Antibacterial activity of isolated compounds from the stem bark of *Calophyllum euryphyllum*

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**Abstract.** *Calophyllum euryphyllum* Lauterb (*Clusiacea* family) has the potential as material for medical industries. To continue our drug discovery of the Indonesian plant, we chose *C. euryphyllum* species for antibacterial sources. Semi polar extract from *C. euryphyllum* was prepared and purified by column chromatographic methods and then identified by spectroscopic methods (LC-MS-MS, IR, UV, and ¹H-NMR and ¹³C-NMR). The pure isolated compound was subjected to antibacterial activities by using *E. coli, S.aureus*, and *B. subtilis*. Semi polar fraction of *C. euryphyllum* showed the antibacterial activity against *E. coli, S.aureus* and *B. subtilis* with MIC about 2.05%, 2.82%, 1.54% for *E. coli, S.aureus* and *B. subtilis*, respectively. One compound was isolated from the semi-polar fraction of the stem bark *C. euryphyllum*. It was identified as a triterpene (with molecular weight 438), a colorless crystal with a melting point was 133-134 °C, and the molecular formula was C₃₀H₄₆O₂.

1. Introduction

Indonesia is the second megadiverse country after Brazil. Forests in Indonesia covers about 88,495,000 ha and have rich biodiversity, particularly in the lowland forests. Indonesia has about 1.75 million different species that include 270,000 species of plant with a high endemic. Biodiversity is especially important to the medical and pharmaceutical industries [1, 2].

Some researchers have discovered many chemicals in rain forest plants that are now used in helpful drugs. One of the most popular drugs for anti-HIV-1, calanolide, was initially made from the bark Calophyllum trees. Calanolide A was isolated from dried fruits and twigs of *Calophyllum lanigerum* var Austrocoriaceum [3]. Medicines that treat some forms of cancer have been made from the rosy periwinkle, a flower that grows on the African Island of Madagascar [2], and also from *Calophyllum teysmannii*. The new chromanone acid from the stem bark of *Calophyllum teysmannii* has also been isolated [4].

The genus Calophyllum consists of 180-200 species. The plant grows in the tropical and humic forest [4]. Clusiaceae/Guttiferae family consists of 14 Genera (e.i Genera *Calophyllum, Caraipa, Clusiella, Endodesmia, Haplocathra, Kayea, Kielmeyera, Lebrunia, Mahurea, Mammea, Marila, Mesua, Neotatea, and genera Poeciloneuron*) [5]

Some species grow in Brazil, South, and Central America, for example. *C. brasiilense*. The bark of *C. brasiilense* Camb is used as anti-inflammation and for the treatment of rheumatism, varicose veins, hemorrhoids, ulcer gastric, and hepatic disturbances [6]. Coumarin from *C. brasiilense* showed in vitro activity against amastigote forms of *Trypanosome cruzi* and *Leishmania infantum* [7].
The extracts and pure isolated actified principles from *C. inophyllum* were evaluated against *S. aureus*, *V. angillarhum*, *E. coli*, and showed the best antibacterial activity [8]. Reyes Chilpa was succeeded isolating coumarin mamea types of A/BA, A/BB, C/OA, and C/OB from *C. Brasiliense*. Those types of mamea reduced the growth of *S. aureus*, *B. subtilis*, and *S. epidermidis*, which were smaller than those induced by the antibiotic chloramphenicol [9].

The polyisoprenylated ketone from *C enervosum* has also succeeded in being isolated. The compounds were evaluated against *B. subtilis*, *E. coli*, (gram-positive), and *P. aeruginosa* and *S. aureus* (gram-negative). One isolated compound was found to have the strongest activity against four test bacteria. However, it is much less active than streptomycin sulfate (positive control with MIC 0.39 x 10⁻⁴ µg/mL) [10].

In this research, bark of *C. euryphyllum* from Palangkaraya, Centre of Kalimantan Island, Indonesia was used. Calophyllum contains many compounds such us xanthone, coumarin, biflavonoid, benzophenone and neoflavonoid, triterpene, and steroid. These compounds have anti-inflammation, antifungal, antihyperglycemic, antiplatelet, antitumor, antibacterial, and antimalarial [11-14]. In this study, we used *C. euryphyllum* species as a source of antibacterial medicine candidate because nobody investigated antibacterial activities from *C. aerophyllum*.

