

Original article

The content of secondary metabolites and total flavonoid levels of Miswak Wood (*Salvadora persica*) extract by kinetic maceration

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Abstract

Miswak wood is empirically used to clean teeth, as it is known to have antibacterial activity mediated by its secondary metabolite. As a potential plant, miswak wood contains various secondary metabolite compounds, such as flavonoid terpenoid, steroid, alkaloid, saponin, and tannin. The levels and types of active compounds extracted from the extraction process depend on the nature of the solvent used. This study compares the total flavonoid levels of miswak wood with methanol, n-butanol, and ethyl acetate solvents. This study used an experimental design from an in vitro laboratory. This study uses samples of miswak wood extract extracted using the kinetic maceration method using methanol, n-butanol, and ethyl acetate solvents. Then, the extraction results will be qualitatively analyzed through phytochemical screening to see the presence or absence of flavonoid compounds, saponins, tannins, phenolics, alkaloids, terpenoids, and steroids, and quantitative phytochemical screening will be carried out to determine the level of flavonoid compounds in miswak wood extract using UV-vis spectrometry. Data analysis used non-parametric Kruskal-Wallis, followed by a post-hoc comparison test. Extract of miswak wood by kinetic maceration method showed positive for secondary metabolite compounds of flavonoids, alkaloids, terpenoids, phenolics, tannins in methanol, n-butanol, and ethyl acetate solvents, while saponins were only negative in ethyl acetate solvent. Determination of flavonoid compound levels showed ethyl acetate contained as much as 99.15 ± 8.14 mgEQ/g, then n-butanol as much as 62.08 ± 6.10 mgEQ/g, and methanol as much as 13.23 ± 7.47 mgEQ/g significance test ($P < 0.05$). Post-hoc pairwise Wilcoxon test showed significant results ($p < 0.05$). This shows the type of solvent that influences flavonoid levels. The types of compounds that can be withdrawn by methanol and n-butanol solvents are more than the ethyl acetate solvent. The highest flavonoid levels were found in ethyl acetate solvent, and the lowest in methanol solvent.

Keywords: ethyl acetate; methanol; n-butanol; secondary metabolite

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Introduction

Miswak wood is empirically used by most Indonesians as a medium for cleaning teeth, which, when associated with its bioactive compounds, has an antibacterial effect. Research findings show that miswak is effective in killing bacteria that cause dental caries (Maulidina et al., 2022). This suggests that miswak has considerable potential as an antibiofilm agent. Dental caries arises from the development of bacterial biofilms, the mechanism of which is to produce acidic substances that can cause damage to tooth enamel (Maulidina et al., 2022).

Miswak contains active compounds that are quite good in the process of destroying biofilm caused by bacteria (Maulidina et al., 2022). Ethnomedicine contains compounds that are unique in both complexity and biological activity. As a potential plant, miswak wood contains various secondary metabolite compounds, such as flavonoid terpenoid, steroid, alkaloid, saponin, and tannin. (Rehman, 2022). Other studies also mention that miswak wood contains various bioactive compounds, such as flavonoids, sterols, saponins, tannins, alkaloids, reducing components, and essential oils (Ramli et al., 2022). The focus of this research is to determine the

content of active secondary metabolite compounds contained in miswak wood and to determine the level of flavonoid compounds contained in miswak wood as the main antibacterial compound.

Flavonoids are active compounds that have antimicrobial activity. These compounds can effectively inhibit the growth of bacteria, viruses, and fungi, thus providing a good therapeutic effect against infections caused by various pathogenic microorganisms, including *Staphylococcus aureus*, *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Candida albicans*, and *Aspergillus flavus* (Huang et al., 2022). Flavonoids are compounds that are highly effective as inhibitors of bacterial cell membranes and energy metabolism by forming complex compounds with extracellular proteins that can lyse bacterial cell membranes (Cushnie & Lamb, 2005). Flavonoids have shown potential as an alternative or adjuvant in modern antibiotic therapy because of their effectiveness against resistant bacteria and their synergistic properties when combined with conventional antibiotics (Song et al., 2022). Flavonoids are found in almost all parts of the plant, namely in the roots, stems, bark, leaves, flowers, and even in the fruit. Flavonoids include phenolic compounds that are rich in hydroxyl groups. (Puspa Yani et al., 2023).

