Isolation and Identification of Ethyl Acetate Extract Secondary Metabolite Compound of Kayu Jawa Bark (L. Coromandelica)

Sudding¹, P Salempa², Nurhikmah³
¹,²,³Chemistry Department of Mathematic and Science Faculty
E-mail: sudding.unm@gmail.com

Abstract. This study aims to isolate the secondary metabolite compounds contained in the ethyl acetate extract in bark of L.coromandelica. Isolation was carried out through several stages, ranging from maceration, fractionation, purity testing, and identification. The isolates obtained was white crystals in the form of needles with a melting point 133-134°C, with Lieberman-Buchard reagents gave positive results. The IR spectrum absorption area showed the existence of several functional groups at wavelength namely 3446.79 cm⁻¹ (O-H), 2935.66 cm⁻¹ (C-H); 2866.22 cm⁻¹ (C-H), 1463.97 cm⁻¹ (C=H), 1377.17 cm⁻¹ (C=H), 1643.35 cm⁻¹ (C=O). Based on the reagent test and IR spectrum data, the crystals obtained are a group of steroid compounds.

1. Introduction

Plants are one of the biodiversity in Indonesia. Plants contain bioactive compounds with certain activities in the form of secondary metabolites. Secondary metabolite compounds are metabolite compounds that are not essential for the growth of an organism. These secondary metabolites in plants play a very important role in protecting themselves from disturbance by other organisms. Examples of secondary metabolite compounds in plants are alkaloids, flavonoids, steroids, terpenoids, tannins and etc. The presence of secondary metabolite compounds found in plants has the potential to provide medicinal effects on the body.

One of the mostly used traditional medicinal plants is Java wood (L. coromandelica), which belongs to the Lannea genus of the Anacardiaceae family. The bark of L. Coromandelica can be used as an astringent, to treat stomach aches, leprosy, peptic ulcers, heart disease, dysentery, and canker sore. The bark of Java wood is used together with the bark of Aegle mermelo, Artocarpus heterophyllus and Sygygium cumini which is useful in treating impotence [1]. The way to use this plant varies depending on the purpose of its use, for example boiling it for the treatment of diarrhea or vomiting and using it directly from its plant parts to accelerate wound healing [2].

L. coromandelica is reported to contain secondary metabolite compounds such as alkaloids, steroids, triterpenoids, phenolics, flavonoids, tannins and saponins [3]. There were some previous researches regarding contained in L. coromandelica among them, [4] reported that the phytochemical filtering results of chloroform extract of the L. coromandelica wood contain chemical compounds of the alkaloid, tannin and steroid class. Ethanol extract and water-alcohol combination of L. coromandelica also reported the presence of dihydroflavonol compounds in the bark [5]. The results of the research on n-hexane extract of L. coromandelica obtained secondary metabolites compounds in the form of steroid class compounds [6].

Several studies on the efficacy include 96% ethanol extract of the L. Coromandelica has antibacterial activity against Staphylococcus aureus, Escherichia coli, Helicobacter pylori, and Pseudomonas aeruginosa [7], methanol extract and n-hexane extract of L. Coromandelica provides antimicrobial activity against the test microbes of E. coli, P. aeruginosa, and Salmonella thypi [8]. 96% ethanol extract

---

Content from this work may be used under the terms of the Creative Commons Attribution 3.0 licence. Any further distribution of this work must maintain attribution to the author(s) and the title of the work, journal citation and DOI.

Published under licence by IOP Publishing Ltd
of *L. coromandelica* is active as an antifungal against *Candida albicans* and *Trichophyton rubrum* [9]. The ethanol extract has anti-oxidant activity [10].

2. **Research methods**

2.1. **Tools**

The tools used in this research are glassware, analytical balance, maceration vessel, evaporator, chamber, Buchner funnel, vacuum liquid chromatography column, flash column, spray bottle, water bath, melting point measuring device, and Infra Red spectrophotometer.

2.2. **Materials**

The materials used are the bark of *L. coromandelica*, and chemicals, including ethyl acetate, n-hexane, chloroform, acetone, methanol, distilled water, silica gel 60, TLC plate (aluminum coated with silica gel G 60 F$_{254}$), aluminum foil, filter paper, and several reagents such as Liebermann-Buchard reagent, 1% FeCl$_{3}$, Dragendorff, and Wagner reagent.

2.3. **Procedures**

2.3.1. **Preparation and extraction of samples**

The outer rough bark of *L. Coromandelica* was cleaned, then cut into small pieces, then smoothed and then dried in the open air. Drying was carried out until the sample was completely dry and changed color from brownish red to brown. A total of 4.2 kg of sample was then macerated with 20 liters of ethyl acetate for 3x24 hours so that the secondary metabolites compound contained in *L. Coromandelica* could be drawn completely. The maserate was then filtered with a soft cloth and then using Whatman filter paper so that the filtrate is completely pure without residue. The filtrate obtained was yellow. The maserate obtained was then evaporated to evaporate the solvent using a rotary evaporator vacuum at 40°C. The evaporation result was obtained 32 grams of brown viscous extract. The viscous extract was then left at room temperature to evaporate all remaining solvent to obtain a dry extract that is shaped like paste.

