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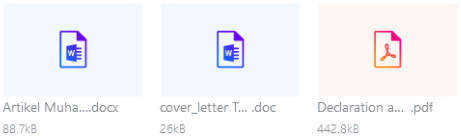


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Title: Antioxidant, Antibacterial, Total phenolic and Flavonoid content of Sungkai leaves (*Paronema canescens*)

Journal: Tropical Journal of Natural Product Research www.tjnpr.org

Corresponding Author: Muharni

Co-authors: Ferlinahayati, Heni Yohandini

Manuscript No: TJNPR JAN746ARN

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Title: Antioxidant, Antibacterial, Total phenolic and Flavonoid content of Sungkai leaves (*Paronema canescens*)

Authors: Muharni*, Ferlinahayati, Heni Yohandini

Decision: Accepted with major corrections

Congratulations

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Abiodun

**Muharni Maryadi** <muharnimyd@yahoo.co.id> Kam, 18 Feb 2021 jam 13.13
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Following my previous email (sent on Feb 8, 2021). I have confirmed my payment regarding manuscript TJNPR JAN746ARN entitled:
Antioxidant, Antibacterial, Total phenolic and Flavonoid content of Sungkai leaves (*Paronema canescens*)
Authors: Muharni*, Ferlinahayati, Heni Yohandini

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Review Comments 3

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Jum, 19 Feb 2021 jam 23.31

Review comments (Antioxidant, Antibacterial, Total phenolic and Flavonoid content of Sungkai leaves (*Paronema canescens*))

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Thank you for the revised MS

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Abiodun

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Professor Abiodun Falodun, PhD

Editor-in-Chief:
Tropical Journal of Natural Product Research (TJNPR)
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Professor of Pharmaceutical Chemistry
Fellow, Fulbright (USA)
Deputy Vice-Chancellor (Academic) 2014-2016
Faculty of Pharmacy
University of Benin
Phone: +234-807-318-4488;
email: faloabi@uniben.edu; abiodun.falodun@fulbrightmail.org
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Include names of all authors, their affiliations, the corresponding email and telephone number in the revised manuscript.

On Tue, 23 Feb 2021 at 07:23, Editor-in-Chief Tjnpr <editor.tjnpr@gmail.com> wrote:

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Professor Abiodun Falodun, PhD

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Tropical Journal of Natural Product Research (TJNPR)
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Email:editor.tjnpr@uniben.edu; editor.tjnpr@gmail.com
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Professor of Pharmaceutical Chemistry
Fellow, Fulbright (USA)
Deputy Vice-Chancellor (Academic) 2014-2016
Faculty of Pharmacy
University of Benin
Phone: +234-807-318-4488;
email: faloabi@uniben.edu; abiodun.falodun@fulbrightmail.org

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## A. MANUSCRIPT

| | |
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| Journal | **Tropical Journal of Natural Product Research** |
| Manuscript Number | TJNPR JAN746AR |
| Type of paper | Original paper |
| Title of paper | **Antioxidant, Antibacterial, Total phenolic and Flavonoid content of Sungkai leaves (*Paronema canescens*)** |
| Name of Authors | |

## B. REVIEWER'S SPECIFIC COMMENTS PER SECTION OF MANUSCRIPT

| | |
|---|---|
| Abstract | Acceptable in current form |
| Introduction | Acceptable |
| Methodology | Acceptable |
| Results | Luck of discussion on almost all results (less citation). Found 4 citation only in their results and discussion<br>Page 6, 12, 13 No standard for antioxidant activity. It is not acceptable without compare with standard such as Quercetin or ascorbic acid. Hence the author can confirm their results as active or not antioxidant. |
| Discussion | Luck of discussion on almost all results (less citation). Found 4 citation only in their results and discussion<br>Page 5, This higher yield is…….<br>It could be correlated to the chemical constituent presence in your sample. Ethyl acetate is semi polar solvent. Hence, the higher yield could be caused by the sample rich with semi polar compounds than nonpolar hexane fraction) and polar compounds (methanol fraction. Please add citation/ref for your discussion.<br>Page 6…<br>Luck of discussion. Please, add some discussion for your results…hight antioxidant could be contribute …… (search similar studies which have reported antioxidant and phenolic content…compare with their report even come from different plant species…or find the same genus or family???) |
| Conclusion | Too short…Write down anything that might be done in the future or further research that could be continued correlated to the results |
| References | Format ok, add more references correlated to results and discussion parts |
| Figures, Tables | Table 2…….page 13<br>Add a note below the table (antioxidant categories…not active if IC50>…..weak if….>….) |

## C. REVIEWER'S GENERAL COMMENTS AND REMARKS

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# Antioxidant, Antibacterial, Total phenolic and Flavonoid content of Sungkai leaves (*Paronema canescens*)


## ABSTRACT

The leaves of the sungkai (*Paronema canescens*) have been used as traditional medicine ~~for the treatment of~~ to treat various diseases. We determined the antioxidant, antibacterial, total phenolic content, and flavonoid

content of solvent extraction fractions of these leaves from *P. canescens*. Fresh leaves were extracted by maceration using solvents of increasing polarity (*n*-hexane, ethyl acetate, and methanol). Each fraction was tested for antioxidant activity using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method. The antibacterial activity was tested using the disc diffusion method using *Escherichia coli*, *Staphylococcus.aureus* and *Salmonella typhi*. Analysis of the total phenolic content was carried out using the Folin–Ciocâlteu reagent and total flavonoid content using $AlCl_3$. The study showed that the ethyl acetate fraction had higher antioxidant activity than the other fractions with an $IC_{50}$ 320 µg/mL. Antibacterial activity test had a minimum inhibitory concentration (MIC) of 62.5 µg/mL against ~~-~~*E. coli* and *S. aureus* at all fractions, and only the *n*-hexane fractions show provided an MIC of 62.5 µg/mL against *S.typhi*. Betulinic acid from the n-hexane fraction also showed antibacterial activity with MIC of 62.5 µg/mL. The analysis of total phenolic and flavonoid, the ethyl acetate fraction contained higher levels of these organics than other fractions, with values of 68.71 ± 0.17 mg Gallic Acid Equivalent (GAE)/g and 2.29 ± 0.05 mg Quercetin Equivalent (QE)/g. ~~It can be concluded that sungkai leaves show weak antioxidant activity, moderate antibacterial activity and that they have much lower levels of flavonoids than phenolics.~~ It can be concluded that sungkai leaves show weak antioxidant activity, moderate antibacterial activity, and much lower flavonoids than phenolics.

***Keywords***: Antioxidant, Antibacterial, Phenolic, Flavonoid, *Paronema canescens*

## Introduction

Indonesia is a tropical country that has high biodiversity, so it is one of the potential countries for obtaining new bioactive compounds. Research on the search for bioactive compounds from traditional medicinal plants is growing, in line with the results of ethnobotanical surveys of various ethnicities, especially in Indonesia. The survey results show that many plants have been used by the community ~~for the treatment of~~ to treat disease but have not been supported by adequate scientific information. Additionally, currently there are also many freely-sold herbal products that are very attractive to the public to treat diseases, ostensibly because they are cheaper, more efficient and better than modern medicine.[1] However, some of these herbal products do not have well-documented scientific information. One of the herbs of traditional medicine is sungkai (*Paronema canescens*). Indonesian people traditionally use *P. canescens* to treat diseases, such as toothache,[2] fever,[3] stomach ache, in

skin care, after childbirth,[4] and malaria.[5] Its use as a medicine is especially prevalent in ~~the area of~~ South Sumatra where the community has, additionally, used sungkai leaves to treat warts,[6] and hypertension.[7]

Based on literature studies some scientific information about the chemical content and biological activity of the *P. canencens* plant has been reported. The phytochemical test of an ethanol extract of the sungkai plant was positive for steroidal, triterpenoidal and phenolic compounds.[8] The compounds of these phenolic groups are known to be antioxidant in nature and have various biological activities, such as antibacterial, antidiabetic, anticancer, antihypertensive and anti-hyperlipidaemia. The methanol extract of the *P. canencens* leaves was reported to have antibacterial activity against *S. mutans*, *S. thyposa*, *B. subtilis*, and *S. aureus*.[4] In another study, it was reported that there were antimalarial compounds existing in the acetone extracts of *P. canencens* leaves.[9] In addition, it was also reported that the sungkai leaf extract was effective as an insecticide against the larvae of *Plusia* sp. The *n*-hexane and ethyl acetate extract of the *P. canescens* stem bark had antioxidant activity with $IC_{50}$ in the *n*-hexane extract –44.55 µg/mL and in the ethyl acetate extract of 43.67 µg/mL,[10] but no scientific information has been divulged regarding the antioxidant and antibacterial activities of the fractions from sungkai leaves.