*C. euryphyllum* produces a colorless, white, or yellow latex. The oppositely arranged leaves have leathery blades, often born on petioles. The leaves are distinctive, with narrow parallel veins alternating with resin canals. The inflorescence is a cyme or a thyrse of flowers that grows from the leaf axils or at the ends of branches. In flower, the sepals and petals may look similar, are arranged in whorls, and have many stamens. The fruit is a drupe with thin layers of flesh over a large seed [15].

Many species of Calophyllum are used for their wood. Some are hardwood trees that can reach 30 meters in height. *C. tomentosum*, *C. elatum* is a tall and big tree grown in the evergreen forest. Calophyllum tends to grow rapidly. The outer sapwood is yellowish, yellow-brown, or orange, sometimes with pink, and the inner heartwood is light reddish to red-brown. The wood has a streaked, ribbed, or zig-zag grain. The wood has been used to build boats, flooring, and furniture, and also made into plywood. Calophyllum wood may be sold under the name *bitangor*. A lot of work has been carried out on this species for its terpenoid and coumarin, flavonoids. This study aims to isolate and characterize the isolated compound from the stem bark of *C. euryphyllum*.

2. Materials and methods

2.1. Plant material
The stem bark of *C. euryphyllum* was collected from Bulungan Research Forest Palangkaraya, Centre of Kalimantan Islands, Indonesia. The sample was determined in Herbarium Research Centre for Biology-LIPI. A voucher specimen was deposited in the Herbarium.

2.2. Isolation
The plant material (2.8 kg) was extracted three times with the technique of maceration using nonpolar, semipolar, and polar solvent. All extracts were evaporated under vacuum to give n-hexane extract (72.9 g as nonpolar extract), 150.9 g as semipolar extract, and 75.9 g as polar extract). Semi polar extract was fractionated to flash column chromatography (silica gel 230-300 mesh 500 g) by using hexane to hexane/ErOAc with 10% increasing polarity to yield some fractions. According to TLC analysis (using TLC plates silica gel 60 F254 thin 0.25 mm, 20x20 cm), the same result was collected to give 16 subfractions. Sub fraction 2 (40 gr) was further separated by CC (column chromatography) packed with Si Gel 60 GF (70-230 mesh) by using hexane: EtOAc increasing polarity to give six subfractions. Sub fraction 2 was subjected to CC using an isothermal eluent system (n-hexane: EtOAc 7:3). The compound was visualized by spraying with 5% v/v H₃SO₄ in methanol and then heated on a plate. The same TLC spot was combined to give six fractions (fraction A-F). Fraction B (1.15 g) was further separated on a column packed with Sephadex LH-20 using CH₂Cl₂/MeOH (2: 1) as a solvent to
give one compound (20 mg). Then, one compound was purified by crystallization methods by using two solvent systems to afford pure compound.

2.3. Antibacterial bioassay test
The agar well diffusion method was used for antibacterial activities. N-hexane and EtOAc extracts of *C. eurypyllum* were prepared and tested for the antibacterial activities. In brief, the bacteria were grown on a Muller-Hinton agar medium (pH 7.3). The agar medium 20 ml was poured into plates to a uniform depth of 5 mm and allowed to solidify. The microbial (*E. coli. S.aureus, B.subtilis, and P. aeruginosa*) suspensions at 5 x 10^6 CFU/mL were streaked over the surface of the media using a sterile cotton swab to ensure the confluent growth of the organism. The wells (6 mm in diameter) were cut from the agar, and 100 µL of the extracts samples were delivered into them. The plates were incubated at 37°C for 24 hours, and the observed growth inhibition zones were measured.