Several things can affect the content of active substances, one of which is that the difference in solvents can affect the active substance content of the extracted material. Extraction solvents have been demonstrated to be effective in the extraction of secondary metabolites from herbal plant tissue extracts. The selection of

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extraction solvents is responsible for the differences in the secondary metabolites produced. Furthermore, the efficacy of extraction methods can be contingent upon the secondary metabolites present, underscoring the necessity of selecting an optimal extraction method (Tella & Oseni, 2019).

The study aims to determine the optimal solvent for extracting active compounds, especially flavonoid compounds, contained in miswak wood obtain using maceration extraction method with different solvents. The advantage of this method is that it minimizes damage to thermolabile compounds. This method does not require high heating so that the compounds contained in the material become well decomposed and do not experience damage due to heating (Susanty & Bachmid, 2016). After making the extract, the researcher will carry out phytochemical screening of the miswak extract using qualitative and quantitative methods. Until now, few studies discuss the content of active compounds qualitatively in miswak wood, and fewer studies quantitatively measure flavonoid compounds.

Methods

Design, place, and time of research

Experimental design using an *in vitro* research design to determine the content of active compounds present in the extract of the miswak plant using three solvents: methanol, n-butanol, and ethyl acetate. This research was conducted in January 2025 - March 2025 at the Laboratory of the Islamic University of Malang.

Sample preparation

The miswak wood trunk is first cleaned and washed to remove dirt until it is clean, then baked first until the moisture content is <10%, the dried miswak wood simplisia is ground until it becomes simplisia powder.

Extract preparation

Miswak simplisia powder was prepared in quantities of as much as 80 grams and then put into an Erlenmeyer flask. Next, 400 ml of solvent (Methanol, ethyl acetate, or n-butanol) was added to the Erlenmeyer containing the miswak simplisia. The mixture was then covered with aluminum foil and left in a shaker water bath for one day. After 24 hours, the extraction results were filtered using a vacuum-assisted filter to separate the filtrate from the rest of the simplisia. The resulting filtrate was then evaporated using a rotary vacuum to separate the extract from the solvent. The extract obtained was collected in a glass jar that had been weighed, then put into the oven at 40-60 ° C and left for 48 hours until the extract became thick or paste-like. The extraction process was repeated three times in different batches.

$$\% \text{ yield} = \frac{\text{weight of extract}}{\text{weight of simplisia}} \times 100\%$$

Phytochemical Screening

Qualitative

Flavonoid

The flavonoid test was carried out with 0.5 g of extract and 96% ethanol up to 5 mL, after which it was warmed for $\pm$ 5 minutes, added 10 drops of concentrated HCl, and 0.2 g of magnesium powder. The solution was shaken. The formation of reddish black, yellow, or orange indicates a positive result of flavonoids (Septia Ningsih et al., 2020).

Terpenoid and steroid

A total of 0.5 g of extract was put into a test tube to which 2 ml of concentrated H₂SO₄ was added. The solution was shaken gently and left for a few minutes. Blue to green color indicates positive results of the steroid test, while brownish red to purple color indicates positive results of the terpenoid test (Septia Ningsih et al., 2020).

Alkaloid

The alkaloid test was conducted with 0.5 g of extract, 2 ml of chloroform, 10 ml of ammonia, and 10 drops of H₂SO₄. The mixture was shaken and allowed to form two layers. The H₂SO₄ layer formed was transferred to three test tubes with a volume of 2.5 mL each. The solutions were tested with Mayer's reagent. The formation of a white precipitate characterizes the positive result of Mayer's reagent (Septia Ningsih et al., 2020).

Saponin

A total of 0.5 grams of extract was put into a test tube and then added to preheated distilled water up to 10 mL. The mixture was shaken vigorously for approximately 1 minute. Next, it was allowed to stand for 10 minutes, and the froth or foam formed, which indicated a positive result for saponin (Septia Ningsih et al., 2020).

Tannin

A total of 1 mL of extract, is mixed with FeCl₃ 10%. The presence of tannins is indicated by a change in color to dark blue or greenish black (Puspawati et al., 2023).

Phenolic

A total of 1 mL of extract, is added with 1 mL FeCl₃. The presence of phenolic is indicated by a change from green to solid black, to blue-black (Tjitda & Nitbani, 2019).