**Commented [YA1]:** Re-write the sentence, Splitting it into two sentences

## Materials and Methods

### *Sample Collection*

The fresh leaves of *P. canescens* were collected from the Musi Banyuasin Regency of South Sumatera, Indonesia in October 2019. The plant was identified as *P. canescens* by Dr. Laila Hanum (Voucher specimen VIC 2704), head of the botany laboratory, University of Sriwijaya. The plant was deposited at the Botany Laboratory in the Biology Department at the University of Sriwijaya.

### *Extraction Process~~and Isolation~~*

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The fresh leaves of *P. canescens* (1.2 kg) were extracted using the maceration method with step gradient polarity and the solvents *n*-hexane, ethyl acetate, and methanol. The leaves soaked for three days before filtration. This process (of soaking and filtration) was repeated three times. This filtered –was evaporated using a rotary evaporator at -about 60 °C to -obtained concentrate the product. The concentrated fractions -was weighed and yield -percentage was calculated.

### *Determination of Antioxidant Activity*

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Each fraction was made concentration test series 50; 100; 200; 300; 400; 500; and 1000 µg/mL in DMSO. From each concentration 200 µL was taken into a dark bottle containing 3.8 mL of 0.05 mM DPPH (prepared by dissolving 1.98 mg DPPH in 100 mL of methanol). The mixture was vortexed for 2 min, then incubated at room temperature for 30 min in the dark. The study was conducted three times.[11] The test solution was measured by UV-Vis spectrophotometer at $\lambda_{max}$ 517 nm. The blank used was methanol (4 mL), and as control DPPH (3.8 ml) was added to 200 µL of DMSO. The percentage of inhibition was calculated using the formula:

$$\% \text{ Inhibition} = \frac{\text{Absorbance of Control} - \text{Absorbance of sample}}{\text{Absorbance Control}} \text{ x } 100$$

Based on the data, a linear regression equation curve was plotted between the sample concentration and the percent inhibition of the sample to determine the $IC_{50}$ value.

*Analysis of total phenolic content*

The total phenolics of the fractions were determined using the Folin and Ciocalteu reagent, following the method described by candra et al[12] with slight modifications. Analysis of each fraction's ~~the~~ total phenolic content ~~of each fraction~~ was determined using a spectrophotometric method based on the formation of the complex with Folin-Ciocâlteu reagent. Gallic acid was used as standard. A total of 1.0 mL of sample was added to 2.5 mL of Folin-Ciocâlteu reagent diluted with water (1:10 v/v). After 5 minutes, 10 mL of 7.5% $Na_2CO_3$. The mixture was incubated for 90 min at room temperature, then the absorbance was measured at a wavelength of 760 nm with a UV-Vis spectrophotometer. ~~Making a~~A standard curve ~~,~~ gallic acid was made with concentrations of 18; 20; 23; 25; 27; 30 µg/mL and plotted as in the fractions. The total phenolic content was determined from a calibration curve and expressed as mg gallic acid per 1 g of extract weight.

*Determination of total flavonoid content*

The total flavonoid content was determined by spectrophotometry based on reaction with $AlCl_3$. The fraction (1 ml) was put into a 10 mL measuring flask, plus 4 mL of distilled water and 0.3 mL of a 5% $NaNO_2$ solution, then left for 5 min. To this, was added 0.3 mL of 10% $AlCl_3$ and 2 mL of 1.0 M NaOH and the solution left for 5 minutes. Quercetin standard curves were made with a concentration series of 100; 80; 60; 40 and 20 µg/mL and treated the same as the samples as described above. Absorbance fractions and standards were measured against the blank at 510 nm on a UV-vis spectrophotometer. The blanks ~~is~~are described as all reagents used without

quercetin or sample. Total flavonoid content was determined from a standard quercetin curve and expressed as mg of quercetin per 1 g of extract weight. The sample measurements were replicated thrice.[13]

*Antibacterial Activity Test*

Antibacterial screening was carried out by the disk diffusion method using disc paper with a diameter of 6 mm. The antibacterial activity test was carried out in triplicate. The sample concentrations used in the antibacterial activity test were 4%, 2%, 1% and 0.5%. Disc paper was dipped at each sample concentration, then placed on nutrient agar (NA) media that had been inoculated with *E. coli* (ATCC 25922), *S. aureus* (ATCC 25923) and *S. typhi* (ATCC 19214) bacteria. Incubation was carried out at 37 °C for -24 hours. Observations were made based on the formation of an inhibition zone around the disc paper. Furthermore, was the measurement of the diameter of the zone- of inhibition formed marked by the clear area formed around the disk.[14,15]

**Commented [YA4]:** Furthermore, the measurement of the zone of inhibition formed marked by the precise area formed around the disk

*Determination MIC*

The MIC was determined by dilution method using a plate. Liquid culture (30 µL) from each bacterium was inoculated into a plate that already contained 180 µL (hole 1) and 100 µL (hole 2, etc.) NB medium and stirred. The isolated compounds were prepared with concentrations of 125, 62.5, 31.25, 15.62, 7.81, 3.9, 1.9, 0.97, 0.48 and 0.24 µg / mL against *S. aureus*, *S. typhi* and *E. coli* bacterial samples that were made in various concentrations are inserted into the plate. Plate holes 11 and 12 were used for a DMSO negative control and a tetracycline (10 mg/mL concentration) positive control, respectively. The cultures were incubated for 24 hours at 37 °C. Antibacterial activity was characterized by the formation of a clear solution.[16,17]

*Data analysis*

Measurements were made in triplicate, data are provided as mean ± SD. Data were analysed statistically using ANOVA (α 0.05), followed by the Duncan New Multiple Range Test (DNMRT) at α 0.05 .

**Result and Discussion**

Fresh sungkai leaves (1.2 kg) were extracted by maceration via solvent with increasing polarity, (*n*-hexane, ethyl acetate, and methanol) in triplicate. After being concentrated, the *n*-hexane fraction (15.97 g) was obtained, ethyl acetate extract (62.69 g), and the methanol fraction (56.40 g), with yields of 1.33%, 5.22%, and 4.70%, respectively. The yield of the ethyl acetate fraction was higher than the other fractions. This higher yield is presumably because ethyl acetate is a semi-polar solvent which can dissolve both non-polar and polar compounds.

**Commented [YA5]:** Not necessarily, it could be correlated to the chemical constituent presence in your sample. Ethyl acetate is semi polar solvent, hence, the higher yield could be caused by your sample rich by semi polar compounds than nonpolar compounds(hexane fraction) and polar compounds (methanol fraction)…please add citation for the discussion

Each fraction was then analysed for total phenolic content, flavonoid content, antioxidant activity and antibacterial activity.

