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Article

Flavonoid Compounds from Ethanol Extract of Sungkai Leaves (*Peronema canescens*. Jack) and Antibacterial Activity Test Against *Staphylococcus epidermidis* and *Escherchia coli*

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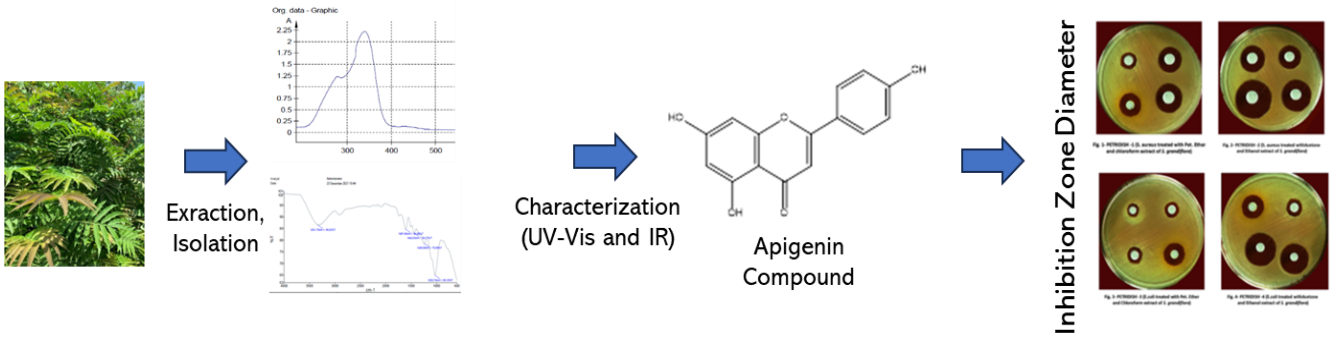
Abstract

The rise of antibiotic-resistant bacteria has intensified the global search for alternative antimicrobial agents, particularly from natural sources. Traditional medicinal plants have been widely recognized for their therapeutic potential, and *Peronema canescens* Jack is well-known to contain bioactive compounds. Among its pharmacological properties, its antibacterial potential has drawn scientific interest. Secondary metabolites such as flavonoids, tannins, and phenolics are believed to contribute to its antimicrobial activity. However, the specific antibacterial compounds in P. canescens remain largely unidentified. This study seeks to isolate and characterize antibacterial compounds from the ethanol extract of Sungkai leaves, aiming to discover new natural antibacterial agents. The research objectives include isolating antibacterial compounds using maceration extraction and fractionation, conducting phytochemical screening to identify metabolite classes, assessing antibacterial activity using the paper disc diffusion method, and characterizing bioactive isolates through UV-Vis spectrophotometry and FTIR analysis. The results indicate that the ethyl acetate fraction of the ethanol extract of *P. canescens* leaves exhibits significant antibacterial activity, particularly at concentrations of 500 and 1000 ppm. UV-Vis spectrophotometric analysis identified flavonoid compounds, including apigenin, flavones, and flavonols, based on characteristic absorption peaks. FTIR analysis confirmed the presence of functional groups associated with flavonoids, supporting their antibacterial potential. These findings highlight *P. canescens* as a promising source of natural antibacterial agents. Further studies focusing on compound purification and in vivo antibacterial testing are recommended to explore its pharmaceutical applications.

