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Antibacterial Compound from n-Hexane Fraction of *Dillenia ochreata* Leaves

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Abstract

Semprawang (*Dillenia ochreata*) belongs to the Dilleniaceae family that has been used by the Musi tribe, Banyuasin, South Sumatra, for scurvy medicine. This study aims to isolate secondary metabolites from *D. ochreata* leaves *n*-hexane extract and test their antibacterial activity against *Escherichia coli* and *Staphylococcus aureus*. The *D. ochreata* leaves were extracted through the maceration method with *n*-hexane solvent, and the isolated compounds were purified using column chromatography. The isolated compounds were analyzed using FT-IR, ^{1}H-NMR, and ^{13}C-NMR spectroscopy and compared the spectroscopic data with the literature. The antibacterial activity was determined against the *E. coli* and *S. aureus* bacteria with the disc diffusion method and MIC value was determined by the microdilution method. Based on the analysis of the spectroscopic data and compared with literature data, it is suggested that the isolated compounds are 3β-glucopyranosyl-lup-20(29)-en-28-oic, which mixes with aromatic compound. The isolated compounds showed antibacterial activity with a minimum inhibitory concentration (MIC) to *E. coli* at 120 µg/mL and *S. aureus* at 60 µg/mL.

Keywords: Antibacterial, Dillenia ochreata, 3β-glucopyranosyl-lup-20 (29) -en-28-oic

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Semprawang (*Dillenia ochreata*) termasuk dalam famili Dilleniaceae yang telah dimanfaatkan oleh masyarakat suku Musi Banyuasin, Sumatera Selatan untuk obat kudis. Penelitian ini bertujuan untuk mengisolasi metabolit sekunder dari ekstrak *n*-heksana daun *D. ochreata* dan menguji aktivitas antibakterinya terhadap *Escherichia coli* dan *Staphylococcus aureus*. Daun *D. ochreata* diekstraksi dengan metode maserasi menggunakan pelarut *n*-heksana dan pemurnian dilakukan dengan metode kromatografi kolom. Senyawa hasil isolasi dianalisis menggunakan spektroskopi FT-IR, ^{1}H-NMR, dan ^{13}C-NMR, dan membandingkan data spektroskopi dengan literatur. Uji aktivitas antibakteri dilakukan dengan menggunakan bakteri uji *E. coli* dan *S. aureus* dengan metode difusi cakram dan penentuan nilai KHM dengan metode dilusi. Berdasarkan analisis data spektroskopi dan dibandingkan dengan data literatur, senyawa hasil isolasi adalah 3β-glucopyranosyl-lup-20(29)-en-28-oic yang tercampur dengan senyawa aromatik. Senyawa hasil isolasi menunjukkan aktivitas antibakteri dengan konsentrasi hambat minimum (KHM) 120 µg/mL terhadap *E. coli* dan 60 µg/mL terhadap *S. aureus*.

Kata Kunci: Antibakteri, *Dillenia ochreata*, 3β-glukopiranosil-lup-20 (29) -en-28- oat

INTRODUCTION

Semprawang (*Dillenia ochreata*), belonging to the family Dilleniaceae, is one of the medicinal plants. These plants are distributed in many Asian countries, in India from the Himalayas to South India. In Indonesia, especially community in Musi Banyuasin, South Sumatra, using the leaves of *D. ochreata* for wounds [1]. Another use of the semprawang plant is its wood as a household appliance. Based on literature studies, information on chemical content and biological activity from *D. ochreata* still needs to be improved. Muharni et al. [2] reported that the leaves of the *D. ochreata* showed antibacterial activity for *Staphylococcus aureus* and *Escherichia coli*. Ethanol

extract of *D. ochreata* leaves contained terpenoids, steroids, saponins, and phenols compounds [2].

