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Anti-cholesterol Activity of Kemuning Leaf Extract (*Murraya paniculata* (L.) Jack.) Using Different Extraction Methods and Solvents

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ABSTRACT

High cholesterol levels may accumulate in blood vessels, causing blockages. Reducing cholesterol levels using biological materials is an essential area of research currently to minimize the impact of chemical drugs. One of the biological materials with potential as an anti-cholesterol is the Kemuning plant (*Murraya paniculata* (L.) Jack). This study aims to determine the content of secondary metabolites, total phenolic content, total flavonoid content, and anti-cholesterol activity of Kemuning leaf extract, with variations in extraction methods and solvents. The extraction methods used were ultrasound-assisted extraction (UAE) and maceration. The solvents used in this study had different polarities, namely 96% ethanol, ethyl acetate, and n-hexane. The extract obtained from variations in extraction methods and solvents was tested for phytochemistry, total phenolics, total flavonoids, and in vitro anti-cholesterol activity using the Liebermann-Burchard method. The results showed that Kemuning leaf extract contained secondary metabolite compounds with anti-cholesterol activity. The best extraction method for secondary metabolites was the UAE method using 96% ethanol; however, the best EC50 value was obtained from maceration with ethyl acetate. The total phenolic content of the 96% ethanol extract of Kemuning leaf using the UAE method was 61.48 mg Gallic Acid Equivalent (GAE/g extract), the total flavonoid content was 100.77 mg Quercetin Equivalent (QE/g extract), and the anti-cholesterol activity had an EC_{50} of 13.03 µg/mL.

Keywords: Cholesterol, Extraction, Phenolic, Flavonoid, Kemuning

1. Introduction

Cholesterol is an amphipathic lipid compound that plays a vital role in the structure and function of cell membranes. Cholesterol can restrict blood flow because it sticks to the walls of blood vessels, leading to stroke and heart disease [1]. Regulating food intake and using synthetic drugs can help manage high cholesterol levels. The use of synthetic drugs to lower cholesterol levels can be harmful to the body if used for an extended period. One way to minimize these negative impacts is to use biological materials with potential anti-cholesterol properties, such as the Kemuning plant (*Murraya paniculata* (L.) Jack).

Kemuning plants are very popular ornamental plants with many benefits. Kemuning plant has analgesic properties, lowers blood cholesterol levels, reduces phlegm, slows down menstruation, lowers fever, vaginal discharge, diarrhea, indigestion, kidney inflammation, rheumatism, stimulates, and the effects of heart depressants [2]. Kemuning leaf contains various compounds, including coumarins, alkaloids, flavonoids, and phenolics [3]. Flavonoids and phenols in the body can lower cholesterol levels by breaking up fatty deposits that accumulate on the walls of coronary arteries, restoring normal blood flow [4]. Phenolics, such as tannins, can inhibit cholesterol absorption by coating the intestinal wall. This action reduces cholesterol entering the blood [5]. Phenolics, flavonoids, and steroids can affect how the body lowers cholesterol levels [6]. Coumarin compounds from *Murraya paniculata* have been investigated for their inhibitory effects on several cholesterol-related

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enzymes, revealing that these compounds can inhibit the enzymes acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) [7].

Proper extraction methods and solvents can increase the effectiveness of Kemuning leaf extract in lowering cholesterol levels. The extracted compounds can be affected by the extraction method and the solvent used. Ultrasound-assisted extraction (UAE) is an extraction method that can be used in addition to maceration. A study on differences in extraction methods from Telang flower extract (*Clitoria ternatea* L.) found that the UAE produced a higher extract yield than maceration [8]. Characterization of grapefruit peel extract (*Citrus maxima*) with different solvents (96% ethanol, ethyl acetate, and water) showed that 96% ethanol was more effective than the other solvents [9]. Several previous studies have shown that the UAE method produces higher extract yields than maceration. Extraction of butterfly pea flowers using UAE yielded higher quercetin levels compared to maceration, although the results were not statistically significantly different [10]. Extraction using the UAE method also shows that the amounts of phenolic compounds extracted from fresh olives are greater than those extracted using the maceration method [11]. However, its application to the extraction of Kemuning leaves has not been widely explored, particularly regarding its potential to reduce cholesterol levels.