Keywords: *Antibacterial, Flavonoids, Sungkai leaves*

Graphical Abstract



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Introduction

The territory of Indonesia is covered by abundant natural wealth. This wealth has the potential to find various types of chemical compounds that are useful for treatment. This can be seen from the many types of plants that are used as traditional medicine. Currently, traditional medicine is growing rapidly, as evidenced by the many studies on natural medicines, including the Sungkai plant (*Peronema canesscens*). *Peronema canesscens* is a wild plant that belongs to the Verbenaceae family. This plant is often referred to as sungkai or jati sabrang, sabrang, ceki, and sekai. Sungkai is widely distributed in western and southern Sumatra, Jambi, West Java, Kalimantan and even the Malaysian peninsula (Barid, 2015; Murningsih et al., 2005). The stems, leaves, flowers or seeds of the sungkai plant can be used as medicines (Elsi et al., 2020). People usually use it by pounding or brewing it for colds, fever, worm medicine, mouthwash, and bruise medicine (Primair, 2013; Hidayat, 2008). In addition, it can also be used as a bath for women after giving birth (Ningsih and Ibrahim, 2013). In several studies, sungkai leaf extract has been shown to inhibit parasite growth. According to Suwandi (1995), ethanol extract of sungkai leaves can inhibit the growth of the Plasmodium berghei parasite in male mice. Sungkai extract has also been shown to be effective in inhibiting the Babesia gibsoni parasite (Subeki, 2004; Murningsih, 2005). In addition, recent studies have shown that ethanol extract of sungkai leaves has anti-hyperuricemia activity which can reduce uric acid levels in mice (Latief et al., 2021). It has been reported by Ibrahim and Kuncoro (2012), that the methanol extract of P. canescens contains secondary metabolites of the alkaloid, terpenoid-steroid, phenolic, flavonoid, and tannin groups. Therefore, many researchers have tried to prove its ability against antimicrobial activity. The results show that sungkai leaves can inhibit Escherchia coli, *Salmonella thyposa* (Ningsih et al., 2013), *Bacillus subtillis*, *Streptococcus mutans*, and *Staphylococcus aureus* (Ningsih and Ibrahim, 2013). Infectious diseases by pathogenic microbes such as bacteria are triggering factors for various diseases that cause high mortality rates, especially in developing countries such as Indonesia. Some bacteria that can cause infections such as Staphylococcus epidermidis and Escherchia coli. Staphylococcus epidermidis is a colony of gram-positive bacteria that infects the mucous membranes and skin of humans. Meanwhile, Escherchia coli is included in the group of gram-negative bacterial colonies that cause acute diarrhea to the death of most babies in the world (Alamsyah et al., 2014).

Several synthetic antibiotics can treat infectious diseases by killing and inhibiting bacterial growth. However, the problem faced by the world of medicine is bacterial resistance to existing drugs. In addition, continuous use of antibiotics can cause side effects on the body which are characterized by the appearance of allergic reactions (Westh et al., 2004).

Based on these problems, new research is needed on plants that are able to produce natural antibiotics that have optimal properties to inhibit antibacterial activity. This can be done by utilizing several plants that contain active antibacterial compounds

Material and Methods

Materials and Instrumentations

The sample used in this study was sungkai leaves (*Peronema canescens* jack). Samples were obtained from Kademangan Village, Jaluko District, Muaro Jambi Regency, Jambi Province, Indonesia. The chemicals were used ethyl acetate, n-hexane, 2N sulfuric acid, Dragendorff reagent, Meyer reagent, Lieberman-Burchard reagent, concentrated HCl, Mg powder, 2N HCl, FeCl3, acetic acid. Anhydrous (Sigma Aldrich), fine silica gel (packing) 0.040 - 0.063 mm and coarse silica gel (imprent) 0.063 -0.200 mm (Merck), Artemia salina larvae eggs, NaCl.

The equipments and instrumentations used in this study were maceration bottles, filter paper, rotary evaporator components, funnels, measuring cups, Erlenmeyer cups, VLC and GCC columns, vacuum pumps, capillary tubes, TLC plates (Merck), drip plates, test tubes, test tube rack, dropping pipette, 1 ml micropipette, 10 ml micropipette, KBR pellet, volumetric flask, vial, stir bar, UV-Vis spectrophotometer (Shimadzu, Japan), FTIR spectrophotometer (Bruker, USA)

Methods

Sample Preparation and Extraction. Prior to the preparation process, the Sungkai plant (*Peronema canescens* Jack) was first identified and confirmed. Subsequently, a 1 kg sample of Sungkai leaves (*Peronema canescens* Jack) was collected, thoroughly cleaned, and washed to eliminate any adhering dust and impurities. The leaves were then cut into small pieces and air-dried for seven days. The prepared samples underwent extraction using the maceration method with ethanol as the solvent for three cycles of 24 hours each, repeated twice. The resulting macerate was concentrated using a rotary evaporator until a thick extract was obtained. This thick extract was further evaporated to remove residual solvent, yielding a dry extract, which was then weighed to determine its yield. The extract was subsequently subjected to partitioning to separate it into three fractions based on the polarity of the secondary metabolite compounds. The partitioning process was carried out sequentially, starting with non-polar compounds, followed by semi-polar and polar compounds, using *n*-hexane, ethyl acetate, and ethanol as solvents, respectively