*Antioxidant activity*

The antioxidant activity of each fraction was determined using the DPPH method. This method is commonly used to evaluate the antioxidant activity of various extracts. The measurements of antioxidant activity are based on the absorbance values of the DPPH that reacts with the extract. A maximum wavelength of DPPH of 517 nm was determined and utilized. The antioxidant activity of each fraction was expressed as percentage of inhibition as shown in Table 1. Percentage inhibition implies the number of DPPH radicals absorbed by the test sample. The higher the % inhibition, the more active the test sample. In Table 1, it can be seen that the higher the test concentration, the greater the inhibition value. At the same concentration (1000 ppm), % inhibition of ethyl acetate extract was 76.14 ± 6.20% and was higher than other fractions. Based on the statistical analysis of the methanol fraction and *n*-hexane fraction, the % inhibitions were not significantly different ($p > 0.05$), but were significantly different from the ethyl acetate fraction ($p < 0.05$).

**Commented [YA6]:** Add citation or reference

**Commented [YA7]:** No standard for this activity???? This results are not acceptable without compare with standard such as Quercetin or ascorbic acid. Hence you can conclude your results as non-active, weak, or active

**Commented [YA8]:** Luck of discussion. Please, add some discussion for your results...hight antioxidant could be contribute .....(search similar studies which have reported antioxidant and phenolic content...compare with their report.... Even from different species or find from the same genus or family???)

**Table 1**

Based on the % inhibition, the concentration that can reduce 50% of the DPPH radicals ($IC_{50}$) is determined. The $IC_{50}$ value determination ~~The determination of the $IC_{50}$ value~~ is based on the linear regression curve for the relationship between concentration (x) and the percent inhibition value (y). The data in Table 2 show that the methanol fraction had the smallest $IC_{50}$ value compared to other fractions (320 ppm). The smaller the $IC_{50}$ value, the more active the fraction. An extract is categorized as (potentially) a strong antioxidant if it has an IC value of < 200 ppm, moderate if between 200 and 1000 ppm and inactive if > 1000 µg/mL. [18] Based on the data obtained, only the methanol fraction was classified as moderate antioxidant, while the other two fractions were inactive. The fraction belonging to the moderately active category of antioxidants still has the potential to be used as a source of antioxidant because sometimes the compounds in pure form are more active than in their fractional form. Based on the literature, Rosdiana[10] reported that the *n*-hexane and ethyl acetate extracts of *P. canescens* stem bark had bioactivity as an antioxidant with an $IC_{50}$ in the *n*-hexane extract of 44.55 µg/mL and ethyl acetate of 43.67 µg/mL. In the leaves of sungkai, target of this study, the $IC_{50}$ value was much greater. This indicates that the antioxidant activity of the sungkai stems is much stronger than that of the leaves.

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**Commented [YA9]:** No standard for this activity???? This results are not acceptable without compare with standard such as Quercetin or ascorbic acid. Hence you can conclude your results as non-active, weak, or active

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**Table 2**

*Total phenolic and flavonoid content*

Determination of the total amount of phenols was carried out in each fraction by using spectrophotometry and the Folin–Ciocâlteu reagent. Gallic acid was used as the phenolic standard. The total phenolic value of each fraction was determined from the linear regression of the standard curve of gallic acid. The total phenolic value was expressed as the number of mg of gallic acid per dry weight of sample (mg GAE / g), shown in Table 3. The ethyl acetate fraction shows the highest total phenolic content of 68.71 ± 0.17 mg GAE/g compared to other fractions.

**Table 3**

Determination of total flavonoids by colorimetry was based on the formation of a flavonoid–$AlCl_3$ complex, with maximum absorption wavelength of 510 nm. Colorimetric reactions are widely used to determine the total flavonoid content in medicinal plants because they are easy to do. Complex formation occurs in the presence of $NaNO_2$ in an alkaline medium and is based on the nitration of the aromatic ring of flavonoids with free hydroxyl groups. $AlCl_3$ also forms acid-stable complexes with the *C*4 keto group and the *C*3- or *C*5-OH groups of the flavonoids. The total flavonoid content (TFC) of the extract was determined from the regression equation of the quercetin calibration curve and the flavonoid value in mg quercetin to dry weight of sample (mg QE / g). The highest level of flavonoids was indicated by the ethyl acetate fraction with a total flavonoid value of only 2.29 ± 0.05 mg QE/g and also the flavonoid content of the ethyl acetate fraction of the *P. canestens* extract was higher than other fractions. The data in Table 4 also show that the total phenolic content was much higher than the flavonoid content in every fraction. The higher the phenolic fraction content, the higher the total flavonoid levels. Flavonoids include phenolic compounds in addition to other compound groups. Data in Table 3 show that the low levels of flavonoids from each fraction were very low. Based on literature studies, the levels of flavonoids in the sungkai leaf fractions were very low. Agbo et al.[13] reported 13 samples from parts of medicinal plants from Nigeria with a total phenolic content of 10 including 11.18 ± 0.30–97.77 ± 0.77 and a total flavonoid content of 10.33 ± 0.00–648.67 ± 12.3 and three other samples of 3.67 ± 0.00–7.00 ± 0.00. The ratio of flavonoids to total phenolic content was determined as the fraction that contains the most flavonoid compounds. The data showed that the three fractions gave a ratio of < 1, indicating ~~a low levels of flavonoids~~low level of flavonoids.

**Commented [YA11]:** Re-write or splitting it into 2 sentences

Flavonoids are known to have various biological effects, including antibacterial, antioxidant, antidiabetic, antihypertensive, anti-tumour, anticancer activity among others. The high phenolic and flavonoid content in a fraction usually indicates high antioxidant activity. Studies reveal that there is a high correlation between antioxidant activity and the content of phenolics and flavonoids. Kristinaningsih et al[19] also to– finding the consistent -the results of phytochemical screening -phenolic -with antioxidan activity value at the -ethanol extract of *Aleurites. moluccana* leaves. In this study showed the low levels of flavonoids were in line with the low antioxidant activity of each fraction.

*Antibacterial activity*

Determination of the antibacterial activity was carried out using the disc diffusion method. The activity value is expressed as the combat zone diameter (clear zone). The test sample was dissolved in DMSO, thus DMSO was used as a negative control. Anti-bacterial screening was carried out on the three fractions using *E. coli*, *S. aureus*, and *S.typhi* as shown in Table 3. The results showed that the antibacterial activity of all fractions had an inhibition zone diameter ranging from 7.7 ± 0.1–9.1 ± 0.4 mm at test concentrations of 0.5% to 4%. DMSO, the negative control, had no inhibition zone.

**-Table 4**

In general, the diameter of the inhibition zone is greater if the concentration is higher. However, in several test concentrations, it was seen that the difference in concentration did not have a significant difference in zone diameter values ($p > 0.05$). The antibacterial activity of the methanol extract was lower than ethyl acetate and *n*-hexane extracts; however, the *n*-hexane and ethyl acetate fractions' inhibition zone diameter were not significantly different ($p > 0.05$). The data in Table 4 also show that the fractions of methanol, ethyl acetate and *n*-hexane had antibacterial activity that was not significantly different ($p > 0.05$) against the three tested bacteria. Based on the literature, antibacterial activity was classified into three categories: strong activity if the inhibition zone was 10–20 mm, moderate activity if an inhibition zone of 5–10 mm, and weak if it had an inhibitory zone diameter of < 5 mm.[20] Based on this criterion, all the fractions of *P. canestens* leaves were classified as moderately antibacterial for the three bacteria *E.coli*, *S. aureus*, and *S. thypii*. The methanol extract of the *P. canescens* leaves was reported to have antibacterial activity against *S. mutans*, *S. thyposa*, *B.subtilis*, and *S. aureus* with an MIC in *S. mutans* of 10%, an MIC of 15% for *S. thyposa* and *B. subtilis*, and 20% MIC for *S.aureus*.[5] In this study, it

was found that all fractions still provided resistance to the three tested bacteria (*E. coli*, *S. aureus*, and *S. typhi*) up to a test concentration of 0.5%.

