted and reflected in a sample (Batubara et al., 2020). The maximum wavelength is determined by looking at the relationship curve of the DPPH absorbance value at the specified wavelength (Apriana et al., 2017). Sample measurements were carried out at the maximum wavelength to obtain maximum sensitivity and minimize error (Agustiarini and Wijaya, 2022). The maximum wavelength obtained from 0.4 mM DPPH solution in this study was 515 nm with an absorbance of 0.3093 (Figure 1).

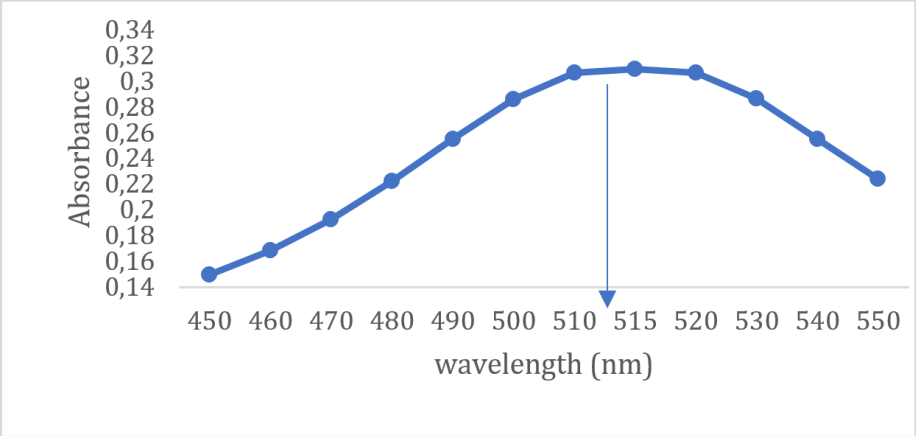

Figure 1. DPPH Maximum Wavelength Determination

These results were consistent with research which states that the maximum wavelength of the DPPH solution was at 515 nm and had entered the DPPH wavelength range of 515-520 nm (Agustiarini and Wijaya, 2022; Amelia and Nasution, 2022). The operating time or incubation time used in this study was the absorbance of quercetin-DPPH from 26-34 minutes. The determination of the operating time was indicated by the absence of a further decrease in absorbance (Vifta et al., 2019). The operating time was indicated by the absorbance, which starts to be constant on the graph a few minutes after the sample was reacted (Figure 2).

In table I it can be seen that the results of measurements using a UV-Vis spectrophotometer with a wavelength of 515 nm obtained percent inhibition of quarcetin with concentrations of 2, 4, 6, 8 and 10 ppm, namely 25.528, 40.093, 61.201, 79.08 and 89.947. The regression equation results obtained from the relationship between the concentration of the quercetin reference solution and the inhibition percentage were y=8.391x + 8.822 with a correlation coefficient (r) of 0.995. The result of inhibition of DPPH free radicals was 50% in comparison to quercetin, namely 4.907 ppm. These results belong to the very strong category of inhibiting free radicals (Molyneux, 2004).

The relationship between the concentration of kefir grain and the IC50 peanut kefirs value can be seen in Table II.

The results of the calculation of the percent inhibition show that the concentration of kefir grains that can reduce the DPPH free radicals is 50%, namely a 0% concentration of peanut extract without the addition of kefir seeds of 64.929 ppm, a concentration of 1% kefir seeds of 57.675 ppm, a concentration of 2% kefir seeds of 54.742 ppm, and the concentration of 3% kefir grains was 51.870 ppm. Peanut kefir with a concentration of 1-3% kefir grains is classified as active (50-100 ppm) (Gordon and Hudson, 1990). The results of the statistical analysis of the IC50 value of variations in the concentration of kefir grains in the One-Way ANOVA test and the Post Hoc test showed that the analysis results between all concentrations of kefir grains obtained a value $p<0.05$. These results indicate that there are significant differences in the results of the analysis. So it can be concluded that there is an effect of the addition of kefir grains on the antioxidant activity of peanut kefir. Based on the IC50 value, the concentration of 3% kefir grains was the highest concentration variation and will be used to determine the IC50 value of peanut kefir with variations in fermentation time. The greater the provision of kefir grains, the higher the total microbial population because more bacteria and yeast are found in kefir (De Souza et al., 2020).

The results of the calculation of the fermentation time that can reduce DPPH free radicals by 50% can be seen in Table III.

Based on the IC50 value obtained in determining the best fermentation time, it can be seen that the 60-hour fermentation time has the highest antioxidant activity with the lowest IC50 value. The IC50 value for 60 hours of fermentation is 51.384 ppm, classified as active (50-100 ppm) (Gordon and Hudson, 1990).

The statistical analysis results of the IC50 value of variations in the time of peanut kefir fermentation in the One-Way ANOVA test obtained a significance value of 0.000 ($p <0.05$). The Post Hoc analysis results between fermentation times were 0 to 12 hours, and 48 to 60 hours obtained a value of $p\geq0.05$ which indicated no significance in the analysis result. Meanwhile, the results of other fermentation times showed a $p<0.05$, indicating significant differences in the analysis results.