Secondary Metabolites Analysis. The cytotoxicity test treatment was carried out on four repetitions. Artemia salina larvae were prepared by incubating the eggs 48 hours before testing and prepared mother liquor and serial concentrations of 1000 ppm, 100 ppm, and 10 ppm solvent used according to the fraction. Sea water was used as a negative control. Added 5 ml of engineered seawater and 1 ml of each test solution which had been evaporated for 24 hours into a test tube and then homogenized, then ten larvae were added. Observations were made (24 hours) on the death of shrimp larvae with the help of a magnifying glass

Isolation. **Thin Layer Chromatography (TLC):** A TLC plate measuring 1 × 5 cm was prepared with a lower boundary of 1 cm and an upper boundary of 0.5 cm, allowing an eluent migration distance of 3.5 cm. The extract was applied to the lower boundary of the plate using a capillary tube and subsequently eluted with the designated mobile phase. Once the solvent front reached the upper boundary, the elution process was halted. The resulting spots were then observed directly under a UV lamp.

Column Chromatography: Vacuum liquid column chromatography (VLC) was performed using silica gel as the stationary phase, with a sample-to-silica gel ratio of 2:1. The sample extract was impregnated with silica gel and introduced into a column preloaded with the stationary phase. Elution was conducted using a gradient system, progressing from non-polar to semi-polar and finally to polar solvents. The resulting fractions were collected in vials based on either the separation of visible bands or the volume of eluent used, followed by evaporation. The eluates obtained from column chromatography were further analyzed using TLC.

The purity of the collected eluates was assessed via TLC using different solvent systems, including ethyl acetate: ethanol (4:6), acetone: ethanol (6:4), and dichloromethane (DCM): acetone (2:8). If TLC results yielded a single distinct spot, the isolate was considered pure. The purified isolate was subsequently subjected to further analysis, including melting point determination, phytochemical screening, antibacterial activity evaluation, and structural characterization.

Antibacterial Activity. All equipment and materials used in the study were thoroughly cleaned and dried. Sterilization was performed using 70% ethanol, followed by autoclaving at 121°C for 15 minutes. Paper discs made from Whatman paper were impregnated with Sungkai leaf extract at varying concentrations of 100, 500, and 1000 µg/mL (ppm). For each fraction obtained from column chromatography, test solutions were prepared at concentrations of 100, 125, and 150 µg/mL (ppm), while isolates were tested at concentrations of 6, 8, and 10 µg/mL (ppm) in ethanol solvent. The antibacterial test was conducted in duplicate (duplo) following the methodology described by Yanti and Mitika (2017).

A positive control using Chloramphenicol and a blank solvent control were included (Yanti and Mitika, 2017). The bacterial strains used in the study were *Staphylococcus epidermidis* and

Escherichia coli, which were inoculated from pure cultures onto petri dishes containing Nutrient Agar (NA) medium. The bacterial cultures were transferred using an inoculating loop and streaked onto the prepared media, followed by incubation for 24 hours.

Antibacterial Activity was determined by paper discs-diffusion were immersed in the test solution, positive control, and blank control, then placed onto the agar media. The plates were incubated at 37°C for 24 hours, after which the diameter of the inhibition zone, representing the area free from bacterial growth, was measured using a caliper or ruler. The inhibition zone diameters were compared with those of the positive and blank controls. The antibacterial activity test was conducted in duplicate, and the inhibition zone was reported as the average of two independent measurements (Waluyo and Pasaribu, 2015).

Isolate Characterization. UV-Vis Spectrophotometry: A pure isolate (0.5 mg) was dissolved in 3 mL of ethanol. Prior to analysis, a baseline correction was performed using ethanol as a blank solution. The sample solution was then transferred into a cuvette and placed in the spectrophotometer. The absorbance spectrum was recorded and analyzed within the wavelength range of 200–800 nm to determine the maximum absorption wavelength.