Quantitative

Preparation of quercetin standard solution

To prepare a stock solution, 10 mg of quercetin was added to 100 mL of ethanol p.a to prepare a 100 ppm quercetin standard solution. Quercetin standard solution from the stock solution was made into 10, 20, 30, 40, and 50 ppm concentrations in a 10 ml volumetric flask. Then each concentration series was pipetted into a test tube in three replicates, quercetin standard solution as much as 1 mL added, 1 mL AlCl₃ 10%, CH₃COONa 1 mL, and 2 mL distilled water. Then it was vortexed and incubated for 30 minutes, and measurements were taken with UV-Vis spectrophotometry to determine the maximum wavelength of 427 nm and measure the absorbance of the standard solution.

Preparation of quercetin standard curve

The standard curve was made by correlating the absorbance results obtained from the measurement of the

maximum wavelength of 427 nm using a UV-Vis spectrophotometer with the concentration of quercetin standard solution.

Determination the level of total flavonoid

Each extract of the three solvents was weighed as much as 5 mg, and 10 mL of ethanol p.a was added to a 10 mL volumetric flask to obtain a concentration of 500 ppm. Each solution was pipetted into a test tube with three replicates, 1 mL of extract solution added, 1 mL of 10% AlCl₃, 1 mL CH₃COONa, and 2 mL distilled water. Then, it was vortexed and incubated for 30 minutes. After 30 minutes, measurements were taken with UV-Vis spectrophotometry to determine the maximum wavelength and measure the absorbance of the standard solution. Total flavonoids from miswak wood extract were calculated using a linear regression equation from the previously measured quercetin calibration curve. Total flavonoid content was expressed as the quercetin equivalent per gram of extract (mg QE/g extract).

Data analysis

Data are expressed as mean $\pm$ SD. After normality and homogeneity tests were carried out, where the test results were not normal, therefore the Kruskal-Wallis non-parametric statistical test was used, then continued with the post-hoc test comparison to determine differences between solvents. The results are said to be significantly different when the value ($p < 0.05$).

Results

Yield of Miswak Wood Extract

The average results of the extracts obtained from each solution Table 1.

Table 1. Yield of Miswak Wood Extraction

Solvent	Average Extract SD $\pm$ (n=3)	Average Extract Yield
Metanol	9.72 $\pm$ 1.61 gram	12.15%
EA	0.99 $\pm$ 0.51 gram	1.24%
Butanol	1.12 $\pm$ 0.37 gram	1.40%

The weight of the miswak wood simplicia used was 80 g in 400 ml of solvent. The yield results show that different types of solvents have varying extraction effectiveness. Among them, methanol gave the highest yield of 12,15%, n-butanol 1,4% and ethyl acetate 1,24%.

Qualitative phytochemical screening of miswak wood extraction

The results of phytochemical tests of miswak wood extracts in methanol, n-butanol, and ethyl acetate solvents are listed in Table 2.

Table 2. Results of Phytochemical Screening Compound Content

Compound type	Color Reaction	Result					
		Methanol		Ethyl acetate		n-Butanol	
		O1	O2	O1	O2	O1	O2
Flavonoid	Reddish black, yellow or orange	+	+	+	+	+	+
Terpenoid	Brownish red to purple	+	+	+	+	+	+
Alkaloid	White sediment	+	+	+	+	+	+
Steroid	Blue to green	-	-	-	-	-	-
Saponin	Foam that forms	+	+	-	-	+	+
Tanin	Dark blue or greenish black	+	+	+	+	+	+
Phenolic	Greenish, pitch black, to blue-black	+	+	+	+	+	+

Note: (+) identified, (-) not identified, (O1) observer 1, (O2) observer 2

In Table 2, The results of the qualitative phytochemical screening demonstrate that the extraction of metabolites is more efficient when using methanol and n-butanol extracts as opposed to ethyl acetate.

Quantitative measurement of flavonoids

The results of the total flavonoid content are shown in Figure 1, which is obtained from the calibration of the standard curve shown in Figure 2.

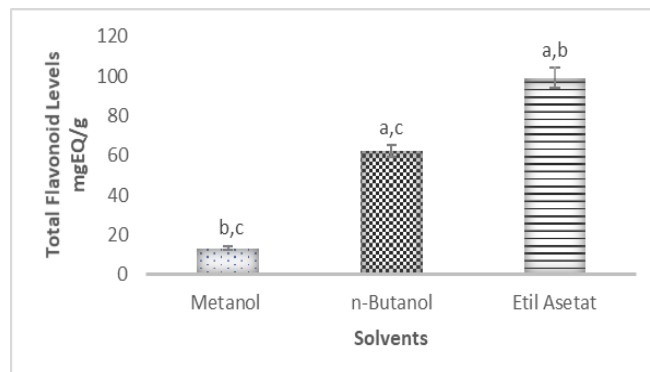


Figure 1. Graph of Total Flavonoid Levels From The Extract Solvents Different Notation Show Significant ($p < 0.05$).