2.3.2. **Fractionation**

Before fractionation, the ethyl acetate extract was analyzed by thin layer chromatography (TLC) using several eluents in various comparisons to determine the type of solvent and the appropriate ratio used in vacuum liquid column chromatography (VLCC). The ethyl acetate extract in the form of paste is firstly impregnated with silica gel G 60 until it is evenly mixed. Then, slowly put silica gel 60 GF$_{254}$ into the column which is the stationary phase until it is tight and dense. After it was tight, Whatman filter paper and impregnated samples were inserted. Furthermore, it was eluted using a solvent which increases its polarity from nonpolar to polar in a gradient.

Elution was started using 100% n-hexane and then increased gradually to 100% ethyl acetate, followed by 100% acetone and 100% methanol. The fractionation results were then TLC with the appropriate eluent.

Fractions having the same stain profile were combined and evaporated at room temperature. The results are then considered for which combined fractions are possible to be forwarded to the column. And so on until a possibly pure compound is obtained, which shows a single spot on the TLC results.

2.3.3. **Purity test**

a. Observation of crystal forms

The purity of crystals obtained from recrystallization is determined by observing the crystal shape under a microscope, pure compounds show uniform crystalline forms, while mixed compounds have various crystal forms.

b. Melting point determination

The melting point range of pure compound crystals is narrower than compound crystals which is not yet pure.
c. Spectroscopy infra-red absorption measurement
Identification of isolates using the FTIR Prestige-21 Shimadzu spectro-photometer to determine the functional groups contained in these compounds.

d. Group test
Isolates were tested using 1% FeCl₃ reagent, Dragendorff, Wagner, Mayer, and Liebermann-Buchard reagents to determine the class of the obtained secondary metabolite compounds.

3. Findings and discussion
3.1. Preliminary test
The ethyl acetate viscous extract which was obtained was subjected to phytochemical testing to identify the secondary metabolite compounds contained in the viscous extract. Iron (III) chloride (FeCl₃), Dragendorff, Wagner and Liebermann-Buchard reagent data are shown in Table 1 and Figure 1.

![Figure 1. The test results of the secondary metabolite compound](image)

**Table 1. Preliminary Test Results of Bark *L. coromandelica* Ethyl Acetate Extract.**

| No | Reagent         | Result   | Information  |
|----|-----------------|----------|-------------|
| 1  | Libermann Burchart | Green    | + Steroid   |
| 2  | FeCl₃ 1%        | Yellow   | - Flavanoid |
| 3  | Dragendorf      | Orange   | + Alcaloid  |
| 4  | Wagner          | Brown sediment | + Alcaloid |

3.2. Fractionation and purification
A total of 8.07 grams of ethyl acetate viscous extract was fractionated by using vacuum liquid column chromatography (VLCC). The TLC results obtained that the eluent n-hexane: ethyl acetate with a ratio (1:9) showed a good stain separation pattern as seen in Figure 2.

![Figure 2. Ethyl-acetate extract chromatogram before VLCC](image)

**Table 2. Fractionation Results Using Vacuum Liquid Column Chromatography (VLCC).**

| Fraction | Eluent                   | Ratio    |
|----------|--------------------------|----------|
| 1        | n-hexane                 | 100%     |
| 2        | n-hexane: ethyl acetate  | 7.5:2.5  |
| 3-5      | n-hexane: ethyl acetate  | 5:5      |
| 6-8      | n-hexane: ethyl acetate  | 2.5:7.5  |
| 9-11     | ethyl acetate            | 100%     |
| 12       | Ethyl acetate: acetone   | 7.5:2.5  |
| 13-14    | Ethyl acetate: acetone   | 5:5      |
Based on the TLC results from 18 fractions. The fractions having the same stain profile are combined as shown in Figure 3. The fractions that have the same stain profile are combined to obtain 7 combined fractions as seen in Table 3.

**Figure 3.** The chromatogram of the VLCC results in 18 fractions

| Fractions | Combined fractions | Mass (g) |
|-----------|-------------------|----------|
| 1-2       | A                 | 0.0883   |
| 3-5       | B                 | 2.8966   |
| 6         | C                 | 0.0237   |
| 7-8       | D                 | 0.9015   |
| 9-10      | E                 | 0.9473   |
| 11-14     | F                 | 0.0431   |
| 15-18     | G                 | 0.0198   |

Based on the combined fraction, the VLCC results obtained were then selected for further fractionation. The eluent used was n-hexane:ethyl acetate with several comparisons/ratio. The results obtained that were the ratio of n-hexane:ethyl acetate with the ratio(7:3) with which the best stain separation.

