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Phytochemical Screening, Antioxidant and Antibacterial Activities of Ethanol Extract and Fractions of *Aleurites moluccana* (L.) Willd. Leaves

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ABSTRACT

Aleurites moluccana (L.) Willd. (Candlenut) possesses great health benefits and is one of the most widely used plants in Indonesia. The activities of its leaf extract as antioxidant and antibacterial agent is yet to be investigated. Therefore, the present study was aimed at determining the antioxidant and antibacterial activities of ethanol extract and fractions of candlenut leaves. Samples were extracted using 96% ethanol and multilevel fractionation was done using water, hexane, and ethyl acetate. The crude ethanol leaf extract and the fractions were phytochemically screened to determine bioactive constituents. Antioxidant activity of extract and fractions was determined using the DPPH (2,2-diphenyl-1-picrylhydrazyl reagent) scavenging method, while antibacterial activity was tested against *Staphylococcus aureus* and *Escherichia coli* by employing the disc diffusion method. The results of the phytochemical screening revealed the presence of two or more of alkaloid, flavonoid, saponin, triterpenoid, steroid, tannin and polyphenol in each of the ethanol leaf extract and fractions. Antioxidant activity (IC_{50}) test indicated that the ethanol extract, hexane, ethyl acetate and water fractions of candlenut leaves were 141.26, 469.45, 65.68 and 47.91 µg/mL, respectively. The test solutions showed antibacterial activity against both *S. aureus* and *E. coli* in the order of water fraction > hexane fraction > ethanol extract > ethyl acetate fraction. The findings from the research suggest the potential use of water fraction of ethanol *A. moluccana* leaf extract in controlling resistant bacteria.

Keywords: *Aleurites moluccana*, Antioxidant, Antibacterial, Phytochemical, Candlenut.

Introduction

Infectious diseases are disorders caused by microorganisms such as bacteria, viruses, fungi, or parasites. Every year, around 3.5 million people die from infectious diseases; mostly poor children, especially those living in low-income countries.[1] Infectious diseases caused by bacterial pathogens are treated with antibiotics. However, misuse and overuse of antibiotics have contributed to antibiotic resistance. The use of traditional herbs as the main source of medicinal products is currently gaining ground for people to live a healthy lifestyle.[2] *Aleurites moluccana* (L.) Willd (Candlenut tree) is a medium-sized tree and up to 10 m tall in height. It is called *kemiri* in Indonesian cuisine. *A. moluccana* has been used for a long time as a spice and in folk medicine. The most widely used part of the candlenut plant is the nut, while the other parts of the plant can be used in traditional medicine. In traditional medicine, the stem barks and leaves are used for the treatment of a range of diseases, including fever, headache, inflammation, hepatitis, tumours, diarrhea, asthma, ulcers, nausea, gonorrhea, dysentery and more.[3,4] Based on previous research, the candlenut has been found to have several properties. These include the stem being reported as a wound healer,[5] while the bark possesses anticancer[6] and antibacterial[7] activities. In the leaf part of candlenut, activities that have been associated with it include Analgesics,[8] anti-inflammatory, antipyretic,[9] hyperlipidemic[10] and antibacterial.[7] Aqueous, methanol and acetonitrile extracts of *A. moluccana* stem and husk have antibacterial activity against *Staphylococcus aureus* and *Pseudomonas aeruginosa*.

Phytochemical screening of methanol extract from candlenut leaves showed that it contains alkaloids, flavonoids, tannins and sterols.[11] In previous studies, alkene hydrocarbons, steroids, terpenes and flavonoids have been identified and isolated by chromatographic separation method. Triterpenes α-amyrenone, *β*-amyrenone, glutinol, α-amyrin, and β-amyrin were isolated from dichloromethane fraction of *A. moluccana* leaf extract. Swertisin and 2'-O-Rhamnosylswertisin are flavonoid that have been isolated from methanol *A. moluccana* extract using silica gel CC and flash chromatography.[8] Sitosterol, n-Hentriacontane and stigmasterol are hydrocarbons and sterols that have been isolated from hydroalcoholic extract.[13] There is a dearth of information in the literature with regard to the activity of candlenut leaf extract as antioxidant and antibacterial agent. Therefore, the aim of the study was to identify the ethanol fractions of candlenut leaf extract with the highest antioxidant and antibacterial activities against *S. aureus* and *E. coli*.