**Table 5**

MIC values were determined in Table 4 for each fraction against the three. The test bacteria showed that all fractions gave an MIC value of 62.5 µg/mL for *E. coli* and *S. aureus*, while for *S. typhi* bacteria only the n-hexane fraction gave an MIC value of 62.5 while the other fractions gave an MIC value of 125. The fraction is stated as antibacterial if it gives an MIC value <100 µg/mL.[21] Based on this data, the three fractions are categorized as active against bacteria e. coli and S. aureus with MIC values 62.5 µg/mL while for *S. typhi* bacteria only n-hexane extract was categorized as antibacterial, while ethyl acetate and methanol fractions showed the weak ones with MIC values> 100 µg / mL, namely 125 µg / mL.

Separation and purification of the n-hexane fraction using column chromatography techniques obtained pure compounds and identified them as betulinic acid.[22] The antibacterial activity test of the isolated compound is shown in Table 5. The MIC values for all three fractions against the three bacteria are presented in Table 5. The test bacteria showed that all fractions had an MIC of 62.5 µg/mL for *E. coli* and *S. aureus*. For the *S. typhi* bacterium only the *n*-hexane fraction had an MIC value of 62.5; the others had an MIC value of 125 µg/mL. Separation and purification of the *n*-hexane fraction using column chromatography obtained a pure compound, identified as betulinic acid. The antibacterial activity of the isolated compound is shown in Table 6.

**Table 6**

In Table 6, we show that the diameter of the inhibition zone increases with the higher concentration of the test compound. Statistical analysis showed that at different concentrations of the inhibition zone values were not significantly different ($p > 0.05$). The isolated compound had antibacterial activity of 125 µg/mL with an inhibition zone diameter of 7.5 ± 0.1–8.2 ± 1.2 mm against the three tested bacteria. Statistical analysis showed that there was no significant preference by the compound for any one bacterium ($p > 0.05$). When the antibacterial activity of the pure compound is compared to the impure *n*-hexane fraction, it can be seen that the antibacterial activity of the isolated compound is higher than that of the fraction. This shows that the compound is more active in its pure form than when present in a fraction. The MIC of the pure compound was determined by the dilution method (Table 7).

**Table 7**

The MIC value was determined based on the formation of a clear solution. Table 7 shows that until concentration of 62.5 µg/mL, a clear solution was formed for the three tested bacteria, while at a concentration of 31.25 µg/mL, a cloudy solution was formed. Based on this data, it was concluded that the isolated compound had an MIC value of 62.5 µg/mL against the three tested bacteria (*E. coli*, *S. aureus*, and *S. typhi*).

## Conclusion

All ~~organic solvent~~ fractions of the leaves from sungkai (*P. canescens*) show weak antioxidant activity and moderate antibacterial activity. The sungkai leaves also have much lower levels of flav onoids than phenolics.

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## Conflict of interest

The authors declare no conflict of interest


## Acknowledgement

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**Table 1.** The influence of sample's concentration on DPPH inhibition of leaves fractions methanol, ethyl acetate, and n-hexane *P. canescens*

| Concentration (µg/mL) | Inhibition percentage (% I) ± SD | | |
|---|---|---|---|
| | n-Hexane | Ethyl acetate | Methanol |
| 50 | 24.69 ± 0.25$^{e}$ | 28.13 ± 4.51 | 15.95 ± 1.65$^{g}$ |
| 100 | 25.30 ± 0.38$^{e}$ | 43.86 ± 2.56 | 19.41 ± 0.14$^{f}$ |
| 200 | 25.92 ± 0.07$^{e}$ | 47.42 ± 3.19 | 24.81 ± 0.28$^{e}$ |
| 300 | 27.64 ± 0.77$^{d}$ | 50.73 ± 1.44 | 27.40 ± 0.60$^{d}$ |
| 400 | 28.50 ± 0.18$^{d}$ | 53.80 ± 7.65 | 28.13 ± 0.33$^{d}$ |
| 500 | 31.33 ± 1.28$^{c}$ | 63.27 ± 3.14 | 28.87 ± 0.87$^{d}$ |
| 1000 | 40.90 ± 3.40$^{a}$ | 76.14 ± 6.20$^{b}$ | 42.26 ± 1.96$^{a}$ |

Numbers follow by same subscript indicate not significant different according to Duncan New Multiple Range Test (DNMRT) 5%.; The experiments were repeated at three times, SD: Standart deviation

**Tabel 2.** Inhibition concentrations of 50% of DPPH•

| Fraction | $IC_{50}$ (µg/mL) | Antioxidant category |
|---|---|---|
| n-Hexane | 1567 | No active |
| Ethyl acetate | 320 | Weak active |
| Methanol | 1281 | No active |

**Commented [YA13]:** No standard for this activity???? This results are not acceptable without compare with standard such as Quercetin or ascorbic acid. Hence you can conclude your results as non-active, weak, or active

**Commented [YA14]:** Add a note below the table (antioxidant categories…not active if IC50>…..weak if….>….etc)

**Tabel 3.** Total phenolic and flavonoids contents of fractions *P. canescens*

| Fractions | Total Phenolic (mgGAE/g) | Flavonoid (mgQE/g) | Ratio F/P |
|---|---|---|---|
| n-Hexane | 9.28±0.08 | 0.77 ±0.13 | 0.083 |
| Ethyl acetate | 68.71±0.17 | 2.29 ±0.05 | 0.033 |
| Methanol | 24.30 ±0.17 | 1.92±0.03 | 0.079 |

The experiments were repeated at three times, SD: Standart deviation, F : Flavonoid P: Phenolic

**Table 4.** Antibacterial activity of fractions the leaves of *P.canescens*

| Fractions | Concentration (µg/mL) | Zone of inhibition (mm) ± SD | | |
|---|---|---|---|---|
| | | ***E. coli*** | ***S. aureus*** | ***S. typhi*** |
| Methanol | 4 % | 8.6 ± 0.1 | 8.8 ± 0.3 | 10.1± 0.6 |
| | 2 % | 8.3 ± 0.5 | 8.6 ± 0.2[a] | 9.2 ± 0.2 |
| | 1% | 8.1 ± 0.2 | 8.5 ± 0.1[a] | 7.9 ± 0.9 |
| | 0,5 % | 7.7 ± 0.1[b] | 7.9 ± 1.1[b] | 7.3 ± 0.2 |
| Ethyl acetate | 4 % | 8.9 ± 0.1[d] | 8.0 ± 0.8[c] | 8.7± 0.1 |
| | 2 % | 8.8 ± 0.1[d] | 7.9 ± 0.4[c] | 8.1± 0.5 |
| | 1 % | 8.6 ± 0.6 | 7.7 ± 0.5 | 7.5 ± 0.4 |
| | 0,5 % | 8.0 ± 0.4 | 7.6 ± 0.1 | 7.1± 0.1 |
| Hexane | 4 % | 9.1 ± 0.4[d] | 8.8 ± 0.4 | 9.2 ± 0.8 |
| | 2 % | 8.9 ± 0.3[d] | 8.5 ± 0.3 | 8.7 ± 0.2 |
| | 1 % | 8.2 ± 0.5 | 8.3 ± 0.2 | 8.2 ± 0.1 |
| | 0,5 % | 7.8 ± 0.3 | 8.1 ± 0.2 | 7.9 ±0.6 |
| Control | | NI* | NI* | NI* |

Control : 10 % DMSO, NI* : No inhibition
Numbers follow by same subscript indicate not significant different according to Duncan New Multiple Range Test (DNMRT) 5%.; Experiments were repeated at least three times Data are presented as Mean ± SD , SD: Standart deviation

**Tabel 5.** Determination MIC value of fractions leaves of *P. canescens*

| **Fractions** | **Bachterial** | **Concentrations (µg/mL)** | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | **125** | **62.5** | **31.25** | **15.62** | **7.8** | **3.90** | **1.95** | **0.97** |
| Methanol | *E.coli* | + | + | - | - | - | - | - | - |
| | *S. aureus* | + | + | - | - | - | - | - | - |
| | *S.typhi* | + | - | - | - | - | - | - | - |
| Ethy acetate | *E.coli* | + | + | - | - | - | - | - | - |
| | *S. aureus* | + | + | - | - | - | - | - | - |
| | *S.typhi* | + | - | - | - | - | - | - | - |
| n-Hexane | *E.coli* | + | + | - | - | - | - | - | - |
| | *S. aureus* | + | + | - | - | - | - | - | - |
| | *S.typhi* | + | + | - | - | - | - | - | - |