The purpose of the study was to determine the content of secondary metabolite compounds, total phenolic levels, total flavonoid levels, and anti-cholesterol activity of Kemuning leaf extract using a variety of extraction methods (UAE and maceration) and solvents (96% ethanol, ethyl acetate, and n-hexane). This study supports the sustainable use of indigenous Indonesian medicinal plants in attempts to manage cardiovascular health by determining the most effective combination of extraction techniques and solvent systems. The study's specific objectives are to ascertain the amounts of secondary metabolites, total phenolic and flavonoid content, and anti-cholesterol activity of Kemuning leaf extract made using a variety of extraction techniques, including maceration with solvents of varying polarities (96% ethanol, ethyl acetate, and n-hexane) and Ultrasonic Assisted Extraction (UAE).

2. METHODS

2.1. Material

The ingredients used in this study were Kemuning leaf *(Murraya paniculata* (L.) Jack.) that was collected from Jatijajar village, Tapos district, Depok city, iron (IIII) chloride 1% ($FeCl_3$), aquadest, hydrochloric acid (HCl), ether, anhydrous acetic acid ($C_4H_6O_3$), concentrated sulfuric acid (H_2SO_4), ethanol 96%, ethyl acetate ($C_4H_8O_2$), *n*-hexane (C_6H_{14}), chloroform ($CHCl_3$), magnesium powder, quercetin solution (Himedia), aluminum chloride ($AlCl_3$10%), cholesterol powder, ciocalteu reagent, sodium carbonate (Na_2CO_3), filter paper.

The tools used in the study were analytical scales, aluminum foil, blenders, spatulas, *rotary evaporators, ultrasonic homogenizers (*TU-650E4), ovens, sieves, stirring rods, evaporative cups, a UV-Vis spectrophotometer (Shimadzu UV-1780), and other glassware.

2.2. Sample Preparation

The Kemuning leaves were cleaned, washed, and dried using an oven at 40 °C for 24 hours. The dried Kemuning leaves are crushed using a blender and sifted using an 80 mesh sieve to produce the simplicial powder [12].

2.3. Kemuning Leaf Extraction by Maceration Method

Simplicial of Kemuning leaf, as much as 100 g, was macerated with each solvent (96% ethanol, ethyl acetate, and n-hexane) in a ratio of 1:10 for 24 hours, filtered, and remacerated 2 times. The filtrate obtained from the maceration results is combined, evaporated with a rotary vacuum evaporator, and yields a viscous extract [13].

2.4. Kemuning Leaf Extraction by UAE Method

Simplicial of Kemuning leaf as much as 25g was added with solvent (96% ethanol, ethyl acetate, and n-hexane) in a ratio of 1:4. The solution of Kemuning leaf extract was placed in an ultrasonic bath. Extraction with UAE was carried out for 30 minutes at 65% amplitude and 40 °C. The filtrate was evaporated with a water bath at a temperature of 40 °C until a viscous extract of Kemuning leaf was obtained [14].

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2.5. Phytochemical Tests [15]

2.5.1. Identification of Flavonoid Compounds

1 mL of Kemuning leaf extract was placed in a test tube, Mg tape was added, and five drops of concentrated HCl were added. The formation of yellow, orange, or red colors indicates a positive result.

2.5.2. Tannin Compound Identification

1 mL of Kemuning leaf extract was placed in a test tube, 5 mL of Aquadest was added, and the mixture was heated. The solution was added to $FeCl_3$ in 3 drops. Positive results if it produces characteristic blue, blue-black, green, or blue-green and sediment.