2.4. Elucidation analysis
Elucidation analysis was done by using LC-MS/MS, FTIR, UV, and NMR. LC-MS/MS system was made up of a combination of chromatographic separation Waters HPLC and mass analyzer type G2-XS QTOF LC/MS Waters Technologies equipped with electrospray ionization (ESI) source system, index scan XEVO, consisting of a binary pump and vacuum degasser. The chromatographic separation was done on Waters Rapid Resolution High Definition (RRHD) column C18, 2.1 mm id x 150 mm length (particle size 1.8 µm). The mobile phase system used solvent A and solvent B, which consisted of 1% formic acid in water and 1% formic acid in acetonitrile, respectively. The combination of both solvents in the LC system was set at a ratio of solvent A: solvent B, 100:0 with gradient elutions as presented in Table 1.

| Time (min) | Flow Rate (mL/min) | Composition A (%) | Composition B (%) | Curve |
|-----------|--------------------|-------------------|-------------------|-------|
| 0.00      | 0.300              | 95.0              | 5.0               | Initial |
| 1.00      | 0.300              | 95.0              | 5.0               | 6     |
| 8.00      | 0.300              | 60.0              | 40.0              | 6     |

Xevo G2-XS QTOF experiment: Function 1- MS^E^, Ionization type: ESI, polarity +, acquisition start time 0 min, acquisition end time 16.00 min, tart mass 50.00 m/z, end mass 120.00 m/z. The sample was diluted in methanol. The Spectrum of sample using LC-MS/MS analysis.

FTIR spectra were recorded on FTIR Prestige 21 Shimadzu spectrophotometer, √ in cm⁻¹. The FTIR absorbance spectrum of the sample at wavelengths was between 4000 and 950 cm⁻¹. As can be seen in Figure 1, FTIR spectra showed important peaks explaining the stretching, bending, double-bond of the isolated compound.

UV spectra were detected on Hitachi U2000 spectrophotometers. The UV –Vis absorption maxima at 294.0 nm and 270 nm suggested the presence of a double bond in triterpene (Figure 2).

The NMR spectra were recorded in acetone solution on a JEOL JNM 500 MHz instrument and reported in δ ppm (chemical shift) values relative to TMS as an internal standard.

¹H-NMR (500 MHz) and ¹³C-NMR (125 MHz) spectra were recorded on a Jeol spectrophotometer using acetone-d6 as solvent and TMS internal standard. The NMR experiments were conducted using the standard Jeol software for COSY and DEPT. High-resolution mass spectra were measured on a Jeol ECA. With chemical shifts (δ) are given in (ppm), and J coupling constants in H [16]. Another chemical (e.i., Si gel 60, Merck) was used for column chromatography, and precoated silica gel plates (Merck, Kieselgel 60 F-254, 0.25 mm) were used for TLC/analysis spot compound, and 1 mm for PTLC. They were visualized by spraying with 5% v/v H₂SO₄ in methanol and then heated on a plate. Electrothermal fisher (scientific serial 9030056) was also used to determine the melting point of the isolated compound.
3. Results and discussion

3.1. Phytochemical result of C. aeruphyllum

The phytochemical result is shown in Table 2. The semipolar and polar fractions contained saponin, tannin, flavonoid, alkaloid, and terpenoid. Unfortunately, the nonpolar fraction did not contain saponin. The nonpolar fraction contained the highest terpenoid (+++) compared to a semipolar fraction (+). However, the polar fraction did not contain terpenoid (-). Highest concentration of tannin was in polar fraction (+++) compared to semipolar (++) and nonpolar fraction (+).

| Fraction  | Saponin | Tannin | Flavonoid | Alkaloid | Terpenoid |
|-----------|---------|--------|-----------|----------|-----------|
| Nonpolar  | -       | +      | ++        | +        | +++       |
| Semipolar | +++     | ++     | ++        | +        | +         |
| Polar     | ++      | +++    | +++       | +        | -         |

The semipolar fraction (EtOAc fraction) from stem barks of C. aeruphyllum contained terpenoid. In this research, we succeeded isolating the terpenoid compound (F4). Terpenoid showed antibacterial activity against *E.coli*, *B. subtilis* and *S. aureus* (9 mm, 8 mm and 9 mm of inhibition to *E.coli*, *B. subtilis* and *S. aureus* respectively) at 4% concentration of terpenoid compound (Table 3). This
research proved that semipolar fraction (EtOAc fraction) of *C. aerophyllum* had an ability as an antibacterial drug.