FTIR Spectrophotometry: A pure isolate (0.5 mg) was mixed with 50 mg of potassium bromide (KBr) and finely ground until a homogeneous mixture was obtained. Baseline calibration was conducted using air as a blank reference. The prepared sample was then placed into a KBr cell and inserted into the FTIR spectrophotometer. Spectral analysis was carried out across the wavelength range of 2.5–25 microns (corresponding to a wavenumber range of 4000–400 cm^{-1}) to identify functional groups present in the compound.

Characterization and Data analysis. Isolate was characterized using a UV-Vis spectrophotometer and FTIR spectrophotometer. Cytotoxicity data analysis was carried out by knowing the mortality of Artemia salina larvae, then looking for the probit number using the probit analysis program SPSS version 25 (SPSS Inc., Chicago, IL, 250 USA).

Results and Discussions

The resulting extraction is a thick colored liquid called macerate. The macerate is then filtered to separate the extract from the residue which is the remains of the maceration. Furthermore, the ethanol extract of sungkai leaves is evaporated using a rotary evaporator. This process aims to separate the extract from the solvent so that a thick extract is obtained. After that, the thick extract is partitioned liquid-liquid with three solvents that have different polarity properties. These solvents are n-hexane, ethyl acetate, and ethanol which are sequentially non-polar, semipolar, and polar. This process aims to group the types of compounds based on their polarity properties, so that three groups of compounds are obtained called fractions. Then each fraction is determined for its yield mass and secondary metabolite compound content. % Yield was calculated using equation 1.

$$\% \text{ Yield} = \frac{\text{Extract (gr)}}{\text{Simpilicia (gr)}} \times 100\% \quad (1)$$

The mass percentage value of the ethanol extract yield and each fraction obtained is shown in Table 1.

Table 1. Mass and yield of dried crude extracts

Fraction extracts	Mass (g)	Yield (%)
Ethanol Extract	35.95	7.65
Fraction of n-Hexane	2.34	0.49
Fraction of Ethyl Acetate	6.45	1.37
Fraction of ethanol	27.15	

Based on Table 6, the percentage of ethanol extract yield mass is 7.649% from 470 gr of simplex. According to this value, the yield mass value in each fraction can also be determined. In the n-hexane fraction, the value is 0.49802%, the ethyl acetate fraction is 1.372%, and the ethanol fraction is 5.77% from 470 gr of simplex mass. Based on this comparison, the ethanol fraction has the highest yield value. This shows that in sungkai leaves there are more polar secondary metabolite compounds.

Secondary Metabolites

Phytochemical screening is a preliminary test to determine the content of secondary metabolite compounds in a sample qualitatively. The test includes determining the content of compounds such as alkaloids, flavonoids, saponins, tannins, and steroids/terpenoids. In this case, the ethanol extract and the three fractions of sungkai leaves will be determined. The ethanol extract of sungkai leaves showed positive results for tannins, steroids, and flavonoids (Table 2).

Comparison of the three test fractions showed that the ethyl acetate fraction had a more dominant positive result than the other fractions. This indicates that the secondary metabolite compounds contained are easily soluble in ethyl acetate solvent because they have the same polarity properties. According to Hasma and Winda (2019), heating the fraction using a water bath can also affect the results of phytochemical screening. This is because uncontrolled heating damages several secondary metabolite compounds, thus showing negative results.

Table 2. Phytochemical screening results of ethanol extract and sungkai leaf fractions

Secondary Metabolites	Crude Extract	Fraction		
		Hexane	EtAce	Ethanol
Alkaloids	-	-	-	-
Saponins	-	-	-	-
Tanin	+	-	+	-
Steroids	+	+	+	-
Terpen	-	-	-	-
Flavonoids	+	+	+	-

Antibacterial activity was tested using the paper disc diffusion method, where the paper discs were induced with sample solutions. The samples were fractions that had been made into solutions with various concentrations. As a comparison, chloramphenicol was used as a positive control. The induced paper discs were attached to the surface of nutrient agar containing bacteria. The test was carried out with two repetitions (duplo). A minimum of 1x24 hours was needed to see antibacterial activity. Samples that had antibacterial activity were indicated by the formation of a clear zone on the disc.