+ : clear -: blurry

**Tabel 6.** Antibacterial activity of betulinic acid from the n-hexane fraction leaves of *P.canescens*
.

| **Sample** | **Concentration (µg/mL)** | **Zone of inhibition (mm) ± SD** | | |
|---|---|---|---|---|
| | | ***E. coli*** | ***S. aureus*** | ***S. typhi*** |
| Betulinic acid | 1000 | $10.3 \pm 1.3^{b}$ | $9.8 \pm 1.1^{d}$ | $9.8 \pm 0.3^{d}$ |
| | 500 | $10.1 \pm 1.6^{b}$ | $9.2 \pm 1.4^{d}$ | $8.9 \pm 0.1^{c}$ |
| | 250 | $7.8 \pm 0.1^{a}$ | $8.8 \pm 0.8^{c}$ | $8.4 \pm 0.1^{c}$ |
| | 125 | $7.5 \pm 0.1^{a}$ | $8.7 \pm 1.9^{c}$ | $8.2 \pm 1.2^{c}$ |

Numbers follow by same subscript indicate not significant different according to Duncan New Multiple Range Test (DNMRT) 5%.; Experiments were repeated at least three times Data are presented as Mean ± SD , SD: Standart deviation

**Tabel 7.** Determination MIC value of betulinic acid

| **Sample** | **Bachterial** | **Concentrations (µg/mL)** | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | **125** | **62.5** | **31.25** | **15.62** | **7.8** | **3.90** | **1.95** | **0.97** |
| Betulinic acid | *E.coli* | + | + | - | - | - | - | - | - |
| | *S. aureus* | + | + | - | - | - | - | - | - |
| | *S.typhi* | + | + | - | - | - | - | - | - |

+ : clear -: blurry

## Response to Reviewer comment

1. Result : Almost all results (less citation). Found 4 citation only in their results and discussionPage 6, 12, 13

**Response:** Additional citation has been added in manuscript ( becomes 11 Refference)

2. No standard for antioxidant activity. It is not acceptable without compare with standard such as Quercetin or ascorbic acid. Hence the author can confirm their results as active or not antioxidant.

**Response**: Standard of antioxidant activity has been added i.e. ascorbic acid

3. Page 5, This higher yield is……. It could be correlated to the chemical constituent presence in your sample. Ethyl acetate is semi polar solvent. Hence, the higher yield could be caused by the sample rich with semi polar compounds than nonpolar hexane fraction) and polar compounds (methanol fraction. Please add citation/ref for your discussion.

   **Response** : Reference has been added ref. no. 19

4. Add some discussion for your results…hight antioxidant could be contribute …… (search similar studies which have reported antioxidant and phenolic content…compare with their report even come from different plant species…or find the same genus or family.

   **Response**: Reference has been added ref.no 23

5. Conclusion Too short…Write down anything that might be done in the future or further research that could be continued correlated to the results

   **Respon** : has been added in manuscript

6. add more references correlated to results and discussion parts

   **Respon:** has been added

7. Table 2 Add a note below the table
   **Response** : has been added in manuscript

**Editorial comments**

1. Abstract is not expected to be in a table or in a text box. Please remove and write on blank page.

   **Respon** : have been revised in the manuscript

2. Include the voucher number of the plant material.
   **Respon** : already in the article

3. Authors' Declaration :
   The authors hereby declare that the work presented in this article are original and that any liability for claims relating to the content of this article will be borne by them.

   **Response** : has been add in the manuscipt

4. 50% reference 2016-2020

   **Response** : has beed added in manuscript

5. The revised and corrected manuscript should be subjected to plagiarism checker (17% allowed in TJNPR) and English language editing.

   **Response** : plagiarism checker has been attached using turnitin program, and English language has been editing using Grammarly and proofread by scribendi.com (attached)

All corrections/changes made in the manuscript have been highlighted in yellow colour in the manuscript in the revision.

# Antioxidant, Antibacterial, Total phenolic and Flavonoid content of Sungkai leaves (*Paronema canescens*)

**ABSTRACT**

The leaves of the sungkai (*Paronema canescens*) have been used as a traditional medicine to treat various diseases. We determined the antioxidant, antibacterial, total phenolic content, and flavonoid content of solvent extraction fractions of these leaves from *P. canescens*. Fresh leaves were extracted by maceration using solvents of increasing polarity (*n*-hexane, ethyl acetate, and methanol). Each fraction was tested for antioxidant activity using the DPPH method, antibacterial activity was tested using the disc diffusion method, analysis of the total phenolic using the Folin–Ciocâlteu reagent, and total flavonoid using $AlCl_3$. The study showed that the ethyl acetate fraction had higher antioxidant activity than the other fractions with an $IC_{50}$ 320 µg/mL. The antibacterial activity test had a minimum inhibitory concentration (MIC) 62.5 µg/mL against *E. coli* and *S. aureus* at all fractions. Only the *n*-hexane fractions show provided a MIC 62.5 µg/mL against *S. typhi*. Betulinic acid from the *n*-hexane fraction also showed antibacterial activity with MIC 62.5 µg/mL. In the analysis of total phenolic and flavonoid, the ethyl acetate fraction contained higher organics levels than other fractions, with values of 68.71 ± 0.17 mg Gallic Acid Equivalent (GAE)/g and 2.29 ± 0.05 mg Quercetin Equivalent (QE)/g. It can be concluded that sungkai leaves show moderate antioxidant activity, moderate antibacterial activity, and much lower flavonoid than phenolic.

***Keywords***: Antioxidant, Antibacterial, Phenolic, Flavonoid, *Paronema canescens*

## Introduction

Indonesia is a tropical country with high biodiversity, so it is one of the potential countries for obtaining new bioactive compounds. Research on the search for bioactive compounds from traditional medicinal plants is growing, in line with ethnobotanical surveys of various ethnicities, especially in Indonesia. The survey results show that many plants have been used by the community to treat disease but have not been supported by adequate scientific information. Additionally, many freely-sold herbal products are currently very attractive to the public to treat diseases, ostensibly because they are cheaper, more efficient, and better than modern medicine.[1] However, some of these herbal products do not have well-documented scientific information. One of the herbs of traditional medicine is sungkai (*Paronema canescens*). Indonesian people traditionally use *P. canescens* to treat diseases, such as toothache,[2] fever,[3] stomach ache, skincare, after childbirth,[4] and malaria.[5] Its use as a medicine is especially prevalent in South Sumatra, where the community has, additionally, used sungkai leaves to treat warts,[6] and hypertension.[7]

Based on literature studies, some scientific information about the chemical content and biological activity of the *P. canencens* plant has been reported. The phytochemical test of an ethanol extract of the sungkai plant was positive for steroidal, triterpenoid and phenolic compounds.[8] The compounds of these phenolic groups are antioxidant and have various biological activities, such as antibacterial, antidiabetic, anticancer, antihypertensive and anti-hyperlipidemia. The methanol extract of the *P. canencens* leaves was reported to have antibacterial activity against *S. mutans*, *S. thyposa*, *B. subtilis*, and *S. aureus*.[4] In another study, it was reported that antimalarial compounds existed in the acetone extracts of *P. canencens* leaves.[9] Besides, it was also reported that the sungkai leaf extract was useful as an insecticide against the larvae of *Plusia* sp. The *n*-hexane and ethyl acetate extract of the *P. canescens* stem bark had an antioxidant activity with $IC_{50}$ in the *n*-hexane extract 44.55 µg/mL and in the ethyl acetate extract of 43.67 µg/mL.[10] In this study, we reported antioxidant and antibacterial activities of the fractions from *P. canescens* leaves.

