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The antibacterial activity of triterpenoid acid from the ethyl acetate extract of *Dillenia ochreata* leaves

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ABSTRACT: *Dillenia ochreata* is a medicinal plant that has been used traditionally as a scabies remedy. Ethanol extract of *D. ochreata* has been reported to have antibacterial activity, and contains triterpenoids, steroids, tannins, and phenolic compounds. The aims of this study were to isolate and identify the chemical compound from the ethyl acetate extract of *D. ochreata* leaves and to evaluate their effects on antibacterial activity. The *D. ochreata* leaves were extracted with the maceration method, separation and purification were carried out using chromatography methods, and IR and NMR spectroscopy were used to characterize the isolated compound. The antibacterial activity was conducted using the disc diffusion method against the *E. coli* and *S. aureus* bacteria, and the dilution method was used to determine the minimum inhibitory concentration value. A pure compound was isolated from the ethyl acetate extract of the *D. ochreata* leaves as a white solid (90 mg). Based on the spectroscopic data and a comparison with the literature, the isolated compound was a triterpenoid group sentulic acid with the molecular formula $C_{30}H_{46}O_4$. The sentulic acid compound showed antibacterial activity with a minimum inhibitory concentration value for *E. coli* and *S. aureus* of 60 µg/ml and 15 µg/ml respectively. Our study is the first to report sentulic acid from *D. ochreata* leaves.

KEYWORDS: *D. ochreata*; triterpenoids; sentulic acid; *E. coli; S. aureus.*

1. INTRODUCTION

Plants are widely used by the people of Indonesia as traditional medicine. One such plant is the genus *Dillenia* [1, 2]. The genus *Dillenia* belongs to the family Dilleniaceae, which is known by the names simpur, sempur, and sempu. This genus has about 100 species, which can grow in tropical and subtropical areas in South Asia, Australia, and islands in the Indian Ocean, especially Indonesia. One of the species of the genus *Dillenia* is the *Dillenia ochreata*, which is known as semprawang, simpur, and simpor (Indonesia), Simpoh (Malaysia), simpor (Brunai), Mai-masam (Burma), san-masan (Thailand), and kadmon (Philippines) [3]. Communities, especially the Musi Banyuasin tribe of South Sumatra, have used the leaves of the *D. ochreata* as a scabies medicine [4], and the wood of *D. ochreata* is used as household furniture. Based on a study of the literature, scientific information of *D. ochreata* is very limited. Muharni et al., (2017) [5] reported that the ethanolic extract of *D. ochreata* leaves was antibacterially active. Phytochemical analysis of the ethanol extract of *D. ochreata* leaves has been found to be positive for triterpenoids, steroids, tannins, and phenolic compounds.

Secondary metabolites from other species of the *Dillenia* genus to be rich with triterpenoids and flavonoids and some compounds have been found to be active antibacterials [6]. The terpenoid group compounds that are commonly found in the genus *Dillenia* are lupene type triterpenes such as betulinic acid, betulin, betulinaldehyde, masagonic and lupeol from *D. indica*. These compounds which have variety pharmacological activities [6, 7]. In addition, oleanene group triterpenoids were also found, such as 3-oxoolean-12-en-28-oic acid, 2-oxo-3β-hydroxyolean-12-en-30-oic acid, 3 oxoolean-1,12-dien-30-oic acid. and 1α-hydroxy-3-oxoolean-12-en-30-oic acid. These compounds are antibacterial [8]. In this study, we aimed to

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isolate, determine the structure, and test the antibacterial activity of secondary metabolites from the ethyl acetate fraction of *D. ochreata* leaves against *S. aureus* and *E. coli* bacteria.

2. RESULTS AND DISCUSSION

2.1 Chemistry

The extracted (500 g) of *D. ochreata* leaves by the maceration method using a solvent with increasing polarity (n-hexane, ethyl acetate) after the leaves had been concentrated using a rotary evaporator. The obtained concentrated extract of n-hexane was 6.54 g, and the concentrated extract of ethyl acetate was 56.64 g with yields of 1.31% and 11.32%, respectively. After the ethyl acetate extract had been separated and purified, a white solid (90 mg) remained. TLC analysis following the appearance of the serium sulfate stain was positive, which was indicated by a single red spot. This showed that the isolated compound was an acidic triterpenoid type.