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Materials and Methods

Source of plant material

Leaves of *A. moluccana* used in this study were obtained from trees growing in Pandansari Village, Lumajang, Indonesia. The plant where the leaves were collected was identified by Ir. Lilik Mastuti, M.P., a Pharmacognosist at Plant Laboratory State Polytechnic of Jember, Indonesia with herbarium number A02052019. The leaves were washed and dried at room temperature. The dried leaves were ground into fine powder.

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Sources of chemical reagents and microorganisms

Ethanol, hexane, ethyl acetate, Mueller Hinton Agar (MHA), Nutrient Agar (NA), hydrogen chloride, sodium chloride, Wagner reagent, ammonium hydroxide, chloroform, Dragendorff, magnesium, ammonia vapor, sulfuric acid, anisaldehyde, $FeCl_3$, gelatine, sterile distilled water, 2,2-diphenyl-1-picrylhydrazyl (DPPH), dimethyl sulfoxide (DMSO) and gentamicin (10 µg) were all purchased from Riedel-de Haën, Germany. The pathogens, *Staphylococcus aureus* ATCC 6538 and *Escherichia coli* ATCC 25922 used in this study were obtained from Microbiology Laboratory of Dental Hospital, University of Jember, Jember, Indonesia.

Plant extraction and fractionation

A 300 mg leaf powder of *A. moluccana* was extracted by ultrasonic maceration using 2.25 mL of 96% ethanol at 35°C. The extract was evaporated using a rotary evaporator (Stroglass Strike 300) at 50 °C and concentrated in the oven at 40°C. The concentrated extract was weighed and percent yield was calculated. Fractionation of extract was carried out by a stratified liquid-liquid partition method using water, hexane, and ethyl acetate solvents. Five (5) grams of ethanol extract of candlenut leaves were dissolved in 50 mL of water. The resulting solution was partitioned by the addition of 50 mL of hexane, shaken vigorously in a separating funnel and then allowed to separate into two layers. The hexane layer was removed and the water layer was fractionated again with 50 mL of ethyl acetate. All the fractions: hexane, ethyl acetate and water were collected and concentrated. The fractionated products were weighed for determination of yield.

Phytochemical screening of ethanol extract and fractions

Phytochemical screening was performed to determine the bioactive components in the ethanol extract and fractions for the following secondary metabolites: alkaloids, flavonoids, saponins, steroids, terpenoids, polyphenols and tannins.[11] The screening was carried out by employing the test tube or colour test and Thin Layer Chromatography (TLC) methods.

Antioxidant activity of ethanol extract and fractions

Antioxidant activity test was carried out using 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay. An aliquot of 1.2 mL of DPPH (0.1 mM) was added to 0.3 mL test solution and the preparation was then incubated at room temperature for 30 minutes. Absorbance was measured by Uv-Vis Spectrophotometer (Hitachi) at 514 nm. Percentage reductions were calculated using the following equation:

$$Inhibitory\ percentage = \frac{(absorbance\ of\ DPPH - absorbance\ of\ sample)}{absorbance\ of\ DPPH}\ x\ 100$$

The half maximal inhibitory concentration (IC_{50}) values, where the concentration of sample required to scavenge 50% DPPH were determined by plotting percentage inhibition versus sample concentration.[14]

Antibacterial activity of ethanol extract and fractions

Preliminary tests were carried out using the disk diffusion method. *S. aureus* ATCC 6538 and *E. coli* ATCC 25922 bacterial stocks in physiological condition of 0.9% NaCl were prepared and then swapped using cotton buds on Mueller Hinton's media in petri dishes. The disk was prepared by adding 10 µL of test solution of each concentration. The negative and positive controls used were 10% DMSO solution and 10 µg gentamicin antibiotics, respectively. Each culture was then incubated at 37°C for 20 hours. Antibacterial activity was observed the next day, based on the diameter of zone of inhibition marked by the clear area formed around the disk.[15]

Statistical analysis

All experiments were repeated at least three times. Data were presented as mean ± standard deviation (SD). Statistical analysis was performed using SPSS software trial version. The data were tested using the Kruskal-Wallis method, followed by the Mann-Whitney test. Diameters of inhibitory zones are said to be significantly different if it had significance value of $P < 0.05$.