### Table 3. Antibacterial bioassay data.

| Concentration (%) | E. coli | B. subtilis | S. aureus |
|-------------------|---------|-------------|-----------|
| 0                 | 6       | 6           | 6         |
| 1                 | 7       | 6           | 8         |
| 2                 | 7       | 7           | 8         |
| 3                 | 8       | 7.5         | 8.5       |
| 4                 | 9       | 8           | 9         |

### 3.2. Isolation

Triterpene was isolated from the bark of *C. euryphyllum Lauterb* via two times Column chromatography (CC) (silica gel 230-300 mesh 500 g) by using hexane/EtOAc with 10% increasing polarity to yield some fractions. Sub fraction 2 were separated one time with CC using an isothermal eluent system (*n*-hexane: EtOAc 7:3). Subfraction B (1.15 g) was then separated on a column packed with Sephadex LH-20 using CH$_2$Cl$_2$/MeOH (2:1) to yield one compound (20 mg), then one compound was purified by crystallized methods using two solvent systems to afford white crystal, melting at 263-264°C. Triterpene compound was indicated by the positive 5% v/v H$_2$SO$_4$ test.

### Table 4. Minimum inhibition concentration (MIC) result of the isolated compound.

| N0 | Bacterial   | MIC    |
|----|-------------|--------|
| 1  | *E. coli*   | 2.05%  |
| 2  | *B. subtilis* | 2.82%  |
| 3  | *S. aureus* | 1.54%  |

### Figure 3. Antibacterial activity of isolate compound from *C.euryphyllum*.

### 3.3. Bioassay results

One triterpene isolated from the bark of *C. euryphyllum Lauterb* had been tested on *E.coli, B. subtilis, S. aureus*. The obtained result demonstrated that triterpene showed antibacterial activities. The result of the test and MIC are presented in Figure 3 and Table 4.

The semipolar fraction (EtOAc fraction) from stem barks of *C. aerophyllum* contained terpenoid. Terpenoid from stem barks of *C. aerophyllum* showed antibacterial activity against *E.coli, B subtilis* and *S. aureus* (9 mm, 8 mm and 9 mm of inhibition to *E.coli, B subtilis* and *S. aureus* respectively) at 4% concentration of terpenoid compound (Table 3). The antibacterial bioassay result for isolated compounds is shown in Figure 3.
3.4. Identification results of the active compound with LC-MS/MS, FT-IR, UV-Vis, and NMR

3.4.1. LC-MS/MS. The LC-MS-MS analysis showed a \([\text{M+H}]^+\) ion peak at \(m/z \ 439.35624\) and a \([\text{M-COOH}]^+\) ion peak at \(m/z \ 394.35624\). It indicated \([2\text{M}]^+\) ion peak at \(m/z \ 876.63964\). According to LC-MS-MS data, the ion peak at \(m/z \ 439.35624\) corresponds to the formula of \(C_{30}H_{46}O_2\). Isolated compounds have a molecule weight of 438.35624 (calcld 438.668), as can be seen in Figure 4. The isolated compound (C.Aep) was a white crystal.

![Figure 4. LC-MS spectrum of isolated compound (CAep) from C.aerophyllum.](image)

3.4.2. FT-IR. FTIR spectra were recorded on FTIR Prestige 21 Shimadzu spectrophotometer, \(\sqrt{\text{cm}}\) in \(\text{cm}^{-1}\). FTIR spectra showed important peaks explaining the stretching, bending, double-bond of the isolated compound. The spectrum showed an adsorption band at 3468.01 and 1685.79 \(\text{cm}^{-1}\) ascribable to hydroxyl (OH-carboxylic acid) and carbonyl functions (C=O of carboxylic acid), respectively. The C-H stretching of absorption for CH\(_3\) and CH\(_2\) occurred at wavelengths of 2939.52 and 2868.15, respectively. The C-H bending of absorption for CH\(_3\) was at 1453.33, and CH\(_2\) was at 1379.10. The ester functional group was observed in the isolated compound at 1658.79 \(\text{cm}^{-1}\) (C=O) and 1193.94 \(\text{cm}^{-1}\) (C-O). FTIR spectrum showed that isolated compounds contained a COOH group (Figure 1). FTIR data conformed the LC-MS-MS data.

3.4.3. UV-Vis. The UV – Vis absorption maxima at 294.0 nm and 270 nm suggested the presence of a double bond in triterpene moiety (Figure 2).