Secondary metabolites are rich in triterpenoids, flavonoids, tannins, and other phytoconstituent groups reported from other *Dillenia* species [3, 4]. *Dillenia pappuana* leaves have been found to contain dillenia acid, dillenia B, dillenia C, dillenia D, dillenia E, and 3-oxolean-1,12-dien-30-oic acid, which have antibacterial properties against *Bacillus subtilis, E. coli* and *Micrococcus luteus*. In addition, the roots of *Dillenia suffructicosa* contained catonic acid compound, koetjapic acid and antibacterial compound betulinic acid [5]. Betulinic acid was also found in *D. indica* fruit [6], stem extract, and other compounds such as lupeol, botulinaldehyde, and stigmasterol [7]. Flavonoid compound dillenetin, myricetin, naringenin, quercetin derivatives and kaempferol glucoside have been reported from the stem bark of *D. indica* [6, 8]. Other species, *D. serrata*, was reported to contain triterpenoid compounds of the oleanane type: koetjapic acid and 3-oxoolean-12-en-30-oic acid, as well as lupene derivatives and betulinic acid [9]. Koetjapic acid is also known as a seco-triterpenoid and is the main compound of the genus *Dillenia*. Tor et al. (2015) were reported six compounds from *D. suffruticosa*: 3-epimaslinic acid, kaempferol, kaempferide, protocatechuic acid, gallic acid and β-sitosterol-3-O-β-D-glucopyranoside [10]. The research for obtaining new antibacterial compounds need to be carried out along with the amount of bacteria resistant to existing antibacterial drugs. The goal of this study was to isolate and identify the structure of secondary metabolite compounds from *D. ochreata* leaves. Furthermore, the isolated compound was tested for antibacterial activity and minimum inhibition concentration (MIC) against *S. aureus* and *E. coli*.

MATERIALS AND METHODS

Materials

Sample Preparation

The fresh leaves of *D. ochreata* were collected from the Sekayu, Musi Banyuasin Regency, South Sumatera, in October 2019. The sample was identified as *Dillenia ochreata* (Miq) Teijsm & Binn. ex Martelli at Herbarium Bogoriense Research Center for Biology, Indonesian Institute of Science Bogor, with register number B-82/IV/D1.01/i/2021. The *D. ochreata* leaves fresh were cut to obtain leaves with a small piece (2x0.3 cm).

Methods

Extration

The fresh leaves (1 kg) were extracted using the maceration method using *n*-hexane solvent for 2x24 hours and then filtration. The maceration was carried out with three repetitions [11]. All the filtrated were concentrated under reduced pressure using a rotary evaporator at a temperature of 70 °C and obtained the crude extract of *n*-hexane.

Separation and purification

Extract of n-hexane (6 g), separated by gravity column chromatography technique. The column was prepared using silica gel 60G (70-230 mesh) as a stationary phase and made suspension with n-hexane as solvent. The sample was pre-adsorbed using 60 G (70-230 mesh) silica gel with a ratio of 1:1. Elution was carried out using solvents with increasing polarity n-hexane, mixtures of n-hexane: ethyl acetate (100:0, 90:10, 80:20, 70:30, 60:40 each of 100 mL, 50:50, 40:60 each of 150 mL, and 0:100 100 mL), respectively. The eluate was collected in the bottle (volume 10 mL) and then analyzed using TLC silica gel 60 F_{254} with mobile phase n-hexane: ethyl acetate 7:3. Spot pattern was visualized using a UV lamp at a λ 254 nm. The TLC results with the same chromatogram pattern was combined into one fraction and obtained five fraction F1 (0.89 g), F2 (0.54), F3 (0.75), F4 (0.68) and F5 (0.59). The selected fraction (Fraction 1, which shows the formation of crystals) was purified until a pure compound was obtained. The isolated compound was tested for purity by TLC using several eluents mixture of n-hexane: ethyl acetate (8:2), n-hexane: ethyl acetate (5:5), ethyl acetate: methanol (9:1). Identification of the isolated compounds was carried out using Infrared (IR) spectrophotometer and nuclear magnetic resonance spectroscopy (^{1}H-NMR, and ^{13}C-NMR).