2.5.3. Identification of Saponin Compounds

1 mL of Kemuning leaf extract was added to 5 mL of Aquadest, and the mixture was heated to 80 °C for 5 minutes. The solution was cooled and homogenized. The onset of foam up to 10-minute intervals indicates the presence of saponins.

2.5.4. Phenolic Compound Identification

1 mL of Kemuning leaf extract was placed in a test tube, 5 mL of Aquadest was added, and the mixture was heated. The solution was added to $FeCl_3$ in 3 drops. Positive results if it produces characteristic blue, blue-black, green, or blue-green and sediment.

2.5.5. Identification of Alkaloid Compounds

1 mL of Kemuning leaf extract was placed in a test tube, then 5 mL of chloroform and 5 mL of ammonia were added. The mixture solution was filtered, and the obtained filtrate was then treated with a few drops of Mayer, Wagner, and Dragendorff reagents. Positive results were characterized by the formation of red deposits in the Dragendorff reagent test, white deposits in the Mayer reagent test, and brown deposits in the Wagner reagent test.

2.5.6. Identify Steroid Triterpenoid Compounds

1 mL of Kemuning leaf extract was added to 1 mL of anhydrous acetone and 1 mL of concentrated sulfuric acid. Positive results are characterized by red coloration, indicating the presence of triterpenoids, and by blue or green coloration, indicating the presence of steroids.

2.6. Determination of Total Phenolic Content [16]

10 mg of Kemuning leaf extract was dissolved in 10 mL of ethanol p.a., then homogenized to obtain a 1000 mg/L solution. The 1000 mg/L Kemuning leaf extract solution was taken up with a pipette to 1 mL, mixed with 2.5 mL of 10% Folin-Ciocalteu, and left for 5 minutes at room temperature. The solution was added with 2.5 mL of 7.5% sodium carbonate and left for 45 minutes. The absorbance was measured at 740 nm using a UV-visible spectrophotometer. The total phenolic content was calculated using a linear regression equation from the previously measured gallic acid calibration curve. The total phenolic content of the sample extract is expressed in mg gallic acid/g Kemuning leaf extract (mg GAE/g extract) with the formula (1).

$$\text{Total Phenolic (mgGAE)/g} = c\frac{V}{m} \quad (1)$$

Information:
c = Total phenolic of the standard curve (mg/L)
V = Sample Volume (L)
m = Sample Weight (g)

2.7. Determination of Total Flavonoid Levels [17]

A total of 5 mg of Kemuning leaf extract was weighed and transferred into a 10 mL measuring flask, which was then filled with ethanol to the calibration mark. From this solution, 1 mL was pipetted into a new 10 mL measuring flask, along with 0.2 mL of 10% $AlCl_3$, 0.2 mL of 1 M potassium acetate, and 3 mL of ethanol. The mixture was then adjusted to the calibration mark with distilled water and left to stand for 30 minutes. The absorbance of the solution was subsequently measured at the maximum wavelength. The resulting absorbance values were used in the regression equation of the quercetin standard curve to calculate the total flavonoid content using the formula (2).

$$\text{Total Flavonoid (mg QE/g)} = c\frac{V}{m} \quad (2)$$

Information:
c = Total flavonoid of the standard curve (mg/L)
V = Sample Volume (L)
M = Sample Weight (g)

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2.8. Anti-cholesterol Activity of Kemuning Leaf Extract [15]

The anti-cholesterol activity test involved varying the concentration of Kemuning leaf extract (10, 15, 20, 25, and 30 µg/mL). A solution of the extract was prepared in a 10 mL flask and mixed with a positive control, a standard cholesterol solution at 175 µg/mL. This mixture was added 0.1 mL of concentrated sulfuric acid and 2 mL of anhydrous acetic acid, followed by chloroform to the mark. The solution was incubated until it turned green. The absorbance was measured by UV-Vis spectrophotometry and compared with that of a standard cholesterol solution to determine the percentage reduction in cholesterol. The reduction was calculated using a specific formula (3).