**Figure 4.** Fraction chromatogram of eluen n-hexane:ethyl acetate (7:3)

Fraction B was then eluted several times with Eluen n-hexane : ethyl acetate (7:3) to produce 86 fractions (Table 4). Each fraction of TLC for merging as shown in Table 4.

**Table 4.** Merger of KKT result factions.

| Fractions | Combined fractions | Mass (g) |
|-----------|--------------------|----------|
| B1        | 7-16               | 0.4103   |
| B2        | 17-25              | 0.2308   |
| B3        | 26-43              | 0.1721   |
| B4        | 72-74              | 0.0583   |
Figure 5. TLC results Chromatogram of 86 KKT fractions (fraction B)
In fractions 7-16, yellowish white crystals were formed and needle-shaped. However, the results obtained in fraction B1 were not pure yet, marked by several stains.

Figure 6. Combined Fractions Chromatogram B (a. UV irradiated, b without UV irradiation)

The fraction B1 was further chromatographed column on the pressed to be obtained. The results of the pressed column chromatography (PCC) showed that 39 fractions was obtained and then combined into 3 fractions, namely B1a, B1b, B1c. In B1b, white, needle-shaped crystals were formed.

Figure 7. TLC results chromatogram from Fraction B

Table 5. Results of KKF Fraction B1.

| Fractions | Combined fractions | Mass (g)  |
|-----------|--------------------|----------|
| B1 a      | 1-7                | 0.0054   |
| B1 b      | 8-19               | 0.1392   |
| B1 c      | 20-39              | 0.0084   |

Figure 8. Chromatogram of Combined Fraction B1 (a), Crystal form of the B1b (b)
3.3. Identification
The B1\textsubscript{b} fraction was then tested for purity using the three eluent system method and melting point test. In the three eluents system method, the purity of the isolates was indicated by the appearance of a single stain on each TLC plate. The three types of eluents used were n-hexane:chloroform with a ratio 6:4 (a), n-hexane:ethyl acetate with a ratio 7:3 (b) and ethyl acetate:chloroform with ratio 6:4 (c) and the results in the form of a single stain were obtained. As in Figure 9.

![Figure 9. The 3 eluent system TLC chromatogram](image)

Melting point test using Melting Point SMP 11. Shows that the isolates obtained are pure, characterized by having a melting point route of not more than 2°C [11]. The melting point test conducted showed that the isolates began to melt began to melt at 133°C and melted altogether at 134°C.

The pure isolates obtained were further identified by group test and IR spectroscopy test. The group test was carried out using LB, 1% FeCl\textsubscript{3}, and Wagner reagents. The results of the group test can be seen in Table 6 and Figure 10.

**Table 6. Pure isolate group test results.**

| No | Reagent      | Results | Information         |
|----|--------------|---------|---------------------|
| 1  | Lieberman-Burchard | Green   | + Steroids          |
| 2  | FeCl\textsubscript{3} | Yellow  | - Flavonoids        |
| 3  | Dragendroff  | Brown   | - Alkaloids         |
| 4  | Wagner       | Orange  | - Alkaloids         |

![Figure 10. Pure isolate group test results](image)

Further identification was carried out using a Shimadzu Prestige-21 IR spectrophotometer to determine the functional groups of the pure isolates obtained. FTIR spectrum analysis of the B1b fraction provided absorption in the area 3446.79 cm\textsuperscript{-1} wave number which was characterized by a sharp band with a strong intensity identified as the –OH group (3000 cm\textsuperscript{-1}-3750 cm\textsuperscript{-1}) [12] which was supported by the presence of absorption with moderate intensity in the area 1047.35 cm\textsuperscript{-1} wave number which indicated the presence of a C-O group (1000 cm\textsuperscript{-1}-1300 cm\textsuperscript{-1}) [13]. The sharp absorption in the wave numbers 2935.66 cm\textsuperscript{-1} and 2866.22 was identified as the C-H stretching vibration CH\textsubscript{2} and CH\textsubscript{3} aliphatic groups (2850 cm\textsuperscript{-1}-3000 cm\textsuperscript{-1}) [14] which was supported by absorption with moderate intensity at wave number 1463.97 cm\textsuperscript{-1} as CH\textsubscript{2} and absorption at wave number 1377.17 cm\textsuperscript{-1} as CH\textsubscript{3} which is thought to be an indication of the existence of dimethyl geminal group -CH(CH\textsubscript{3})\textsubscript{2} which is a characteristic of steroid compounds [6]. Then in the
absorption area of 1643.35 cm\textsuperscript{-1} with a weak intensity indicated the existence of C=C group (1600 cm\textsuperscript{-1} - 1680 cm\textsuperscript{-1}) [14]. Based on the FTIR result data, it shows that the B1\textsubscript{b} fraction is a steroid compound. For more details, see Table 7 and Figure 11.