## Materials and Methods

### *Sample Collection*

The fresh leaves of *P. canescens* were collected from the Musi Banyuasin Regency of South Sumatera, Indonesia, in October 2019. The plant was identified as *P. canescens* by Dr Laila Hanum, head of the botany laboratory,

University of Sriwijaya. A voucher specimen has been deposited at the Botany Laboratory in the Biology Department at the University of Sriwijaya with Voucher specimen VIC 2704.

*Extraction Process*

The fresh leaves of *P. canescens* (1.2 kg) were extracted using the maceration method with step gradient polarity and the solvents *n*-hexane, ethyl acetate, and methanol. The leaves soaked for three days before filtration. This process (of soaking and filtration) was repeated three times.[11] This filtered was evaporated at about 60 °C to obtained concentrate the product. The concentrated fractions were weighed, and the yield percentage was calculated.

*Determination of Antioxidant Activity*

The antioxidant activity of extracts was evaluated using the DPPH method according to a previous method of Xu et al.[12] and some modifications. Each fraction was made concentration test series 50; 100; 200; 300; 400; 500; and 1000 µg/mL in DMSO. From each concentration, 200 µL was taken into a dark bottle containing 3.8 mL of 0.05 mM DPPH. The mixture was vortexed for 2 min, then incubated at room temperature for 30 min in the dark. The study was conducted three times. UV-Vis measured the test solution at 517 nm. Ascorbic acid was used as a positive control. The blank used was methanol (4 mL), and as control DPPH (3.8 ml) was added to 200 µL of DMSO. The percentage of inhibition was calculated using the formula:

$$\% \text{ Inhibition} = \frac{\text{Absorbance of Control} - \text{Absorbance of sample}}{\text{absorbance Control}} \text{ x } 100$$

Based on the data, a linear regression equation curve was plotted between the sample concentration and the per cent inhibition of the sample to determine the $IC_{50}$ value. (13)

*Analysis of total phenolic*

The fractions total phenolics were determined using the Folin Ciocalteu reagent, following the method described by candra et al[14] with slight modifications. Analysis using a spectrophotometric method and gallic acid was used as standard. 1.0 mL sample was added to 2.5 mL of Folin reagent after 5 minutes, 10 mL of 7.5% $Na_2CO_3$, incubated for 90 min at room temperature, then the absorbance was measured $\lambda_{max}$ 760 nm. A standard curve gallic acid was made with concentrations of 18; 20; 23; 25; 27; 30 µg/mL and plotted as in the fractions.

*Determination of total flavonoid content*

The total flavonoid content was determined by spectrophotometry based on reaction with $AlCl_3$ following the method described by Amalitc et al [15] with slight modifications. The fraction 1 ml plus 4 mL of distilled water and 0.3 mL of a 5% $NaNO_2$, then left for 5 min. To this, was added 0.3 mL of 10% $AlCl_3$ and 2 mL of 1.0 M NaOH and the solution left for 5 minutes. Quercetin standard curves were made with a concentration series of 100; 80; 60; 40, and 20 µg/mL and treated the same as the samples described above. Absorbance fractions and standards were measured at $\lambda_{max}$ 510 nm. The blanks are defined as all reagents used without quercetin or sample, and the measurements were replicated thrice.

*Antibacterial Activity Test*

The antibacterial screening was carried out by the disk diffusion method using disc paper with a diameter of 6 mm. The sample concentrations used in the antibacterial activity test were 4%, 2%, 1% and 0.5%. Disc paper was dipped at each sample concentration, then placed on nutrient agar (NA) media that had been inoculated with *E. coli* (ATCC 25922), *S. aureus* (ATCC 25923) and *S. typhi* (ATCC 19214) bacteria. Incubation at 37 °C for 24 hours. Observations were made based on the formation of an inhibition zone around the disc paper. Furthermore, the zone of inhibition formed marked by the precise area formed around the disk.[16]

*Determination MIC*

The minimal inhibitory concentrations (MIC) determined by the micro-dilution method were conducted according to the Clinical and Laboratory Standards Institute[17] with slight modifications. Liquid culture (30 µL) from each bacterium was inoculated into a plate that already contained 180 µL (hole 1) and 100 µL (hole 2, etc.) NB medium and stirred. The isolated compounds were prepared with concentrations of 125, 62.5, 31.25, 15.62, 7.81, 3.9, 1.9, 0.97, 0.48 and 0.24 µg / mL bacterial samples that were made in various concentrations are inserted into the plate. Plate holes 11 and 12 were used for a DMSO negative control and tetracycline (10 mg/mL concentration) positive control. The cultures were incubated for 24 hours at 37 ºC. Antibacterial activity by the formation of a clear solution.[18]

*Data analysis*

Measurements were made in triplicate. Data are provided as mean ± SD. Data were analyzed statistically using ANOVA (α 0.05), followed by the Duncan New Multiple Range Test (DNMRT) at 0.05 .

## Result and Discussion

Fresh sungkai leaves (1.2 kg) were extracted by maceration via solvent with increasing polarity in triplicate. After being concentrated, the *n*-hexane fraction (15.97 g) was obtained, ethyl acetate extract (62.69 g), and the methanol fraction (56.40 g), with yields of 1.33%, 5.22%, and 4.70%, respectively. The product of the ethyl acetate fraction was higher than the other fractions. The higher yield at ethyl acetate extract could be caused by leaves of *P. canescens* contained more elevated semi-polar compounds than non-polar and polar compounds. Besides that, the higher product is presumably because ethyl acetate is a semi-polar solvent that can dissolve both non-polar and polar compounds.[19]

### *Antioxidant activity*

The measurements of antioxidant activity based on the absorbance values of the DPPH that reacts with the extract. A maximum wavelength of DPPH of 517 nm was determined and utilized. The antioxidant activity of each fraction was expressed as a percentage of inhibition, as shown in Table 1. Percentage inhibition implies the number of DPPH radicals absorbed by the test sample. The higher the % inhibition, the more active the test sample.[20] Table 1 it can be seen that the higher the test concentration, the greater the inhibition value. At the same concentration (1000 µg/mL), the % inhibition of ethyl acetate extract was 76.14 ± 6.20. It was higher than other fractions, while ascorbic acid as a standard at concentration 100 µg/mL shows the % inhibition of 95.19 ± 0.51 (Table 2). Based on the statistical analysis of the methanol fraction and *n*-hexane fraction, the % inhibitions were not significantly different ($p > 0.05$) but were significantly different from the ethyl acetate fraction ($p < 0.05$). The antioxidant activity was influenced by the flavonoid and phenolic content in the extract. The Flavonoids will donate hydrogen atoms or electrons to free radicals to stabilize radical compounds so that the higher the flavonoid content in the extract, the higher the antioxidant activity.[21] The total flavonoid content was an influence on antioxidant activity.[12]

**Table 1**

**Table 2**

Based on the % inhibition, the concentration that can reduce 50% of the DPPH radicals ($IC_{50}$) is determined. The $IC_{50}$ value determination is based on the linear regression curve for the relationship between concentration (x)

and the per cent inhibition value (y). The data in Table 3 show that the ethyl acetate fraction had the smallest $IC_{50}$ value compared to other fractions (320 µg/mL) while ascorbic acid as a standard antioxidant activity 10.69 µg/mL. The smaller the $IC_{50}$ value, the more active the fraction. An extract is categorized as (potentially) a strong antioxidant if it has an IC value of < 200 µg/mL, moderate if between 200 and 1000 µg/mL and inactive if > 1000 µg/mL.[22] Based on the data obtained, only the ethyl acetate fraction was classified as a moderate antioxidant, while the other two fractions were inactive. The fraction belonging to the moderately active category of antioxidants still can be used as a source of antioxidant because sometimes the compounds in pure form are more active than in their fractional form. Based on the literature, Rosdiana[10] reported that the *n*-hexane and ethyl acetate extracts of *P. canescens* stem bark had bioactivity as an antioxidant with an $IC_{50}$ *n*-hexane extract of 44.55 µg/mL and ethyl acetate of 43.67 µg/mL. In the leaves of sungkai, the target of this study, the $IC_{50}$ value was much greater. This indicates that the antioxidant activity of the sungkai stems is much stronger than that of the leaves.