$$A(\%) = \frac{(C-B)}{C} \times 100 \quad (3)$$

Information:
A (%) = % Reduced Cholesterol
B = Cholesterol Absorbance + Sample
C = Positive Control Absorbance

EC_{50} (Effective Concentration 50) in cholesterol research is used as a benchmark to determine the concentration of an extract needed to lower cholesterol levels by 50%. The EC_{50} value is calculated using a linear regression equation that describes the relationship between the concentration of Kemuning leaf extract (X) and the average cholesterol-lowering activity (Y) from a series of measurements. The value of EC_{50} is determined by the formula (4).

$$EC_{50} = \frac{(y-a)}{b} = X \quad (4)$$

Information:
a: intercept (from curve)
b: slope (from curve)
Y= 50.

3. Results and Discussion

3.1. Secondary Metabolites from Kemuning Leaf Extract

Phytochemical screening was performed to identify the compounds present in the Kemuning leaf extract. The analyzed compounds included alkaloids, flavonoids, phenolics, saponins, and triterpenoids/steroids. The results of the phytochemical screening for Kemuning leaf extract are presented in Table 1.

Table 1. Phytochemical Screening for Kemuning Leaf Extract

Parameter	Secondary Metabolites in Variations of Solvents and Extraction Methods					
	Ethanol 96%		Ethyl Acetate		n-Hexane	
	A	B	A	B	A	B
Flavonoids	+	+	+	+	-	+
Alkaloids						
-Wagner	+	+	+	+	+	+
-Dragendroff	+	+	+	-	+	-
-Mayer	+	+	+	+	+	+
Phenolics	-	+	+	+	+	+
Saponins	+	+	-	+	+	+
Triterpenoids	+	-	+	+	-	+
Steroids	-	+	-	-	+	-

A: Maceration
B: UAE
(+): Contains secondary metabolite compounds
(-): Does not contain secondary metabolite compounds

Secondary metabolites extracted using the Ultrasound-Assisted Extraction (UAE) method exhibit greater efficacy than those obtained via traditional maceration. This advantage is mainly due to the benefits of the UAE method, which include shorter extraction times and a higher yield of bioactive compounds [18].

Table 1 shows that the UAE method, when applied with nearly all solvents, can successfully extract a diverse array of secondary metabolites, including flavonoids, alkaloids, phenolics, saponins, and triterpenoids/steroids. In contrast, the maceration method utilizing a 96% ethanol extract fails to yield phenolic compounds, a shortcoming likely attributed to the prolonged extraction time. Extended extraction durations can lead to reduced concentrations of tannins being extracted [19].

Additionally, saponin compounds are not obtained from the ethyl acetate extract, as this solvent is semi-polar and therefore ineffective in extracting

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polar saponins. Similarly, n-hexane extracts do not yield flavonoid compounds. This observation is supported by qualitative Thin Layer Chromatography (TLC) results, which did not detect flavonoids in the n-hexane extract [20]. This lack of extraction is due to the nonpolar nature of n-hexane, which is unable to dissolve polar flavonoids effectively.

3.2. Total Phenolic Content of Kemuning Leaf Extract

Total phenolic content was measured by UV-Vis spectrophotometry at the selected wavelength. The absorbance measurements yielded a linear regression equation of y = 0.0179x - 0.0026, with a linearity value (R^2) of 0.9992. In the maximum wavelength test for 250 mg/L of gallic acid, measurements were taken over 400-800 nm, yielding a maximum at 740 nm.