Note: (a) Significant to methanol, (b) Significant to n-butanol, (c) Significant to ethyl acetate

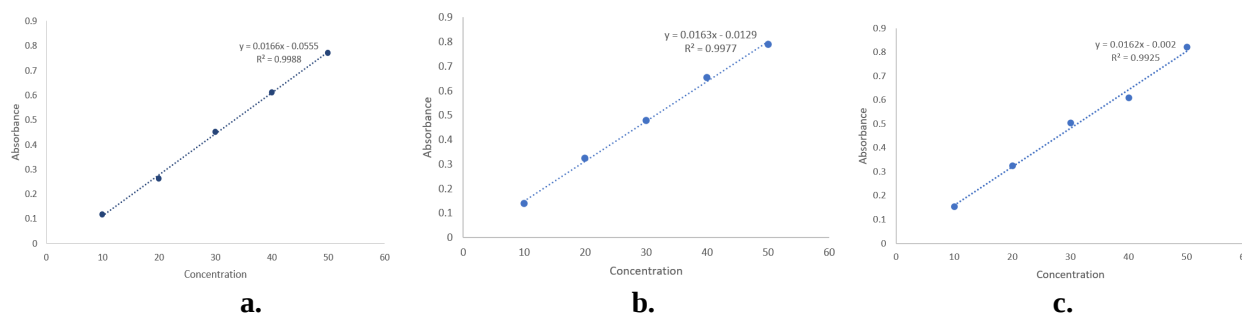


Figure 2. Standard curve. Note: Figure a standard curve of batch 1 with correlation coefficient $R^2 = 0.9988$, figure b standard curve of batch 2 with correlation coefficient $R^2 = 0.9977$, figure 3 standard curve of batch 3 with correlation coefficient $R^2 = 0.9925$.

Based on Figure 1, the average total flavonoid content of extracts with methanol solvent was 13.23 ± 7.47 mgEQ/g, n-butanol was 62.08 ± 6.10 mgEQ/g, and ethyl acetate was 99.15 ± 8.14 mgEQ/g. Kruskal-Wallis significance test ($p < 0.05$) shows that the total flavonoid content is influenced by the type of solvent, the average difference of the three solvents shows significant results expressed in ($p < 0.05$).

Discussion

Yield of Miswak Wood Extract

The yield results of miswak wood extracts show significant differences, where the highest yield results are found in extracts in methanol solvents, then n-butanol, and the lowest in ethyl acetate. Several factors, such as solvent type, solvent concentration, simplisia particle size, extraction method, and extraction time, affect the yield. Extraction is the separation of parts in solution based on differences in their solubility. During the extraction process, the active ingredients will be dissolved by the appropriate solvent according to their polarity properties (Nursamsiar et al., 2023). Click or tap here to enter text. Click or tap here to enter text.

The yield results are due to differences in the polarity of the extraction solvent which causes a large difference in the levels of bioactive compounds in the extract, methanol is a polar solvent that is commonly used because it has the ability to penetrate cell walls better so that the extraction efficiency is higher than n-butanol and ethyl acetate (Septia Ningsih et al., 2020). In addition, the nature of the compounds contained in herbal ingredients determines how much yield is produced, so that polar compounds are more extracted in methanol.

Methanol has a lower molecular weight compared to other types of solvents, methanol solvents can form a hydrogen bond and dissolve in H_2O at an infinite solubility level, which means it can bind to the -OH group in a large ratio. Because the hydroxyl group (-OH), which has a very high electronegativity value of oxygen, methanol has a very strong polar group. The smallest yield was found in the ethyl acetate solvent, which indicates that there are few semi-polar bioactive compounds extracted in miswak wood (Septia Ningsih et al., 2020).