**Table 3**

*Total phenolic and flavonoid content*

Determination of the total amount of phenols was carried out in each fraction by using spectrophotometry and the Folin–Ciocâlteu reagent. The total phenolic value of each fraction was determined from the linear regression of the standard curve of gallic acid. The total phenolic value of the sample (mg GAE / g), shown in Table 4. The ethyl acetate fraction shows the highest total phenolic content of 68.71 ± 0.17 mg GAE/g than other fractions.

**Table 4**

Determination of total flavonoids by colourimetry was based on the formation of a flavonoid–$AlCl_3$ complex. The total flavonoid content (TFC) of the extract was determined from the regression equation of the quercetin calibration curve and the flavonoid value in mg quercetin to dry weight of the sample (mg QE / g). The highest level of flavonoids was indicated by the ethyl acetate fraction compare to the other fractions with a total flavonoid value of 2.29 ± 0.05 mg QE/g. The total polyphenol and total flavonoid highest also shown for extracts from the fresh sarcocarp of *C. multiflorus* is extracted with ethyl acetate compared to n-hexane extract. The highest the total polyphenol and total flavonoid is significant, which might indicate that most of the polyphenols, flavonoids were more soluble in a less polar solvent, such as ethyl acetate.[23] This told that the polyphenol and flavonoid are more soluble in a semi-polar solvent such as ethyl acetate.

The data in Table 4 also show that the total phenolic content was much higher than the flavonoid content in every fraction. The higher the phenolic fraction content, the higher the total flavonoid levels. Flavonoids include phenolic compounds in addition to other compound groups. Data in Table 3 show that the low levels of flavonoids from each fraction were deficient. Based on literature studies, the levels of flavonoids in the sungkai leaf fractions were deficient. Agbo et al[24] reported 13 samples from parts of medicinal plants from Nigeria with a total phenolic content of 10, including 11.18 ± 0.30–97.77 ± 0.77 and a total flavonoid content of 10.33 ± 0.00–648.67 ± 12.3 and three other samples of 3.67 ± 0.00–7.00 ± 0.00. The ratio of flavonoids to total phenolic content was determined as the fraction that contains the most flavonoid compounds. The data showed that the three fractions gave a ratio of < 1, indicating a low level of flavonoids. Flavonoids are known to have various biological effects, including antibacterial, antioxidant, antidiabetic, antihypertensive, anti-tumour, anticancer activity, among others. The high phenolic and flavonoid content in a fraction usually indicates high antioxidant activity. Kristinaningsih et al[25] also found consistent the results of phytochemical screening phenolic with antioxidant activity value at the ethanol extract of *Aleurites moluccana* leaves. In this study showed the low levels of flavonoids were in line with the low antioxidant activity of each fraction.

*Antibacterial activity*

The activity value is expressed as the combat zone diameter (clear zone). The anti-bacterial screening was carried out on the three fractions, as shown in Table 5. The results showed that the antibacterial activity of all fractions had an inhibition zone diameter ranging from 7.7 ± 0.1–9.1 ± 0.4 mm at test concentrations of 0.5% to 4%. DMSO, the negative control, had no inhibition zone. Antibacterial properties of flavonoids and organic acids widely distributed in plants, with their activity was quite diverse. The antibacterial properties of the extract of *P. canescens* are also thought to be related to the chemical content of flavonoids and phenolics in the extract.[26]

**Table 5**

In general, the diameter of the inhibition zone is greater if the concentration is higher. However, in several test concentrations, it was seen that the difference in concentration did not have a significant difference in zone diameter values ($p > 0.05$). The antibacterial of the methanol extract was lower than ethyl acetate and *n*-hexane extracts; however, the *n*-hexane and ethyl acetate fractions' inhibition zone diameter was not significantly different. The data in Table 5 also show that the fractions of methanol, ethyl acetate and *n*-hexane had an

antibacterial activity that was not significantly different ($p > 0.05$) against the three tested bacteria. Based on the literature, antibacterial activity was classified into strong activity if the inhibition zone was 10–20 mm, moderate activity 5–10 mm, and weak if it had an inhibitory zone diameter of < 5 mm. [27] Based on this criterion, all the fractions of *P. canestens* leaves were classified as moderately antibacterial for all bacteria test. The methanol extract of the *P. canescens* leaves was reported to have antibacterial activity against *S. mutans*, *S. thyposa*, *B.subtilis*, and *S. aureus* with a MIC in *S. mutans* of 10%, an MIC of 15% for *S. thyposa* and *B. subtilis*, and 20% MIC for *S.aureus*.[5] In this study, it was found that all fractions still provided resistance to the three tested bacteria up to a test concentration of 0.5%.

**Table 6**

MIC values were determined in Table 6 for each fraction against the three. The test bacteria showed that all fractions gave a MIC value 62.5 µg/mL for *E. coli* and *S. aureus*, while for *S. typhi* bacteria, only the n-hexane fraction gave a MIC value 62.5 while the other fractions gave a MIC value of 125. The fraction is stated as antibacterial if it gives MIC value <100 µg/mL.[28] Based on this data, the three fractions are categorized as active against bacteria *E. coli* and S. aureus with MIC values 62.5 µg/mL while for *S. typhi* bacteria, only n-hexane extract was categorized as antibacterial, while ethyl acetate and methanol fractions showed the weak ones with MIC values > 100 µg / mL, namely 125 µg/mL.

Separation and purification of the n-hexane fraction using column chromatography techniques obtained pure compounds and identified them as betulinic acid.[29] The antibacterial activity test of the isolated compound is shown in Table 6. The MIC values for all three fractions against the three bacteria are presented in Table 6. The test bacteria showed that all fractions had a MIC 62.5 µg/mL for *E. coli* and *S. aureus*. For the *S. typhi* bacterium, only the *n*-hexane fraction had MIC value 62.5; the others had MIC value 125 µg/mL. Separation and purification of the *n*-hexane fraction using column chromatography obtained a pure compound, identified as betulinic acid.

**Table 7**

In Table 7, we show that the inhibition zone's diameter increases with the higher concentration of the test compound. Statistical analysis showed that values were not significantly different at different concentrations of the inhibition zone ($p > 0.05$). The isolated compound had the antibacterial activity of 125 µg/mL with an inhibition zone diameter of 7.5 ± 0.1–8.2 ± 1.2 mm against the three tested bacteria. Statistical analysis showed

no significant preference by the compound for anyone bacterium ($p > 0.05$). The antibacterial activity of the pure compound is compared to the impure *n*-hexane fraction. It can be seen that the antibacterial activity of the isolated compound is higher than that of the fraction. This shows that the compound is more active in its pure form than when present in a fraction. The MIC of the pure compound was determined by the dilution method (Table 8).

**Table 8**

The MIC value was determined based on the formation of a clear solution. Table 8 shows that until the concentration of 62.5 µg/mL, a clear solution was formed for the three tested bacteria, while at a concentration of 31.25 µg/mL, a cloudy solution was formed. It was concluded that the isolated compound had a MIC value of 62.5 µg/mL against the three tested bacteria (*E. coli*, *S. aureus*, and *S. typhi*).

## Conclusion

All fractions of the leaves from sungkai (*P. canescens*) show weak antioxidant activity and moderate antibacterial activity. The sungkai leaves also have much lower levels of flavonoids than phenolics. Because of the complexity of natural phytochemicals in the extract, other methods were required to assess the overall antioxidant potential and using bacterial others to evaluate the antibacterial activity potential of *P. canescens* leaves.

## Conflict of interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article are original and that any liability for claims relating to the content of this article will be borne by them.