Antibacterial activity was seen in the ethyl acetate fraction. At concentrations of 500 and 1000 ppm, a clear zone was formed indicating the presence of active compounds. Based on the measurement values obtained, the activity of the compound was categorized as a compound that had weak to moderate activity. The positive control was resistant to E. coli bacteria, meaning that no clear zone was formed on the disc. This can happen because the composition of the E. coli bacteria is more complex than that of S. epidermidis bacteria (Alamsyah et al., 2014).

At this stage, isolation is carried out using the Vacuum Liquid Chromatography (VLC) method, in which the active fraction of ethyl acetate will be eluted with variations in the eluent ratio. The variation starts from a ratio of 100% non-polar eluent, then increased to semi-polar, to polar. The elution process is carried out in a gradient manner which is stored in a 100 ml vial bottle. This is done to separate the compound groups specifically based on their polarity properties. Then a container is obtained in the form of eluates of 52 vials. The next stage is the grouping of the isolated fractions using the Thin Layer Chromatography (TLC) method. This method is based on the position of the stain pattern produced on a thin plate from the elution process. Stain patterns that have the same position will be grouped into 1 fraction. In this case, the fractions obtained were 6 fractions (Tabel 5).

Table 1. Grouping of fractions based on vial order

Fractions	Vials
I	1
II	2-10
III	11-33
IV	34-42
V	43-47
VI	48-52

Table 2. Potential antibacterial activity of ethyl acetate fraction

Samples	Inhibition Zone Diameter (mm)			
	S. epidermidis		*E. coli*	
	Averages	**Activity**	**Averages**	**Activity**
F1	1.25 ± 0.05	Weak	0.95 ± 0.05	Weak
F2	1.0 ± 0.00	Weak	1.5± 0.06	Weak
F3	1.7 ± 0.10	Weak	1.65± 0.06	Weak
F4	1.8± 0.00	Weak	0.8± 0.00	Weak
F5	0.1± 0.05	Weak	0.8± 0.00	Weak
F6	1.75± 0.04	Weak	0.8± 0.00	Weak
+	2.8± 0.04	Weak	2.65± 0.06	Weak
-	0± 0.0	-	0± 0.00	-

The next stage is testing the antibacterial activity to see the active potential in each fraction. This activity test is done by the paper disc method, where each fraction will be tested with 150 µg/mL (ppm). Based on table 6, it can be concluded that each fraction has antibacterial activity with weak intensity. Among the existing fractions, the third fraction (F3) has the highest average value. In addition, the third fraction (F3) also produces more isolate crystals than other fractions, so that the F3 isolate will be continued to the characterization stage.

Phytochemical screening of Isolate

Phytochemical Screening: Phytochemical screening was conducted on isolate F3, which exhibited relatively strong antibacterial activity compared to other fractions. The results confirmed that isolate F3 belongs to the flavonoid class of secondary metabolites, as indicated by a characteristic color change to reddish or orange when reacted with hydrochloric acid (HCl) and magnesium (Mg) powder.

The purity of the isolate was assessed using TLC, where a pure isolate is identified by the formation of a single spot pattern on the TLC plate during the elution process. The elution was performed using solvent systems with the following ratios: ethyl acetate: ethanol (4:6), acetone: ethanol (6:4), and dichloromethane (DCM): acetone (2:8). The corresponding retention factor (Rf) values obtained for each solvent system were 0.77, 0.71, and 0.84, respectively.

Antibacterial activity test of isolate

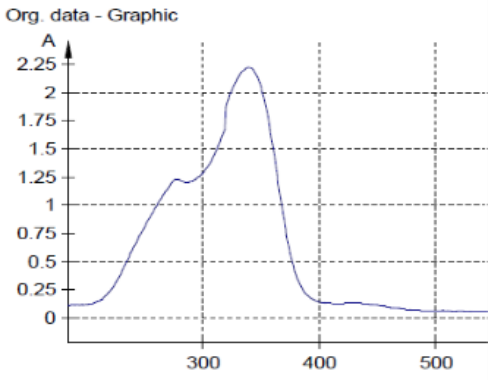
Through the same antibacterial activity testing method on extracts and fractions, isolate F3 was tested with concentration variations of 6; 8; 10 µg/ml (ppm). Table 7 shows the results of the antibacterial activity test of isolate F3.