Table 2. Total Phenolic Content of Kemuning Leaf Extracts

Extract	Total phenolics (mgGAE/g extract)	
	Maceration	UAE
Ethanol 96% Extract	22.21	61.48
n-Hexane Extract	11.14	24.80
Ethyl-Acetate Extract	20.38	22.48

As shown in Table 2, the total phenolic content of Kemuning leaf extract obtained by the UAE method is higher than that obtained by the maceration method. Ultrasonic waves in the UAE technique enhance the mass transfer rate and shorten the extraction time [21]. The cavitation phenomenon associated with this method increases extraction efficiency, enabling the solvent to more effectively extract phenolics and thereby elevating the phenolic content in the extract. Cavitation bubbles induce fragmentation, resulting in a decrease in particle size and causing the bioactive components to dissolve in the solvent [22]. Additionally, the total phenolic content of seaweed (*Gracilari verrucosa*) using the UAE method was greater with UAE (911.35 mg GAE/g extract) compared to maceration (878.08 mg GAE/g extract) [23].

Table 2 demonstrates that the Kemuning leaf extract obtained with 96% ethanol as the solvent, using both maceration and ultrasound-assisted extraction (UAE) methods, exhibits a higher total phenolic content than extracts obtained with other solvents. 96% ethanol is a polar solvent, allowing effective dissolution of phenolic compounds. As a result, 96% ethanol proves to be more efficient in extracting total phenolics than n-hexane and ethyl acetate. In the *Tetracera indica* leaf, the extract with 96% ethanol yielded a superior total phenolic content of 237.271 mg GAE/g extract, outperforming the ethyl acetate extract at 81.958 mg GAE/g extract, and the n-hexane extract, which contained only 30.771 mg GAE/g extract [24]. Noni leaf (*Morinda citrifolia* L) that was extracted with 96% ethanol solvent demonstrated the highest total phenolic (299.73 mg GAE/g extract), followed by aquadest (125.45 mg GAE/g extract), and n-hexane (3.70 mg GAE/g extract) [25]. This evidence underscores the effectiveness of 96% ethanol in extracting phenolic compounds [23].

3.3. Total Flavonoid of Kemuning Leaf Extract

The flavonoid content of Kemuning leaf extract was quantified using a UV-Vis Spectrophotometer at 432.5 nm. This wavelength was obtained by measuring the maximum wavelength of quercetin, a flavonoid standard, when reacted with $AlCl_3$. The absorbance measurements yielded a linear regression equation: y = 0.0097x - 0.0025, with a linearity (R^2) value of 0.9993. This linearity value indicates the correlation between the substance's concentration and the measured absorbance; a value closer to 1 indicates a stronger relationship and better linearity. The results for the standard quercetin content in the Kemuning leaf extract are presented in Table 3.

Table 3. Total Flavonoid Levels of Kemuning Leaf Extract

Extract	Total Flavonoids (mg QE/g extract)	
	Maceration	UAE
Ethanol 96% Extract	8.92	100.77
Ethyl Acetate Extract	44.86	37.25
n-Hexane Extract	21.83	22.47

Table 3 indicates that the 96% ethanol extract of Kemuning leaf and the ethyl acetate extract obtained

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through the UAE method exhibited higher total flavonoid levels than those extracted by maceration. This enhancement is attributed to the ultrasound-assisted extraction (UAE) method, which uses ultrasonic waves to facilitate solvent penetration into plant cells. This increases the mass transfer rate and extraction efficiency, resulting in a greater yield of flavonoids than the traditional maceration technique.

Conversely, the n-hexane extract of Kemuning leaf obtained using the UAE method showed lower total flavonoid levels than maceration. This is likely due to the polar nature of flavonoids, which makes them more soluble in polar and semi-polar solvents such as ethanol and ethyl acetate. N-hexane solvent is less effective in dissolving polar flavonoids, reducing extraction levels [26]. In contrast, ethanol and ethyl acetate, being polar and semi-polar, respectively, are better suited to penetrate plant cell membranes and bind with flavonoids.