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**Table 1.** The influence of the sample's concentration on DPPH inhibition of leaves fractions methanol, ethyl acetate, and n-hexane *P. canescens*

| Concentration (µg/mL) | Inhibition percentage (% I) ± SD | | |
|---|---|---|---|
| | n-Hexane | Ethyl acetate | Methanol |
| 50 | 24.69 ± 0.25$^{e}$ | 28.13 ± 4.51 | 15.95 ± 1.65$^{g}$ |
| 100 | 25.30 ± 0.38$^{e}$ | 43.86 ± 2.56 | 19.41 ± 0.14$^{f}$ |
| 200 | 25.92 ± 0.07$^{e}$ | 47.42 ± 3.19 | 24.81 ± 0.28$^{e}$ |
| 300 | 27.64 ± 0.77$^{d}$ | 50.73 ± 1.44 | 27.40 ± 0.60$^{d}$ |
| 400 | 28.50 ± 0.18$^{d}$ | 53.80 ± 7.65 | 28.13 ± 0.33$^{d}$ |
| 500 | 31.33 ± 1.28$^{c}$ | 63.27 ± 3.14 | 28.87 ± 0.87$^{d}$ |
| 1000 | 40.90 ± 3.40$^{a}$ | 76.14 ± 6.20$^{b}$ | 42.26 ± 1.96$^{a}$ |

Numbers followed by the same subscript indicate not significantly different according to Duncan New Multiple Range Test (DNMRT) 5%.; The experiments were repeated at three times, SD: Standard deviation

**Table 2.** The influence of standard ascorbic acid concentration on DPPH inhibition.

| Concentration (µg/mL) | % inhibisi |
|---|---|
| 6,25 | 40.1 0 ± 1.32[a] |
| 12,5 | 52.16 ± 0.41[b] |
| 25 | 75.05 ± 1.23[c] |
| 50 | 93.15 ± 0.61[d] |
| 100 | 95.19 ± 0.51[d] |

Numbers followed by the same subscript indicate not significantly different.;
The experiments were repeated at three times, SD: Standard deviation

**Tabel 3.** Inhibition concentrations of 50% of DPPH•

| Fraction | $IC_{50}$ (µg/mL) | Antioxidant category * |
|---|---|---|
| n-Hexane | 1567 | inactive |
| Ethyl acetate | 320 | moderate active |
| Methanol | 1281 | inactive |
| Standard Ascorbic acid | 10.69 | Strong active |

*strong : IC value < 200 µg/mL, moderate $IC_{50}$ : 200 and 1000 µg/mL and inactive $IC_{50}$ : > 1000 µg/mL.

**Tabel 4.** Total phenolic and flavonoids contents of fractions *P. canescens*

| Fractions | Total Phenolic (mg GAE/g) | Flavonoid (mg QE/g) | Ratio F/P |
|---|---|---|---|
| n-Hexane | 9.28 ± 0.08 | 0.77 ± 0.13 | 0.083 |
| Ethyl acetate | 68.71 ± 0.17 | 2.29 ± 0.05 | 0.033 |
| Methanol | 24.30 ± 0.17 | 1.92 ± 0.03 | 0.079 |

The experiments were repeated at three times, SD: Standard deviation, F: Flavonoid P: Phenolic

**Table 5.** Antibacterial activity of fractions the leaves of *P.canescens*

| Fractions | Concentration (µg/mL) | Zone of inhibition (mm) ± SD | | |
|---|---|---|---|---|
| | | ***E. coli*** | ***S. aureus*** | ***S. typhi*** |
| Methanol | 4 % | 8.6 ± 0.1 | 8.8 ± 0.3 | 10.1± 0.6 |
| | 2 % | 8.3 ± 0.5 | 8.6 ± 0.2[a] | 9.2 ± 0.2 |
| | 1% | 8.1 ± 0.2 | 8.5 ± 0.1[a] | 7.9 ± 0.9 |
| | 0,5 % | 7.7 ± 0.1[b] | 7.9 ± 1.1[b] | 7.3 ± 0.2 |
| Ethyl acetate | 4 % | 8.9 ± 0.1[d] | 8.0 ± 0.8[c] | 8.7± 0.1 |
| | 2 % | 8.8 ± 0.1[d] | 7.9 ± 0.4[c] | 8.1± 0.5 |
| | 1 % | 8.6 ± 0.6 | 7.7 ± 0.5 | 7.5 ± 0.4 |
| | 0,5 % | 8.0 ± 0.4 | 7.6 ± 0.1 | 7.1± 0.1 |
| Hexane | 4 % | 9.1 ± 0.4[d] | 8.8 ± 0.4 | 9.2 ± 0.8 |
| | 2 % | 8.9 ± 0.3[d] | 8.5 ± 0.3 | 8.7 ± 0.2 |
| | 1 % | 8.2 ± 0.5 | 8.3 ± 0.2 | 8.2 ± 0.1 |
| | 0,5 % | 7.8 ± 0.3 | 8.1 ± 0.2 | 7.9 ±0.6 |
| Control | | NI* | NI* | NI* |

Control : 10 % DMSO, NI* : No inhibition

Numbers followed by the same subscript indicate not significantly different according to Duncan New Multiple Range Test (DNMRT) 5%.; Experiments were repeated at least three times. Data are presented as Mean ± SD, SD: Standart deviation.

**Tabel 6.** Determination MIC value of fractions leaves of *P. canescens*

| **Fractions** | **Bacterial** | **Concentrations (µg/mL)** | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | **125** | **62.5** | **31.25** | **15.62** | **7.8** | **3.90** | **1.95** | **0.97** |
| Methanol | *E. coli* | + | + | - | - | - | - | - | - |
| | *S. aureus* | + | + | - | - | - | - | - | - |
| | *S. typhi* | + | - | - | - | - | - | - | - |
| Ethyl acetate | *E. coli* | + | + | - | - | - | - | - | - |
| | *S. aureus* | + | + | - | - | - | - | - | - |
| | *S. typhi* | + | - | - | - | - | - | - | - |
| n-Hexane | *E. coli* | + | + | - | - | - | - | - | - |
| | *S. aureus* | + | + | - | - | - | - | - | - |
| | *S. typhi* | + | + | - | - | - | - | - | - |

+: clear -: blurry

**Tabel 7.** Antibacterial activity of betulinic acid from the n-hexane fraction leaves of *P.canescens*
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| **Sample** | **Concentration (µg/mL)** | **Zone of inhibition (mm) ± SD** | | |
|---|---|---|---|---|
| | | ***E. coli*** | ***S. aureus*** | ***S. typhi*** |
| Betulinic acid | 1000 | $10.3 \pm 1.3^{b}$ | $9.8 \pm 1.1^{d}$ | $9.8 \pm 0.3^{d}$ |
| | 500 | $10.1 \pm 1.6^{b}$ | $9.2 \pm 1.4^{d}$ | $8.9 \pm 0.1^{c}$ |
| | 250 | $7.8 \pm 0.1^{a}$ | $8.8 \pm 0.8^{c}$ | $8.4 \pm 0.1^{c}$ |
| | 125 | $7.5 \pm 0.1^{a}$ | $8.7 \pm 1.9^{c}$ | $8.2 \pm 1.2^{c}$ |

Numbers followed by the same subscript indicate not significantly different according to Duncan New Multiple Range Test (DNMRT) 5%.; Experiments were repeated at least three times. Data are presented as Mean ± SD, SD: Standart deviation

**Tabel 8.** Determination MIC value of betulinic acid

| Sample | Bacterial | Concentrations (µg/mL) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | **125** | **62.5** | **31.25** | **15.62** | **7.8** | **3.90** | **1.95** | **0.97** |
| Betulinic acid | *E. coli* | + | + | - | - | - | - | - | - |
| | *S. aureus* | + | + | - | - | - | - | - | - |
| | *S. typhi* | + | + | - | - | - | - | - | - |

+: clear -: blurry