Results and Discussion

Ethanol extract of Aleurites moluccana leaves was fractionated into three different fractions

Extraction of *A. moluccana* leaves was carried out by maceration method, which was achieved by employing ultrasonic procedure. The solvent selected in the extraction was 96% ethanol. This was chosen because, in the extraction of natural materials, ethanol can extract most of the secondary metabolites contained in plant powder. Percentage yield of extract was 14.23%. The ethanol extract was fractionated, stratified by the liquid-liquid partition method using nonpolar solvent (hexane), semipolar solvent (ethyl acetate) and polar solvent (water). The percent yield of these fractions are shown in Table 1. The water fraction produced the highest amount of yield, while the least amount was recorded for the ethyl acetate fraction.

Constituents of ethanol extract and fractions of Aleurites moluccana leaves

The outcome of the phytochemical screening of ethanol extract and fractions of *A. moluccana* (Table 2) revealed that all test samples contained alkaloids. Flavonoid was only detected in ethanol extract, ethyl acetate and water fractions. The secondary metabolites; saponin group and steroid triterpenes were only found in the ethanol extract and water fraction. All test samples, except the hexane fraction contained tannin and polyphenol.

Antioxidant and antibacterial activities of ethanol Aleurites moluccana leaf extract and fractions

Table 3 shows the values obtained for IC_{50} of extract and fractions. The greatest antioxidant activity was observed in the water fraction, followed by the ethyl acetate fraction, ethanol extract, and the hexane fraction. This finding is consistent with the results of phytochemical screening that showed that water and ethyl acetate fractions, as well as ethanol extract of *A. moluccana* leaves contain polyphenols, which contribute to antioxidant activity.

Table 1: Percentage Yield of Ethanol *Aleurites moluccana* Leaf Extract Fractions

Fraction	Yield (%)
Hexane	27.05
Ethyl acetate	23.32
Water	38.90

Table 2: Secondary Metabolites from Ethanol *A. moluccana* Leaf Extract and Fractions

Secondary metabolite	Ethanol extract	Hexane fraction	Ethyl acetate fraction	Water fraction
Alkaloid	+	+	+	+
Flavonoid	+	-	+	+
Saponin, triterpenoid, and steroid	+	-	-	+
Tanin and polyphenol	+	-	+	+

Key: -: not detected; +: detected

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Table 3: Antioxidant Activity of Ethanol Extract and Fractions of *A. moluccana* Leaves

Test sample	IC_{50} (µg/mL)
Ethanol extract	141.26
Hexane fraction	469.45
Ethyl acetate fraction	65.68
Water fraction	47.91

The results obtained for the diameter of inhibitory zones of *A. moluccana* leaf extract and fractions against *S. aureus* are presented in Table 4. At the smallest test concentration of 200 µg/mL, antibacterial activity against *S. aureus* was observed in the following order: water fraction > hexane fraction > ethanol extract > ethyl acetate fraction. On the contrary, in the *E. coli* bacterial culture (Table 5), ethanol extract and hexane fraction did not offer antibacterial activity at the smallest test concentration of 200 µg/mL. However, antibacterial activity was observed in the *E. coli* culture, in the following order: water fraction > hexane fraction > ethanol extract > ethyl acetate fraction.

In the present study, it was observed that the antibacterial activity of ethanol extract and fractions against *S. aureus* was stronger than *E. coli*. The reason for this observation is due to the difference in cell wall arrangement of the Gram-positive and Gram-negative bacteria. The Gram-negative bacteria have a thicker cell wall arrangement, compared to the Gram-positive bacteria that consist of one layer of cell wall, mainly composed of a thick layer of peptidoglycan. In contrast, the Gram-negative bacteria have two layers of cell wall, which consist of peptidoglycan (thinner than the one present in the Gram-positive bacteria) as well as a layer of lipopolysaccharides and proteins located at the top.[16] Moreover, *S. aureus* have 3-5 times greater pressure compared to *E. coli*, making it easier for lysis.[17]