The IR spectrum of isolated compound showed peak of 2940-2850 cm^{-1}(CH aliphatic asymmetric and symmetric), and was supported by the absorption at 1481 cm^{-1} (bending $(CH_2)n$) and 1366 cm^{-1} $((CH_3)_2$-CH-) [9]. In addition, the absorption band of 1700 cm^{-1} represented carbonil group (C=O) in form carboxyclic acid. This data indicated the isolated compound is triterpenoid acid. The ^{1}H-NMR spectrum of isolated compounds showed typical signals for triterpenoid compounds, namely, signals that accumulate in the chemical shift (δH) region below 2 ppm [10].

The spectrum further showed the presence of six high-intensity signals with integration 3, which is a typical signal for the methyl group at chemical shifts δ_H of 0.88; 0.91; 0.95; 0.94; 1.20; and δ_H 1.76 ppm each (3H, s). The methyl signal at δ_H 1.76 (3H, *s*) ppm was thought to be methyl bound to C sp^2 (C = C − CH_3) (Efdi et al., 2012). The ^{1}H NMR spectrum also showed a signal at δ_H 5.27 (1_H, *t*, J = 3 Hz), which indicated the presence of olefinic protons (C = C) and protons in chemical shifts (δH) of 4.70 ppm (1H, *s*) and δ_H 4.88 ppm (1H, *s*), which is a signal for the exocyclic proton-type methine. Next, the signal on the chemical shift (δ_H) was 1.53 (1H, *m*), 2.35 (1H, *m*), 2.15 (1H) and δ_H 2.19 ppm (1H), where the integration of one proton each is a signal for four protons of methine [11].

In the ^{13}C NMR spectrum, six signals were apparent at C >100 ppm: δ_C 181.9, 178.1, 148.9, 145.4, 123.2, and 114.1 ppm, which are characteristic signals for C sp^2. Signals at δ_C 181.9 and 178.1 ppm are typical signals for carbon carbonyl in acid form. This data indicated that the isolated compound had two C = C doubles and two C = O groups. In the area of δC <60 ppm, a signal was visible, and after widening the spectrum, 24 carbon signals were obtained so that the total carbon signal in the ^{13}C NMR spectrum was 30 signals. This data strengthened the understanding that the isolated compounds were triterpenoids.

To determine the type of carbon, namely, whether it was CH_3, CH_2, CH, or C, the DEPT 135 spectrum was measured. Based on the ^{13}C-NMR spectrum analysis, the DEPT 135 isolated compounds had six methyl signals at δ_C 17.9; 20.0; 24.4; 26.4; 33.5 and 33.6 ppm, 11 methylene signal at at δ_C 114.2; 47.3; 35.4; 35.0; 33.8; 32.8; 29.5; 28.9; 25.9; 24.7 and 24.0 ppm, four methine signals at C 123.6; 51.7; 42.9 and 39.3 ppm and nine quaternary carbon signals (C) at δ_C 181.9 ; 178.1; 148.9; 145.3; 47.7; 43.5; 40.4 and 31.7 ppm. Based on the DEPT spectrum, it can be seen that the six sp^2 C signals consisted of one methylene signal (δ_C 114.1), one methine signal (δ_C 123.6), and four quaternary C signals, with two of them being acid carbonyl signals (δ_C 181.9, 178.1). Based on this data, the resulting combination of the two had two copies, which had three vinylic protons

The NMR spectrum data of the isolated compound were compared with the NMR spectrum data for the triterpenoid sentulic acid that has been reported from the harp plant (*Sandoricum koetjape* Merr.), as shown in Table 1. The isolated compound and the comparison compound of sentulic acid were measured using the same solvent (CD_3OD). Based on these data, it can be seen that the NMR data were almost the same as the NMR data for sentulic acid. There was a slight difference in the value of the chemical shift between the NMR spectrum data for the isolated compound and the comparison data for sentulic acid due to the difference in the strength of the NMR instrument used; the isolated compound was measured with a power of 500 MHz for protons and 125 MHz for carbon, while the comparison compound of sentulic acid was measured with a power of 400 MHz for protons and 100 MHz for carbon [11]. The structure of sentulic acid can be seen in Figure 1.