Table 3. Potential antibacterial activity of isolate F3

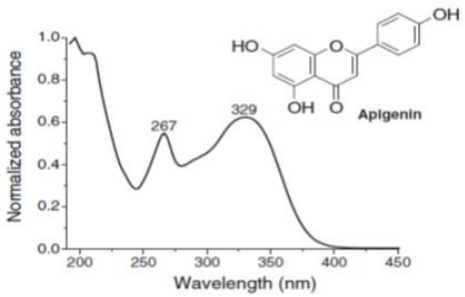
Concentrations (ppm)	Inhibition Zone Diameter (mm)			
	E. coli		*S. epidermidis*	
	Average	**Activity**	**Average**	**Activity**
6	0.95 ± 0.05	Weak	0.85± 0.05	Weak
8	2.25± 0.05	Weak	1.45± 0.02	Weak
10	3.05± 0.05	Weak	3.8± 0.05	Weak
+	4.1± 0.2	Weak	1.0± 0.05	Weak
-	0±0.00	-	0.0± 0.00	-

Isolate Belongs to Apigenin

This characterization is carried out to predict double bonds or aromatic conjugation in a molecule, in this case the test sample obtained is isolate F3. The results obtained are shown in Figure 4 and 5.



(A)

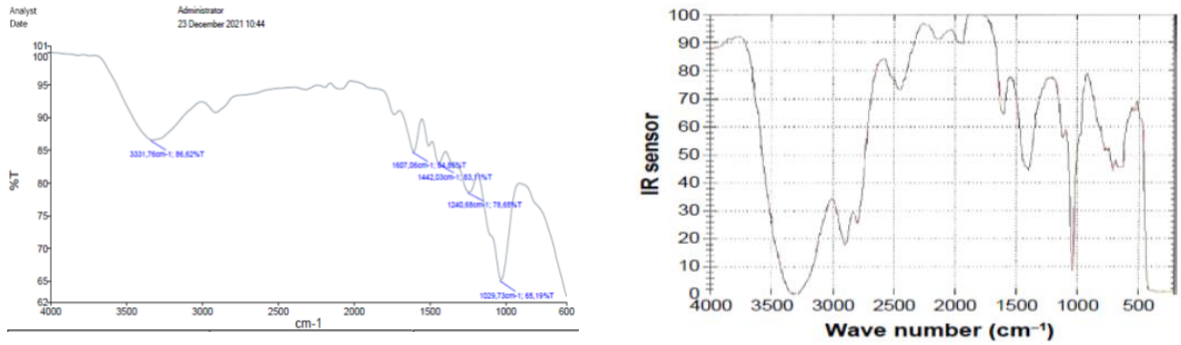


(B)

Figure 4. UV-Vis spectrum of Isolate (A) and Apigenin (B)

Based on Figure 4, two maximum absorption peaks were obtained, namely in band I at a wavelength of 280 nm and band II with a wavelength of 339 nm. The maximum absorption band with a wavelength of 339 nm was identified as the resonance of the cinnamoyl group of ring B. While the maximum absorption band with a wavelength of 280 nm was identified as the resonance of the benzoyl group of ring A (Indarto, 2015). The characteristic of flavonoid compounds has a spectrum consisting of two maximum absorptions in the wavelength range of 230-295 and 300-560 nm (Neldawati, 2013). Based on the characterization that has been carried out, it shows that isolate F3 ethyl acetate has the characteristics of flavonoid compounds of the flavone and flavonol types. Based on a comparison of several literatures, the F3 ethyl acetate isolate has a similar UV-Vis spectrum to the Apigenin compound with a maximum absorption of 329 nm in band I and 267 nm in band II (Moilanen et al., 2013).