Antibacterial Activity Test

The antibacterial activity of the isolated compound was tested using the agar diffusion method to bacteria *S. aureus* (ATCC 25923) and *E. coli* (ATCC 25922). The negative control uses DMSO solvent. The antibacterial activity was carried out three replicated. The paper disc was dripped with five µL of sample test with concentrations of 1000, 500, 250 and 125 µg/mL, then placed on NA media that had been inoculated with the bacteria test and incubated at 37 °C for 24 hours. Observations were made based on forming an inhibition zone around the disc paper. Furthermore, the zone of inhibition formed was determined [12].

Determination of the minimum inhibitory concentration value

The MIC value was determined using the micro-dilution method, conducted according to the Clinical and Laboratory Standards Institute [13]. The NB medium 180 µL was inoculated into microplate (hole 1), and 100 µL (hole 2 - 10) and hole 11 were inserted negative control (DMSO). The isolated compound was diluted with DMSO (120 µg/mL) were inserted into the holes 1 and stirred until homogeny then 100 µL solution from hole 1 was inserted into the hole 2. Do the same thing in succession until the hole 10, so that the final concentration of the sample was obtained 120; 60; 30; 15; 7,5; 3,75; 1,87; 0,93; 0,46; and 0,23 µg/mL. Hole-11 of the microplate was used for a DMSO negative control. The bacterium (liquid culture) 30 µL was inoculated into a hole 1-11 and stirred. The culture was incubated for 24 hours at 37 °C. Antibacterial activities were marked by forming a clear solution [14].

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).

RESULTS AND DISCUSSION

D. ochreata (1 kg) leaves were macerated using *n*-hexane solvent with three replications; after being concentrated using a rotary evaporator, the crude extract of *n*-hexane was obtained at 65.4 g (6.54% yield). The crude extract of *n*-hexane (6 g) was separated using gravity column chromatography. After analysis by thin layer chromatography (TLC) based on the resulting stain pattern, there were five fractions F1 (0.89 g), F2 (0 .54), F3 (0.75), F4 (0.68), and F5 (0.59 g). The F1 fraction showed that crystals were formed, and after purification, the pure compound was obtained in white crystals (23 mg). The purity test of the isolated compounds by thin layer chromatography used three types of eluent with increasing polarity [15]. The solvents used are n-hexane: ethyl acetate (8: 2) (A), n-hexane: ethyl acetate (5: 5) (B) and ethyl acetate: methanol (9: 1) (C). Spot visualization used cerium sulfate (Figure 1). The chromatogram in **Figure** 1 shows a single spot pattern in each eluent. This indicates that the isolated compound was pure**.**

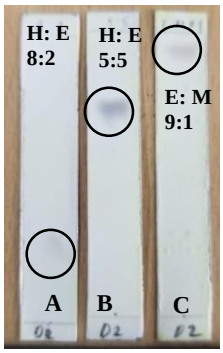

Figure 1. The TLC of the isolated compound

Identification isolated compound

The IR spectrum of the isolated compound showed absorption at 2944 and 2915 cm^{-1}, which is characteristic of asymmetric and symmetric aliphatic Stretching-CH absorption. This is supported by the appearance of signals at 1458 and 1391 cm-1, which is the absorption of bending cyclic (CH_2)n and $(CH_3)_2$-CH- in the cycloaliphatic. IR spectrum also showed absorption in the 1774 cm^{-1} region, which is the absorption for the C=O carbonyl group of acid, and the absorption at 1156 cm^{-1}, which is the absorption for C-O. IR spectral data showed characteristic absorption for triterpenoid compounds. Identification Using ^{1}H NMR (500 MHz, chloroform) shows signals that accumulate in δ_H < 3 ppm, accompanied by the appearance of high-intensity peaks, which are characteristic signals of methyl groups. This indicates that the isolated compound is a cycloaliphatic terpenoid group. In addition, it also showed a signal at δ_H 6–7.ppm was a characteristic signal for aromatic protons. Furthermore, there are many signals in the δ_H 3-5.ppm region, typical signals for the sugar unit's methine proton (CH) [16].