3.4.4. NMR. The \(^1\)H-NMR spectra were recorded on a JEOL JNM 500 MHz spectrometer, operated at 500 MHz (\(^1\)H), and 150 MHz (\(^13\)C) in an acetone-d6 solvent, and reported in \(\delta\) ppm (chemical shift) values relative to TMS as an internal standard. NMR data showed the presence of six methyl proton at 0.75 (3H, s); 0.85 (3H, s); 0.96 (6H, s); 1.05 (3H, s) and 1.70 (3H, s). Two olefinic protons appeared downfield at \(\delta\) 4.72 and \(\delta\) 4.59 ppm, which were identical with the chemical shift of H-2 and H-3, respectively, from the isolated compound. There are eleven methilen (CH\(_2\)), as shown in Figure 5 (methyl in the isolated compound). The \(^1\)HNMR spectrum was recorded as a carboxylic group (\(\delta_c\) 183.3, C-30). Olefinic proton H-2 to C-2 (\(\delta_c\) 126.0 and H-3 to C-3 (\(\delta_c\) 127.4) suggested double bond in C2=C-3 in the terpenoid compound (Figure 5 and 6).
Terpenoid as the isolated compound presence of carboxylic acid, saturated ketone, and hydroxyl groups was seen in the FTIR spectrum (3465, 1685, and 1641). The isolated compound was analyzed for C\textsubscript{30}H\textsubscript{46}O\textsubscript{2} with a molecular weight of 438.35624 (LC-MS/MS), and the melting point was 263-264°C. The \textsuperscript{1}H-NMR and \textsuperscript{13}C-NMR data of terpenoid are summarized in Table 5 and Figure 7.

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**Figure 5.** \textsuperscript{1}H-NMR spectrum of isolated compound.

**Figure 6.** Chemical shift (\textsuperscript{13}C-NMR) of isolated compounds.

**Figure 7.** Structure of isolated compound.
Table 5. $^1$H-NMR and $^{13}$C-NMR of the isolated compound.

| Carbon No | $\partial$C (ppm) | $\partial$H (ppm) |
|-----------|------------------|------------------|
| 1         | 27.4 (t)         | 3.13-3.02        |
| 2         | 126.0 (d)        | 4.73 (1H d J 8 9 Hz) |
| 3         | 127.4 (d)        | 4.59 (1H d J 8 9 Hz) |
| 4         | 145.6 (s)        | -                |
| 5         | 53.2 (d)         | 1.48-1.34        |
| 6         | 33.2 (t)         | 1.48-1.34        |
| 7         | 31.3 (t)         | 1.92             |
| 8         | 50.4 (d)         | -                |
| 9         | 38.4 (s)         | 2.35             |
| 10        | 56.4 (d)         | 1.14-1.28        |
| 11        | 35.2(t)          | 1.14-1.28        |
| 12        | 30.5 (t)         | -                |
| 13        | 41.0 (s)         | -                |
| 14        | 41.9 (s)         | 1.48-1.34        |
| 15        | 30.0 (t)         | 1.48-1.34        |
| 16        | 36.8 (t)         | -                |
| 17        | 29.7 (s)         | 2.26             |
| 18        | 44.8 (d)         | 1.67-1.61        |
| 19        | 30.4 (t)         | -                |
| 20        | 40.5 (s)         | 1.58-1.50        |
| 21        | 29.2 (t)         | 1.58-1.50        |
| 22        | 35.7 (t)         | 4.59-4.71        |
| 23        | 110.6 (t)        | -                |
| 24        | 20.0 (q)         | -                |
| 25        | 16.2 (q)         | -                |
| 26        | 18.8 (q)         | -                |
| 27        | 18.8 (q)         | -                |
| 28        | 32.4 (q)         | -                |
| 29        | 31.2 (q)         | -                |
| 30        | 183.3 (s)        | -                |

4. Conclusion
C. euryphyllum bark is promising to be used as new pharmaceuticals with antibacterial activities. Treatment with C. euryphyllum has been reduced to inhibit the growth of the bacterial pathogen. Isolated compounds have antibacterial activity with MIC 2.05%, 2.82% and 1.54% for E.coli, B. subtilis and S aureus, respectively. Its characterization was a triterpene, as a colorless crystal with a melting point was at 133-134°C, and the molecular formula was C$_{36}$H$_{46}$O$_{2}$.

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