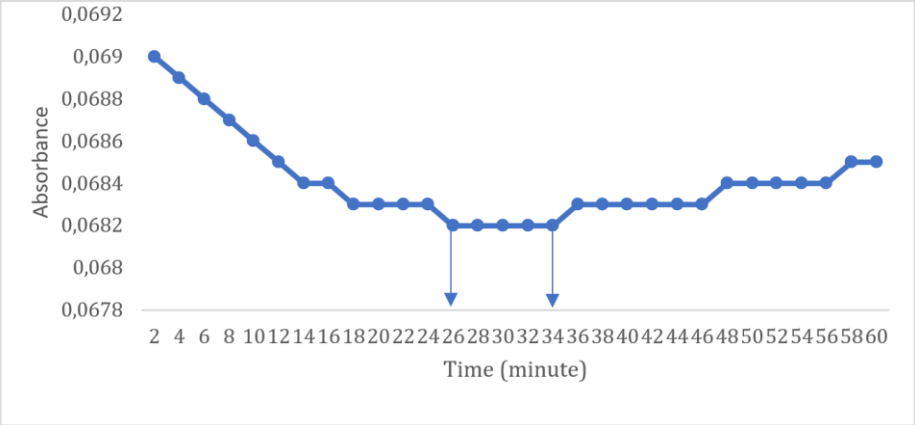

Figure 2. Operating Time Determination

Table I. IC_{50} results from Quercetin reference solution

Concentration (ppm)	Percent inhibition (%)	IC50 (ppm) ± SD	RSD (%)
2	25.528		
4	40.093		
6	61.201	4.907 ± 0,036	0.734
8	79.08		
10	89.947		

Table II. IC50 Results of Peanut Extract Kefir with Various Concentrations

Kefir Grain	IC50 (ppm) ± SD
0%	64.929 ± 0.432
1%	57.675 ± 0.362
2%	54.742 ± 0.307
3%	51.870 ± 0.334

Table III. IC50 Result of Peanut Kefir with Variation of Fermentation Time

Fermentation Time (Hours)	IC50 (ppm) ± SD
0	64.319 ± 1.135[a]
12	62.609 ± 1.014[a]
24	59.376 ± 0.119
36	56.321 ± 0.614
48	51.870 ± 0.334[b]
60	51.384 ± 0.964[b]

DISCUSSION

The peanut (*Arachis hypogaea* L.) sample used in this study was locally shelled peanuts. Processing of kefir using peanuts is a food variation for people allergic to lactose (Lactose intolerance) (Stella, 2019). To make kefir from peanut seeds, D-glucose was added as the carbon source needed by microbes to obtain energy and growth, and as a substrate for fermentation (Kunaepah, 2008). The starter used was kefir seeds with various concentrations of 0%, 1%, 2%, and 3%. The more kefir grains were added, the more microbes were contained in the kefir (Safitri and Swarastuti, 2013). The duration of fermentation in making kefir also greatly affects the quality of the kefir produced. The longer the fermentation, the more the total acid and the lactic acid bacteria will increase (Anjliany et al., 2022).

Antioxidant activity testing uses the DPPH radical capture method. The DPPH method is based on the reduction of a purple DPPH radical solution by a free radical inhibitor (electron donor) into a yellow non-radical compound (Sundu et al., 2022). The principle of the DPPH method is that there is a relationship between antioxidants and DPPH through the transfer of electrons or hydrogen radicals to DPPH which causes DPPH to become non-radical. If all the electrons in the DPPH free

radical have a pair, the color change will occur from dark purple to pale yellow and can be seen on a UV-Vis spectrophotometer. Sample measurements were carried out at the maximum wavelength with the aim of obtaining maximum sensitivity and minimizing errors (Agustiarini & Wijaya, 2022).

The results of the maximum wavelength were followed by operating time testing to determine the time when the reaction was most stable (Rahayu et al., 2021). Operating time measurement was important to determine the stable reaction time between DPPH as a free radical and sample solution. This stability is because quercetin and DPPH have reacted perfectly, as can be seen from the occurrence of color fading, which was originally dark in color and will lose color (Lembong and Lara Utama, 2021). If the solution's absorbance is measured before the operating time, the reaction may not be perfect (Suharyanto and Prima, 2020). Quercetin is a positive control because it has high antioxidant activity and is classified as very strong (Ramadhan et al., 2020). Quercetin belongs to a group of flavonoids with phenolic compounds, is found in almost every plant type, and has keto and hydroxyl groups.

The concentration of 0% kefir grains was peanut extract without adding kefir grains as a starter. The difference in antioxidant activity in peanut extract and peanut kefir can be seen from the increase in the value of antioxidant activity from 0% to 3%. These results indicate an effect of fermentation on the value of antioxidant activity with variations in the concentration of kefir grains. The higher the kefir grains added, the higher the antioxidant activity. It was because the microbes that make kefir produce antioxidant compounds from the results of microbial metabolism in kefir grains. The RSD results for the four concentrations were as follows per the literature, which states that the relative standard deviation (RSD) requirement was ≤ 2.7% (Wulansari and Lubada, 2020). Synthesis of antioxidant components can be produced faster in proportion to the higher concentration of starter in the fermentation. However, because the availability of substrate does not increase, a point will be reached where degradation and synthesis reach optimum. Antioxidant activity has increased, one of which is due to forming organic acids. The longer the fermentation will cause an increase in the production of acids and alcohol, lowering the pH of the kefir. An increase in an acidic environment is known to increase the stability of antioxidants. Acidic compounds such as lactic acid, acetic acid, citric acid, and succinic acid produced during fermentation can also increase and stabilize antioxidant activity (Primurdia, 2014).

CONCLUSION

In the present study of the antioxidant activity using the DPPH method, the highest concentration of peanut kefir seeds was 3% with an IC50 value of 51.870 ppm. The highest antioxidant activity was investigated at 60 hours of the fermentation time of peanut kefir extract with an IC50 of 51.384 ppm.

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