Table 7. IR absorption of pure isolates from ethyl acetate extract of Kayu Jawa bark with its functional groups possibility.

| No | Wave Numbers (cm\textsuperscript{-1}) | Functional Groups | Intensity |
|----|--------------------------------------|-------------------|-----------|
| 1  | 3446.79                              | -OH               | Strong    |
| 2  | 2935.66; 2866.22                      | C-H               | Strong    |
| 3  | 1463.97                              | CH\textsubscript{2} | Moderate  |
| 4  | 1377.17                              | CH\textsubscript{3} | Moderate  |
| 5  | 1643.35                              | C=C               | Weak      |
| 6  | 1047.35                              | C-O               | Moderate  |

Figure 11. Infrared Spectrum of pure isolates

4. Conclusion

Based on the results obtained from the study, it can be concluded that the secondary metabolite compounds isolated from the ethyl acetate extract of bark \textit{L. coromandelica} are steroid group compounds in the form of white needles.

References

[1] Wahid, Arif. 2009. In Vitro Phytochemical and Biological Investigation of Plant Lannea coromandelica (Family: Anacardiaceae). Thesis to Department of Pharmacy, East West University, Bangladesh.

[2] Rahayu, Mulyati., Suhardjo, Prawiroatmodjo. 2006. Keanekaragaman Tanaman Pekarangan Dan Pemanfaatannya Di Desa Lampeapi, Pulau Wawoni Sulawesi Tenggara. \textit{Jurnal Teknik Lingkungan. Vol 6 (2): 360 - 364}.

[3] Kumar, Tekeshwar., Vishal, Jain. 2015. Appraisal Of Total Phenol, Flavonoid Contents, And Antioxidant Potential Of Folkloric \textit{L. coromandelica} using In Vitro And In Viv.\textit{Medan:Universitas Sumatera Utara.}

[4] Joseph, 2013. An Investigation of The Phytochemistry and In Vitro Cytotoxic Effect of The Aqueous Extract of Lannea Coromandelica Bark. \textit{An International Journal Of Pharmaceutical Sciences Vol.4, Issue 4, Supl 1. ISSN : 0976- 7908}. 7
[5] Rao, V.S., Eintein, J.W., Das, K. 2014. Hepatoprotective And Antioxidant Activity Of Lannea coromandelica Linn. On Thiaoacetamide Induced Hepatotoxicity In Rats. *International Letters Of Natural Science J. Vol.3 : 30-43. ISSN : 2300-9675.*

[6] Paramudita, Eka. 2017. Isolasi Dan Identifikasi Senyawa Metabolit Sekunder Ekstrak N-Heksana Kulit Batang Kayu Jawa Lannea Coromandelica (Houtt) Merr. *Jurnal Kimia Vol 18. No.1.*

[7] Rahmadani, Fitri. 2015. Uji Aktivitas Antibakteri dari Ekstrak Etanol 96% Kulit Batang Kayu Jawa (Lannea Coromandelica) terhadap Bakteri Staphylococcus Aureus, Escherichia Coli, Helicobacter Pylori, Pseudomonas Aeruginosa. (Skripsi), Jakarta: UIN syarif Hidayatullah.

[8] Ismail, 2016. Uji Ekstrak metanol dan ekstrak n-heksan kortex Kayu Jawa (L.coromandelica (Houttt.) Merr.) terhadap mikroba uji Escherichia coli, Pseudomonas aeruginosa, dan Salmonella thypi. Jakarta : UIN Syarif Hidayatullah.

[9] Mozer, Hardi. 2015. Uji Aktivitas Antifungi Ekstrak Etanol 96% Kulit Batang Kayu Jawa (Lannea coromandelica) terhadap Candida albicans dan Trichophyton rubrum. Skripsi. Jakarta: UIN Syarif Hidayatullah.

[10] Watri, Ratnasari. 2014. Formulasi dan Uji Efektivitas Sediaan Krim Ekstrak Etanol Korteks Kayu Jawa. Makassar: UIN Alauddin.

[11] Mutiah. Noormand Suriah. 2011. Karakteristik Kekuatan dan Derajat Kristalinitas Polipropilena Teriradiasi. *Jurnal Sains dan Teknologi Nuklir Indonesia. Vol 2 (1).*

[12] Fessenden, R. J dan Joan, S. F. 1982. *Kimia Organik.* Jakarta: Erlangga.

[13] Sitorus, Marham. 2009. *Spektroskopi Eludasi Struktur Molekul Organik.* Yogyakarta: Graha Ilmu

[14] Sastrohamidjojo, H. 2013. *Dasar-Dasar Sperktroskopi.* Yogyakarta : UGM.