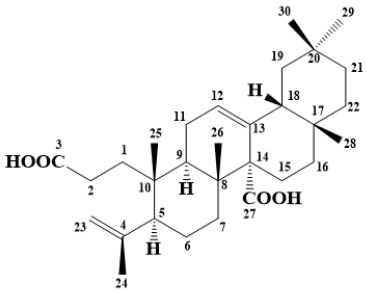

Figure 1. Structure of sentulit acid

Table 1. NMR data isolated compound (CD_3OD), and sentulit acid (CD_3OD), [11]

Position C	Isolated compoud		Sentulit acid [11]	
	δ_C (ppm)	**δ_H (ppm), mult, *J* (Hz)**	**δ_C (ppm)**	**δ_H (ppm), mult, *J* (Hz)**
1	29.5		34.4	
2	35.0	2.15; 2.19	28.1	2.22; 2.29
3	178.1		176.8	
4	148.9		147.2	
5	51.7	2.01	49.4	2.00
6	25.9		24.2	
7	33.8		35.0	
8	40.4		38.9	
9	39.3		37.2	
10	40.4		38.9	
11	24.2		22.8	
12	123.6	5.27 (*t*) *J* = 3.00 Hz	125.3	5.61 (*t*) *J* = 3.00 Hz
13	145.3		137.6	
14	43.5		55.7	
15	24.7		21.9	
16	28.9		27.5	
17	31.7		32.7	
18	42.9		49.3	
19	47.3		43.9	
20	47.7		30.6	
21	32.8	1.53 (*m*); 2.35 (*m*)	34.2	1.60 (*m*); 1.65 (*m*)
22	35.4		36.4	
23	114.2	4.70 (*s*); 4.88 (*s*)	112.9	4.67 (*s*); 4.86 (*s*)
24	24.0	1.76 (*s*)	22.5	1.75 (*s*)
25	20.0	0.95 (*s*)	19.6	0.97 (*s*)
26	17.9	1.20 (*s*)	17.5	1.08 (*s*)
27	181.9		178.5	
28	33.6	0.88 (*s*)	27.5	0.87 (*s*)
29	33.5	0.94 (*s*)	32.5	0.83 (*s*)
30	26.4	0.91 (*s*)	22.7	0.87 (*s*)

Several acidic triterpenoid compounds have been reported from the genus *Dillenia*. among others with a Lupene-type triterpene such as Dillenic acid A -E from leaf and steam of *D. papuana* [12], betulinic acid from leaf *D. indica* [13], koetjapic acid from stem bark and root bark of *D. serrata* [8], katonic acid and Koetjapic acid from root bark of *D. suffruticosa* [14]. Other compounds 3-epi-maslinic acid from root of *D. suffruticosa* [7]. and messagenic acid from *D. philippinensis* leaf. The dillenic acid A-E. betulinic acid are antibacterially active against the bacteria *Bacillus subtilis*. *Micrococcus luteus*. and *E. coli* [15].

Some of these oleanic skeleton triterpenoids are also found in the form of oleanic skeletons that are cleaved in cyclic A. such as koedjape acid from *D. suffroticosa* [16]. Sentulic acid is a triterpenoid with an oleanic skeleton that is cleaved in cyclic A. but it has never been reported from the genus *Dillenia*. especially *D. ochreata*. so the results of this study are the first to reveal sentulic acid from the species *D. ochreata*. Sentulic acid however has been reported from the plant *Sandoricum koetjape* [11].