On the other hand, the IR spectrum of isolate F3 ethyl acetate in Figure 6 obtained has a similar wave absorption pattern to the comparative wave absorption possessed by the apigenin compound in Figure 21. The wave number 3331.76 cm Based on Figure 4, two maximum absorption peaks were obtained, namely in band I at a wavelength of 280 nm and band II with a wavelength of 339 nm. The maximum absorption band with a wavelength of 339 nm was identified as the resonance of the cinnamoyl group of ring B. While the maximum absorption band with a wavelength of 280 nm was identified as the resonance of the benzoyl group of ring A (Indarto, 2015). The characteristic of flavonoid compounds has a spectrum consisting of two maximum absorptions in the wavelength range of 230-295 and 300-560 nm (Neldawati, 2013). Based on the characterization that has been carried out, it shows that isolate F3 ethyl acetate has the characteristics of flavonoid compounds of the flavone and flavonol types. Based on a comparison of several literatures, the F3 ethyl acetate isolate has a similar UV-Vis spectrum to the Apigenin compound with a maximum absorption of 329 nm in band I and 267 nm in band II (Moilanen et al., 2013). with sharp intensity is indicated as a stretching vibration of the OH group. At the wave number 1607.06 cm-1 indicates the presence of a C=O ketone group. At the wave number 1442.03 cm-1 indicates a C=C ring. At the wave number 1240-1029.73 cm-1 with sharp intensity indicates the presence of cyclic C-O.



(a) **(b)**

Figure 6. IR spectrum of isolate F3 (a), IR spectrum of Apigenin compound (Shoubaky et al., 2016)

Tabel 8. Wavenumber of isolate functional groups

Wavenumbers (cm^{-1})		Functional Groups
Isolate	Apigenin (Shoubaky *et al.*, 2016)	
3331,76	3333	O-H stretch
1607,06	1646	C=O
1442,03	1466	C=C
1029,73-1240,68	1024	C-O stretch

In addition to comparative data of FTIR absorption waveform, the suspicion that Apigenin compound contained in isolate F3 is strengthened by the results of phytochemical screening. The test was conducted by reacting the isolate using HCl and Mg powder to produce foam and a reddish or orange color change which is a positive result of flavonoids. Thus, isolate F3 ethyl acetate is suspected to be an apigenin compound (4', 5, 7-trhydroxyflavone) with the molecular formula C15H10O5 (Figure 8).

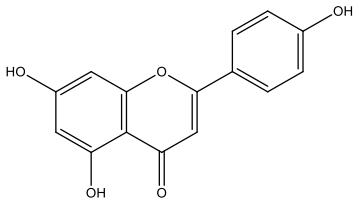


Figure 8. Chemical structure of Apigenin (4', 5, 7-*trhydroxyflavone*)

Apigenin is a secondary metabolite compound of the flavonoid group contained in fruits, vegetables, and other medicinal plants. Apigenin or known as 4', 5, 7-trhydroxyflavone is a yellow crystalline powder that is insoluble in water but soluble in organic solvents. Several studies have shown that apigenin has many molecular targets that can inhibit inflammation. Based on in vivo, in vitro, and clinical experimental studies, apigenin can be an efficacious therapeutic agent for treating diseases such as rheumatoid arthritis, autoimmune disorders, Parkinson's, Alzheimer's, and various types of cancer (Ali et al., 2017).

Conclusion

The ethanol extract of Sungkai leaves exhibits limited antibacterial activity. At concentrations of 500 and 1000 ppm, it forms a clear zone indicative of weak to moderate antibacterial potency. Consequently, the ethyl acetate fraction was further processed through isolation, yielding isolate F3, which demonstrated weak antibacterial activity even at the highest concentration of 10 ppm. The ethyl acetate fraction of isolate F3 possesses characteristics consistent with flavonoid compounds, specifically of the flavone and flavonol classes, with Apigenin as the identified compound

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None

Author Contributions

Conceptualization, NCAS and JA.; Methodology, JA and N; Software, NCAS and ML.; Validation: NCAS and ML; Formal Analysis, JA and ML.; Investigation, ML and N.; Resources, NCAS and ML.; Data Curation, S and MF; Writing – Original Draft Preparation, ZA, MF; Writing – Review & Editing, NCAS and JA; Visualization: ML and N.; Supervision, NCAS and ML; Project Administration, NCAS.

Conflic of Interest

The authors declare no conflict of interest

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