Phytochemical Compounds Contained in Miswak Wood Extract

Based on Table 2, it is evident that the solvents methanol, n-butanol, and ethyl acetate encompass active compounds including flavonoids, terpenoids, alkaloids, tannins, and phenolics, except saponins, which are absent in ethyl acetate. It shows that the three solvents have almost the same ability to attract different secondary metabolite compounds, which is also in line with research conducted by Krisnawan previously, who explained that miswak extracts carried out using maceration methods with ethanol solvents contain active compounds of flavonoids, alkaloids, terpenoids, saponins, tannins, and phenolics (Krisnawan, 2023). The

findings of this study are in partial or complete alignment with those of previous research on the stem of *Salvadora persica*. These prior studies demonstrated that the stem of *S. persica* contains bioactive phytochemical compounds, including terpenoids, flavonoids, tannins, alkaloids, and saponins (Abdallah & Al-harbi, 2015).

The results of phytochemical screening of methanol extracts conducted in the study showed that flavonoids, saponins, tannins, terpenoids, alkaloids, and phenolic compounds can be extracted using methanol, because methanol is a universal solvent with polar groups (-OH) and nonpolar groups (-CH₃), so that polar and non-polar compounds can be extracted using methanol. (Popoola, 2019). No color change is formed in methanol, n-butanol, and ethyl acetate solvents because there is no oxidation reaction of steroid compounds that produce chromophore groups (Asmara, 2017), resulting in a negative steroid test.

The results of phytochemical screening of n-butanol extract showed positive results on flavonoids, saponins, tannins, phenolics, alkaloids, and terpenoids. n-butanol is a solvent that has amphipathic properties because it has hydroxyl groups as polar groups and hydrocarbon chains as non-polar groups so that it can dissolve polar and non-polar compounds.

Phytochemical screening results were carried out to identify the class of compounds contained in the ethyl acetate extract. Alkaloid, flavonoid, tannin, phenol, and terpenoid tests showed positive results. Ethyl acetate, as a semi-polar solvent, has the ability to attract compounds with a wide range of polarities, ranging from polar to nonpolar (Putri et al., 2018). In ethyl acetate, negative saponin results were obtained; this can occur because there is no reaction between the chemical compositions contained in the sample that causes froth or foam to form. The constituent structure of organic saponins consists of hydroxyl and carbon groups, which allow these compounds to dissolve in water and form foams, whereas the ethyl acetate solvent extract does not have this structure (Sintia et al., 2023).

Total Flavonoid Levels

In determining the total flavonoid content, it was found that miswak wood extract with ethyl acetate solvent had higher levels than other solvents. This can occur because several types of flavonoids, including flavones, flavonols, and flavanones, are readily soluble in semi-polar solvents (Puspa Yani et al., 2023). This indicates that the flavonoid compounds contained in miswak wood are more semi-polar in nature, so solvents with higher polarity attract fewer flavonoid compounds. In this study, methanol has the least flavonoid content. This is based on the theory that the effectiveness of compound extraction by solvents is highly dependent on the nature of the solvent. The principle is that each compound will dissolve in solvents with the same polarity properties, and the type of solvent used impacts the phytochemical compounds extracted. (J. Y. Putri et al., 2023).

The mechanism of the color formation reaction is used to determine the total flavonoid content of the sample. The presence of $AlCl_3$ reagent and sodium

acetate causes a colored solution. AlCl_3 reagent will work with hydroxyl and ketone groups on flavonoid compounds. AlCl_3 will react with ketone groups at C4 and OH groups at C3 (flavonol) or C5 on flavone or flavonol compounds to form stable yellow complex compounds. Quercetin has a keto group on the C-4 atom and hydroxyl groups on the C-3 and C-5 atoms. Therefore, quercetin is one of the compounds from the flavonoid group that is used as a standard to determine flavonoid levels (Nursamsiar et al., 2023).

The yield is the percentage of the weight of the extract obtained. In the yield, there are compounds not only flavonoid compounds but also other compounds extracted in the solvent. In this study, the highest total flavonoid content was found in extracts of miswak wood with ethyl acetate solvent, which has the lowest yield. The flavonoid content contained in the extract of miswak wood is quite high, in ethyl acetate n-butanol, and in methanol, so it is likely to have a fairly good effectiveness as an antibacterial, antiviral, and antifungal.

Conclusion

The extraction process using methanol and n-butanol yielded a greater variety of compounds compared to ethyl acetate. However, the highest flavonoid levels were observed in the ethyl acetate extract, while the methanol extract contained the lowest levels of flavonoids.

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