Spectrum of H NMR also showed the signal in the δ_H 7.43 ppm (2H, *d*, *J* = 2 Hz), δ_H 7.40 ppm (1H, *d*, *J* = 1.5 Hz), and δ_H 7.38 ppm. (2H, *d*, *J* = 2 Hz) were five ortho-coupled aromatic protons. Furthermore, the signal for other aromatic protons is also shown at δ_H 7.39 ppm (1H, *s*), and in the δ_H 6.60 ppm (1H, *dd*, *J* = 2.0; 2.5), δ_H 6, 34 ppm (2H, *dd*, *J* = 2.5; 1.0) and δ_H 6.21 ppm (1H, *d*, *J* = 1.0), which are another group of aromatic protons coupled with ortho positions, but a mixture of triterpenoids and other compounds containing an aromatic ring. The Expanded of the ^{1}H NMR spectrum at $_{\delta H}$ 4.3-4.6 ppm and δ_H H 3.1 -3.6 ppm indicated a signal at δ_H 4.77 ppm (1H, *d*, *J* = 4 Hz), δ_H 4.62 (3H, *m*), δ_H 4.52 (1H, *d*, *J* = 4 Hz), and δ_H 4.34 (1H, *d*, *J* = 4 Hz), are characteristic signals of CH methine from sugar units. Signals supported it for other protons of methine at δ_H 3.49 ppm (1H, s), δ_H

3.46 ppm (1H, *s*) and H 0.72 ppm (1H, *s*). The spectrum ^{1}H NMR shows multiplet signals at δ_H 0.94 – 1.35 ppm with the integration of 1 or 2 protons, typical signals for methine and methylene protons in the cycloaliphatic structure of a compound. This signal is commonly characteristic of the triterpenoid or steroid group. It was supported by the singlet signals with the integration of 3 protons with strong intensity at δ_H 0.94 – 1.35 ppm that were signals at δ_H 0.97 (3H, *s*); δ_H 0.98 (3H, *s*); δ_H 1.02(3H, *s*); δ_H 1.16(3H, *d*), δ_H 1.18(3H, *s*); δ_H 1.25(3H, *s*); and δ_H 1.31 (3H, *s*) were signals for the seven methyl proton. The number of methyl signals with singlet multiplicity is a characteristic of triterpenoid compounds. Usually, triterpenoid compounds have eight methyl groups, so one methyl group is suspected of having changed.

The ^{13}C NMR spectrum shows many C (sp^2) signals in δ_C >100 ppm but with different intensities. This indicates that the isolated compound is impure. Carbon signal build-up occurs in areas δ_C < 50 ppm, which were the typical signal for methyl, methylene, methine and quaternary C carbons for cyclo aliphatics (C sp^3) [17]. The signals at δ_C 60-80 ppm were signals for C sp^3 bound to the electronegative group (signals carbon for the sugar unit and signals carbon for the C-3 of triterpenoids that bind oxygen atoms. The ^{13}C NMR spectrum also showed a signal at δ_C 175. 4 ppm for carbonyl acids. Based on 1D IR spectrum data and NMR spectra data (^{1}H NMR and ^{13}C NMR), it is indicated that the isolated compound was the aromatic triterpenoid compound that binds to a sugar group or is a triterpenoid compound mixed with aromatic compounds. Based on literature studies, the most common triterpenoid acids found in the genus *Dillenia* were betulinic acid and its derivatives, such as 3β-glucopyranosyl–lup-20(29)-en-28-oic acid. The NMR data of isolated compounds were compared with the NMR data of 3β-glucopyranosyl–lup-20(29)-en-28-oate, as shown in Table 1.