Interestingly, the total flavonoid content in the 96% ethanol extract of Kemuning leaf produced by the UAE method was higher than the total phenolic content of the same extract, despite the expectation that the phenolic content would surpass that of flavonoids, given that flavonoids are a subset of phenolic compounds. This discrepancy may be influenced by factors such as the duration of the extraction process and the time the extract is stored post-extraction, which can affect the concentration of active compounds in the Kemuning leaf extract.

3.4. Anti-cholesterol Activity of Kemuning Leaf Extract

In the Liebermann-Burchard method for assessing anti-cholesterol activity, changes in cholesterol levels were quantified using a UV-Vis Spectrophotometer. The inclusion of anhydrous acetic acid is crucial, as it ensures that the Kemuning leaf extract is free of water and facilitates the formation of acetyl steroid derivatives. Additionally, using concentrated sulfuric acid results in a green coloration of steroids, including cholesterol [27]. The absorbance value of the resulting color is measured with a UV-Vis spectrophotometer, with the absorbance directly proportional to the concentration of the substance in the solution [28].

Table 4. Anti-cholesterol Activity of Kemuning Leaf Extract

Extract	EC_{50} Extraction Method (µg/mL)	
	Maceration	UAE
Ethanol 96% Extract	8.214	13.03
Ethyl Acetate Extract	2.291	43.396
n-Hexane Extract	6.199	42.715

The EC_{50} value indicates the concentration of extract required to lower cholesterol levels by 50% of the initial total cholesterol level. A lower EC_{50} value indicates more vigorous anti-cholesterol activity. Kemuning leaf extract obtained by the maceration method showed a better EC_{50} result than that obtained by the ultrasonic-assisted extraction (UAE) method (Table 4). The maceration method does not employ high temperatures or controlled stirring, thereby minimizing damage or degradation of active compounds during extraction, so that it helps maintain the quality and anti-cholesterol activity of the extract [29].

The ability of the extract as an anti-cholesterol is related to the content of compounds in the extract. Based on phytochemical tests, the ethyl acetate extract of Kemuning leaf obtained by maceration showed the presence of alkaloids, flavonoids, and phenolics, secondary metabolites that can lower blood cholesterol levels. Flavonoids can help prevent fat accumulation in the walls of blood vessels [30]. The carbonyl group in flavonoids interacts with the hydroxyl group in cholesterol to form a hemiacetal. In contrast, the hydroxyl group in cholesterol forms a hydrogen bond with the ketone group in flavonoids [31]. The unbound compound in the sample is referred to as free cholesterol, which reacts with anhydrous acetic acid and concentrated sulfuric acid [32]. Phenolics inhibit fat absorption in the intestine, interacting with mucosal proteins and intestinal epithelial cells [33]. Consequently, phenolics can reduce cholesterol accumulation in the bloodstream and promote excretion through feces.

In this study, the total phenolic and flavonoid contents were not proportional to the extract's anti-cholesterol properties. This may be due to specific types of compounds acting as anti-cholesterol agents,

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as well as to the influence of other compound classes, such as alkaloids. Further research is needed to identify the specific compounds in the extract that act as anti-cholesterol agents. Although there was no correlation between the total phenolic and flavonoid contents and the EC_{50} value, the anti-cholesterol properties of Kemuning leaf extract are considered good. These leaves can be further developed as an anti-cholesterol herbal raw material.

4. CONCLUSION

Based on the study's findings, the extract of Kemuning leaf, obtained using various solvents and extraction methods, contains secondary metabolites such as flavonoids and phenolics, which exhibit potential anti-cholesterol properties. The extraction method and solvent selection that most effectively extracted these secondary metabolite compounds with cholesterol-lowering potential were 96% ethanol and the Ultrasound-Assisted Extraction (UAE) method. The total phenolic content, total flavonoid content, and EC_{50} values for the 96% ethanol extract of Kemuning leaf obtained by the UAE method were 61.48 mg GAE/g extract, 100.77 mg QE/g extract, and 13.03 µg/mL, respectively. Further, in vivo research is needed to develop the benefits of the Kemuning leaf extract.

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