The yield of *A. moluccana* water fraction was greater than the ethanol extract, hexane and ethyl acetate fractions. Water fraction can dissolve polar compounds in the extract. The result of phytochemical screening showed that the water fraction contained alkaloids, flavonoids, saponins, and tannins. These secondary metabolites were able to provide antibacterial activity by their respective mechanisms of action. The mechanism of action of alkaloids as an antibacterial agent is by disrupting the peptidoglycan component in bacterial cells, so that the cell wall layer is not fully formed, thereby causing bacterial death. Another reported mechanism of the alkaloids is that they act as DNA intercalators and inhibit bacterial cell topoisomerase enzymes.[18] In the case of the flavonoids, the antibacterial mechanism of action is facilitated by inhibiting nucleic acid synthesis, cell membrane function and energy metabolism.[19] Some secondary metabolites such as the saponins exert their antibacterial effect by causing protein and enzyme leakage from within cells. Saponins can reduce the surface tension of bacterial cell walls and reduce membrane permeability; this can occur because their surface-active substances are similar to detergents.[20] Furthermore, tannins are lipophilic compounds that easily bind to cell walls and cause cell wall damage. They exert their antibacterial effect by damaging microbial cell wall and forming bonds with the functional proteins of microbial cells.[21]

Table 4: Zone of Inhibition of Ethanol Extract and Fractions of *A. moluccana* leaves against *S. aureus*

Concentration (µg/mL)	Diameter of Inhibitory Zone (mm)			
	Ethanol extract	Hexane fraction	Ethyl acetate fraction	Water fraction
200	8.06 ± 0.06	7.41 ± 0.02	7.51 ± 0.01	7.82 ± 0.02
400	8.54 ± 0.03$^{(a)(b)(c)}$	8.21 ± 0.03	8.00 ± 0.00	8.81 ± 0.01
600	9.56 ± 0.05$^{(a)(d)(e)[1]}$	9.06 ± 0.06$^{(g)(h)[1]}$	8.56 ± 0.01	9.70 ± 0.01
800	9.69 ± 0.03$^{(b)(d)(f)[2]}$	10.04 ± 0.03$^{(g)(i)[2]}$	9.00 ± 0.01	11.00 ± 0.00
1000	10.05 ± 0.04$^{(c)(e)(f)[3][4]}$	10.53 ± 0.03$^{(h)(i)[3]}$	10.01 ± 0.01	11.35 ± 0.01$^{[4]}$
C+	30.1 ± 0.01			
C-	0.0 ± 0.00			

Data are presented as Mean ± SD. Values with different number superscript are significantly different between the same samples in different concentrations at P<0.05. Values with different alphabet superscript are significantly different between the same concentration in different samples. C+: positive control (Gentamicin 10 µg); C-: negative control (DMSO 10%).

Table 5: Zone of Inhibition of Ethanol Extract and Fractions of *A. moluccana* Leaves against *E. coli*

Concentration (µg/mL)	Diameter of Inhibitory Zone (mm)			
	Ethanol extract	Hexane fraction	Ethyl acetate fraction	Water fraction
200	ND	ND	7.11 ± 0.01	7.20 ± 0.00
400	7.42 ± 0.03$^{[1][2]}$	7.81 ± 0.01	7.50 ± 0.01	7.71 ± 0.01
600	8.42 ± 0.03$^{(a)[1][3]}$	8.58 ± 0.03$^{(a)[4][5]}$	7.71 ± 0.01	8.31 ± 0.01
800	8.62 ± 0.03	9.22 ± 0.03$^{(b)[4][6]}$	7.95 ± 0.01	8.51 ± 0.02$^{(b)}$
1000	9.27 ± 0.24$^{(c)[2][3]}$	10.01 ± 0.01$^{(c)[5][6]}$	8.55 ± 0.00	14.00 ± 0.00
C+	28.03 ± 0.02			
C-	0.00 ± 0.00			

Data are presented as Mean ± SD. Values with different number superscript are significantly different between the same samples in different concentrations at P<0.05. Values with different alphabet superscript are significantly different between the same concentration in different samples. C+: positive control (Gentamicin 10 µg); C-: negative control (DMSO 10%); ND: not detected.

Conclusion

The water fraction of *A. moluccana* leaf extract has antioxidant and antibacterial activities against *S. aureus* and *E. coli*, more than the crude ethanol extract, hexane and ethyl acetate fractions. Our findings suggest its potential use in the control of antibiotic resistant organisms.

Conflict of interest

The authors declare no conflict of interest.

Authors' Declaration

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

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