The NMR data show that the chemical shift values of isolated and comparison compounds are almost identical but have few differences. This difference is because of differences in the instrument's strength used for measurement. The instrument used to measure the compound was 500 MHz for ^{1}H-NMR and 125 MHz at ^{13}C-NMR with CD_3OD solvent) while 3β-glucopyranosyl–lup-20(29)-en-28-oic acid as a comparison compound 400 MHz ^{1}H-NMR and 100 MHz for ^{13}C-NMR in $CDCl_3$ solvent. Based on the analysis of IR and 1D NMR spectroscopy data (^{1}H NMR and ^{13}C NMR) and compared with data in the literature, the isolated compound is 3β-glucopyranosyl–lup-20(29)-en-28-oic (Figure 2).

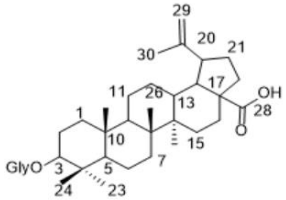

Figure 2. Structure of 3β-glucopyranosyl–lup-20(29)-en-28-oic.

Antibacterial Activity

Antibacterial activity was determined in the inhibition zone against bacterial growth. The antibacterial activity test of the isolated compound (Table 2) at a concentration of 125 - 1000 µg/ml exhibited an inhibition zone diameter of 7.44 –10.13 mm against *E. coli* and 8.78 –9.11 mm against *S. aureus*.

Table 2. Antibacterial activities of isolated compounds against *E. coli* and *S. aureus*

Concentration (µg/mL)	Zones of inhibition (mm) ± SD			
	E. coli			
	1	**2**	**3**	**Average ± SD**
1000	8.60	10.08	11.71	10.13 ± 1.28[a]
500	8.98	8.91	11.93	9.94 ± 1.71[a]
250	8.85	8.23	7.53	8.19 ± 0.72[b]
125	7.79	7.92	6.61	7.44 ± 0.66[b]
	S. aureus			
1000	8.83	7.78	10.73	9.11 ± 1.49[a]
500	8.51	10.77	7.81	9.03 ± 1.54[a]
250	8,66	9.38	9.00	9.01 ± 0.36[a]
125	7.79	9.12	9.44	8.78 ± 0.87[a]

Table 1. Chemical shift (δ) values 1H and 13C NMR spectra for isolated compound (1H-500 MHz, 13C-125 MHz, in CD3OD) and 3β-glucopyranosyl–lup-20(29)-en-28-oate (1H-400 MHz, 13C-100 MHz, in $CDCl_3$, ppm) [18].

C	δ_C [18]	δ_C isolated	δ_H mult. (J Hz)[18]	δ_H, mult. (J Hz) isolated	DEPT
1	38.7	37.8	0.90; 1.69		CH_2
2	27.4	25.3	1.55; 1.62		CH_2
3	79.0	75.3	3.20 *dd* (4.5; 11.5)	3.46 (1H, *s*)	CH
4	38.9	37.9	–		C
5	55.3	57.2	0.68		CH
6	18.3	15.4	1.53		CH_2
7	34.3	34.7	1.40		CH_2
8	40.7	39.8	–		C
9	50.5	50.4	1.26		CH
10	37.2	37.8	–		C
11	20.8	17.1	1.41; 1.43		CH_2
12	25.5	25.3	1.66; 1.68		CH_2
13	38.4	37.9	2.20		CH
14	42.4	45.2	–		C
15	29.7	25.4	1.22; 1.55		CH_2
16	32.1	34.7	1.42; 2.30		CH_2
17	56.3	57.3	–		C
18	49.3	49.5	1.62		C
19	46.9	46.5	3.02 *dt* (10.5; 5.5)	3.20 (1H, *s*)	CH
20	150.4	146.9	–		C
21	30.5	25.7	1.41; 1.99		CH_2
22	37.0	43.9	1.48; 1.96		CH_2
23	27.9	25.3	0.95 (*s*)	1.17 (*s*)	CH_3
24	15.3	16.0	0.74 (*s*)	0.97 (*s*)	CH_3
25	16.1	18.9	0.80 (*s*)	0.98 (*s*)	CH_3
26	16.0	19.0	0.92 (*s*)	1.02 (*s*)	CH_3
27	14.7	12.2	0.96 (*s*)	1.24 (*s*)	CH_3
28	179.8	175.4	–		C
29	109.7	109.9	4.62 *d* (2.0); 4.70 *d* (2.0)	4.75 *d* (4.0); 4.52 *d* (4.0)	CH_2
30	19.4	21.3	1.69 (*s*)	1.31 (*s*)	CH_3
1’	100.7	99.4	4.11 (*d*)	4.34 (*d*, 4.0)	CH
2’	73.4	75.3	3.14 (*m*)	4.63(*m*)	CH
3’	76.9	75.2	3.14 (*m*)	4.63 (*m*)	CH
4’	70.0	66.6	3.14 (*m*)	4.63 (*m*)	CH
5’	76.7	75.6	3.06 (*m*)	3.49 (*m*)	CH
6’	62.8	57.8	2.94 (*m*)	2.65 (*m*)	CH_2