2.2. Antibacterial Activity

The antibacterial activity test of the isolated compound (Table 2) at a concentration of 125-1000 µg/ml exhibited inhibition zone diameter of 8.58–10.45 mm against *E. coli* and 8.44 –10.59 mm against *S. aureus*. Antibacterial activity was determined of the inhibition zone against bacterial growth [17]. Antibacterial activity is categorized as weak if the inhibition zone is <9 mm. Moderate 9–12 mm. strong if the diameter is 13–18 mm. and very strong if it is >18 mm. Accordingly the strength of the inhibitory activity of the isolated compounds against the growth of the *E. coli* and *S. aureus* bacteria at concentrations of 250-1000 µg/ml were assigned to the medium category. Statistical analysis showed the inhibition zone were significantly different ($p<0.05$) at variation concentrations and no significantly different ($p>0.05$) against *E. coli* dan *S. aureus* bacteria.

Table 2. Antibacterial activity of isolated compound

Concentration (µg/ml)	Zone of inhibition (mm)			
	E. coli			
	I	II	III	Average ± SD
1000	10.50	11.10	9.76	10.45 ± 0.67[a]
500	9.82	10.10	9.62	9.84 ± 0.24[b]
250	9.65	9.90	9.00	9.50 ± 0.46[b]
125	8.10	9.00	8.64	8.58 ± 0.45[c]
	S. aureus			
1000	11.25	9.88	10.66	10.59 ± 0.68[a]
500	9.78	9.58	9.32	9.56 ± 0.23[b]
250	9.64	8.85	9.10	9.19 ± 0.40[b]
125	8.56	8.90	7.88	8.44 ± 0.52[c]

Numbers followed by the same subscript indicate no significant difference

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 concentration of 60 µg/ml. a clear solution was formed for *E. coli*. while at *S. aureus* bacteria at concentration 15 µg/ml This indicated the isolated compound (sentulit acid) have antibacterial activity higher against *E. coli* bacteria compared *S. aureus* bacteria. According to Tagousop et al. (2018) [18]. 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 (sentulic acid) was allocated to the strong category of antibacterial activity (MIC value <100 µg/ml). Based on the literature sentulic acid also exhibits anti-leukemic-like activity on human promyelocytic leukemia cells (HL-60) [11] as well as anti-inflammatory activity [19].

Table 3. Determination MIC value of isolated compound against *E. coli* and *S. Aureus* bacteria.

Bacteria	Concentrations (µg/ml)										Negative control (DMSO)
	120	60	30	15	7.5	3.75	1.87	0.93	0.46	0.23	
E. coli	+	+	–	–	–	–	–	–	–	–	–
S. aureus	+	+	+	+	–	–	–	–	–	–	–

(+) Clear, (-) Blury

3. CONCLUSION

In this study. sentulic acid was isolated from the ethyl acetate fraction of *D. ochreata* leaves. Sentulic acid showed strong antibacterial activity against *E. coli* and *S. aureus* bacteria with MIC values of 60 µg/ml and 15 µg/ml. respectively. This is also the first report of sentulic acid obtained from *D. ochreata*

4. MATERIALS AND METHODS

4.1. General Experiment

The tools used in this research: *rotary evaporator* (Dragon Lab RE 100-Pro), spectrometer IR (Perkin Elmer), spectrometer NMR (NMR JEOL JNM-ECZ500R (^{1}H-NMR, ^{13}C-NMR dan DEPT 135), UV lamp (UltraViolet *Observing Cabinet*-02), analytical balance (OHAUS PA4202).

4.2. Methods

4.2.1. Plant materials

Fresh of *D. ochreata* leaves was collected from Musi Banyuasin South Sumatera, Indonesia in October 2019. The sample was identified *Dillenia ochreata* (Miq) Teijsm.& Binn.ex Martelli at Herbarium Bogoriense, Indonesian Institute of Science Bogor, with register number B-82/IV/D1.01/i/202.1

4.2.2. Preparation of Extracts

Fresh of *D. ochreata* leaves (500 g) were extracted by maceration method using a solvent with graded polarity (n-hexane, ethyl acetate) for 2x24 hours each and then filtered. The extraction process was repeated three times [20]. Each filtrated were concentrated using a rotary evaporator at temperature of 70°C to yield the crude n-hexane (6.54 g), and ethyl acetate extract (56.64 g).