Statistical analysis showed that there was no significant difference ($p>0.05$) in the diameter of the inhibition zone for all variation of concentrations at *S. aureus*, while bacteria for *E. coli* showed a significant difference ($p < 0.05$) between the test concentrations of 1000 µg/mL and 500 µg/mL with a concentration of 125 µg/mL and 250 µg/mL. The inhibition zone between concentrations 250 and 125 at bacterial *E. coli* showed no significant difference ($P > 0.05$). The inhibition zone showed no significant difference ($p>0.05$) towards *E. coli* and *S. Aureus*. The aim of determining the MIC value was to find the smallest concentration of isolated pure compound that could inhibit the growth of the bacteria. Table 3 shows that at a concentration of 120 µg/mL, a clear solution was formed for *E. coli*, while at *S. aureus* bacteria at a concentration of 60 µg/mL. This indicated the isolated

compound has higher antibacterial activity against *E. coli* than *S. aureus* bacteria. According to Togousop et al. (2011) [19], the antibacterial activity of compounds from plant extracts can be categorized as strong (MIC <100 µg/mL), moderate (MIC 100–500 µg/mL) and weak (MIC >500 µg/ml. Accordingly, isolated compound was allocated to the strong category of antibacterial activity (MIC value <100 µg/mL) against *S. aureus* and the moderate category against *E. coli* bacteria.

Table 3. MIC values of the isolated compound against *E. coli* and *S. aureus*

Bacteria	Concentration (µg/mL)										
	120	60	30	15	7.5	3.75	1.87	0.93	0.46	0.23	DMSO
E. coli	+	–	–	–	–	–	–	–	–	–	–
S. aureus	+	+	–	–	–	–	–	–	–	–	–

(+) active antibacterial (-) inactive antibacterial

Several antibacterial compounds in the Triterpenoid group also have been reported from other species of the Dillenia genus, such as betulinic acid from the roots of *D. suffruticosa* [5]. Betulinic acid is also contained in *D. phillippinensis* and other compounds 3-oxoolean-12-en-30-oic acid, which is also antibacterial active [20]. Nick et al. (1994) [3] reported dillenia A, dillenia acid B, dillenia acid C, dillenia acid D, dillenia acid E, betulinic acid and aldehyde betulin of *D. pappuana* and showed activity against bacteria against *Bacillus subtilis, E. coli* and *Micrococcus luteus*.

CONCLUSION

The compounds have been isolated from the *n*-hexane fraction of Semprawang leaves in the form of white crystals (23 mg). Based on the analysis of IR and 1DNMR spectroscopy data (^{1}H-NMR and ^{13}C-NMR) and comparing the NMR spectrum data in the literature, it was concluded that the compound isolated was 3β -glucopyranosyl–lup-20(29)-en-28-oat mixed with aromatic compounds. The isolated compound had antibacterial properties with a MIC value of 120 µg/mL against *E. coli* and 60 µg/mL against *S. aureus*.

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