4.2.3. Fractionations and purification

The ethyl acetate extract (50 g) was separated using the vacuum liquid chromatography method. The stationary phase (silica gel 60 G) was placed in a column at a ratio of 30:1 (silica:sample), and the adsorbent was leveled and compacted. The prepared sample was then preadsorbed using silica gel 60 G (70–230 mesh) at a 1:1 ratio and stirred until smooth. The sample was inserted into the column and then leveled. Eluents were added with increasing polarity (n-hexane and mixtures of n-hexane: ethyl acetate). The eluate was collected using a bottle and subsequently concentrated using a rotary evaporator. It was analyzed using TLC 60 F_{254}, and the TLC results were observed using a UV lamp at λ 254 nm and spot viewer sprayed with serium sulfate reagent (1.5%). Base on TLC analaysis five fractions F1 (5.52 g), F2 (6.02 g), F3 (6.90 g), F4 (3.27 g), F5 (20.07 g) were obtained.

The F2 fraction showed the formation of a white solid, so the separation and purification continued for the F2 fraction. The F2 fraction (6.02 g) was separated by applying gravity column chromatography using silica gel 60 G (70–230 mesh). The sample was preabsorbed and inserted into the column, and elutes were added using solvents with gradient polarity n-hexane: ethyl acetate. The analysis was then carried out via TLC, and four fractions F2.1 (1.61 g), F2.2 (0.86 g), F2.3 (0.91 g) and F2.4 (0.62 g) were obtained.

The F2.4 fraction was separated and purified again using gravity column chromatography with eluent n-hexane–ethyl acetate (9:1-7:3). Based on the TLC analysis, four fractions F2.4.1 (0.11 g), F2.4.2 (0.09 g), F2.4.3 (0.09 g), and F2.4.4 (0.10 g) were obtained. The fraction F2.4.2 showed the formation of a white solid after purification using n-hexane to obtained white solid (60 mg). The purity test of the isolated compound was carried out using the TLC method with various eluents: n-hexane: ethyl acetate (7:3 and 5:5) and ethyl acetate: acetone (9:1), and the appearance of a cerium sulfate (1.5%) stain gave a single red color. The isolated compound was characterized using IR and NMR spectroscopy.

4.2.4. Antibacterial activity

The antibacterial activity was carried out by the disc diffusion method against *E. coli* and *S. aureus* bacteria. As nagarive control using DMSO. The antibacterial activity test was repeated three times using seri concentrations 1000, 500, 250, and 125 µg/ml. The disc paper was dipped into each 5 µL of each sample and placed on media that had been inoculated with bacteria. It was then incubated at 37°C for 24 hours. Observations the inhibition zone a round the disc paper. The inhibition zone was expressed in millimeters [21, 22].

4.2.5. Determination the minimal inhibitory concentration (MIC)

The (MIC) determined by the micro-dilution method (CLSI, 2015). The NB medium 180 µL was inoculated into a micro plate (hole 1) and 100 µL (hole 2 - 10) and hole 11 were inserted negative control (DMSO). The sample (20 µL) with concentrations of 120 µg/ml were inserted into the holes 1, and stirred until homogenic 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; 1.67; 0.93; 0.46 and 0,23

µg/ml. The bacterium (liquid culture) 30 µL was inoculated into a hole 1-11, and stirred. The cultures were incubated for 24 hours at 37°C [23].

4.3. Data Analysis

The data antibacterial activity was analyzed using analysis of variance (ANOVA) alfa 0.05, followed by a Duncan new multiple range test (DNMRT) at alfa 0.05 with data processing software *Statistical Product and Services Solutions* (SPSS) version 26.

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