



TOXICITY AND ANTIMICROBIAL ACTIVITY FROM EXTRACT AND OLEANAN DERIVATIVE COMPOUNDS OF THE BARK *MELOCHIA UMBELLATE* (HOUTT) STAPF VAR. DEGRABRATA

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ABSTRACT

Oleanan derivative compound namely 3-acetyl-12-oleanen-28-oic acid, has been isolated from n-hexane fraction of the bark *M. umbellate* (Houtt) Stapf var. degrabrata. The molecular structure was determined by IR spectroscopy, NMR 1D and 2D (¹H-NMR, ¹³C-NMR, DEPT, COSY, HMQC and HMBC). The results of test bioactivity to hexane, chloroform, ethyl acetate, methanol extracts and compound 1 mentioned above showed that they are toxic to *A. salina* (brine shrimp) with LC₅₀ 361.93 to 460 µg/mL. The most toxic one was the compound 1 with the LC₅₀ value of 361.93 µg/mL. At a concentration of 1000 µg/mL, the extract of hexane, methanol and compound 1 showed very high inhibition to the growth of *B. subtilis* and *C. albican*, whereas the extract of ethyl acetate had very high inhibition effect to the growth of *S. aureus* and *A. niger*. The inhibition zone of the three extracts and compound 1 were greater than 14 mm. On the other hand, the extracts of chloroform gave weak inhibition against bacteria and fungi.

KEY WORDS: *M. umbellate*, toxicity and antimicrobial activity, 3-acetyl-12-oleanen-28-oic acid.



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INTRODUCTION

Plant *Melochia umbellate* (Houtt) Stapf var. *degrabrata* is a plant belonging to the genus of *Melochia*. This genus of plants consisting of 65 species widespread in tropical forest regions from West Africa, South America, tropical regions of Asia and Papua New Guinea. Some species of plants *Melochia* are used by local people for traditional medicine. *M. chamaedris* is used by people in Rio Grande do Sul Brazil as an anti hypertensive and cancer agent¹. *Sterculia setigara* Del (*Melochia tomentosa*) is most widely used as a traditional medicine in West Africa to treat dysentery, ulcers, syphilis, epilepsy, asthma and malaria². Dry powder of the leaves of the plant *S. setigara* is usually used by the medicine in the state of Bauchi (Nigeria) for treatment of tuberculosis (chronic cough with blood stains) and HIV/AIDS³. In South Sulawesi, leaves of *M. umbellate* plants are known as *paliasa* has been used for a long time as traditional medicine for treating liver disease, hypertension, diabetes, cholesterol and hepatitis⁴. Some researches on the biological activity of the *Melochia* genus have been reported including, the bark extract, berries and roots of *S. setigera* (*M. tomentosa*) had antibacterial and antifungal activity, especially against *B. subtilis*, *S. aureus*, *P. aeruginosa*, *E. coli*, *A. niger* and *C. albicans*. Ethanol extract of this plant was also shown to effectively inhibit the growth of *P. aeruginosa* bacteria with minimum inhibition concentration (MIC) of 2.0 µg/ml and *B. subtilis* with (MIC) of 1.0 µg/ml^{5,6}. Methanol extracts of the leaves of *M. umbellate* (Houtt) Stapf var. *degrabrata* effectively improved the heart function of mice induced by carbon tetrachloride. Methanol extract of the leaves also had antioxidant activity and were toxic to larvae shrimp of *Artemia salina*^{7,8}.

Secondary metabolites of hexane extract of *M. umbellate* roots have been reported to contain stigmasterol compounds (5.22-stigmastadien-3β-ol) that were potential as antibacterial compounds acting as stigmasterol glycosidated antifungi⁹. It has also been reported that two new compounds

have been successfully been isolated from heartwood tissue extract of *M. umbellate* (Houtt) Stapf var. The first compound was an alkaloid compound, quinolinon (7.8-epoksimelochinon) which was toxic against *A. salina* and murine leukemia cells P-388 and the second one was a flavonoid compound (6.6'-dimethoxy-4.4'-dihydroxy-3'.2'-furano-soflavan) which was not toxic against *A. salina* and murine leukemia cells P-388¹⁰. *M. umbellate* (Houtt) Stapf var. *degrabrata* plant is potential to be developed and utilized as a source natural bioactive materials due nature toxicity against *A. salina* larvae and other bioactive properties and relation to the use of this plant as a traditional medicine by certain people. Based on the literature to date, it has not been widely reported on the use of bioactive compounds from *M. umbellate* (Houtt) Stapf var. *degrabrata* plants, especially the stem bark of the plant tissue.

METHODS

Equipment and Materials

Equipment used in this study were glass tools commonly used in the laboratory, vacuum liquid chromatography equipment, press column chromatography, gravity column chromatography, thin layer chromatography (TLC), a developer room (chamber), micropipette, microplate, antimicrobial test equipment, ultraviolet light (λ, 254 and 360 nm), evaporator tools Buchi, melting point apparatus Fisher Johns, FTIR 8501 Shimadzu and NMR JEOL JMN A 5000. Materials used in this study are a sample the bark of *M. umbellate*. The plants were identified and collected in Bogor-based, Center for Research and Development Biology, LIPI Bogor, with specimen numbers: BO-1912171. Organic solvent used quality p.a and technical; *n*-hexane, chloroform, ethyl acetic, acetone and methanol, silica gel Merck (7730, 7733 and 7734), cerium sulphate solution 2 %, DMSO, *A. salina* brine shrimp, pure cultures of bacteria *B. subtilis*, *S. aureus*, *E. coli*, *P. aeruginosa*, *S.*

thypi, and pure cultures of the fungus *C. albicans*, *M. furfur* and *A. niger*. medium *Mueller Hinton Agar* (MHA), and sterile paper discs.

Extraction and Isolation of Compound 1

The bark powder of *M. umbellate* plants (5.25 kg) was extracted by maceration with methanol 1 X 24 hours (3 times). The methanol extract was concentrated by a low-pressure rotary evaporator and methanol extract (393.58 g) was obtained. The methanol extract 300 g was partitioned with of liquid - liquid extraction using solvents with increasing polarity; n-hexane, chloroform and ethyl acetic. The weight of extracts from each solvent are shown in Table 2. Hexane extract (15.0 g) was fractionated by vacuum liquid chromatography and eluted with n-hexane eluent gradient step polarity with ethyl acetic to produce a combined 16 major fractions. Solid formed in fraction D (1.2205 g) was crystallized using hot methanol several times to obtain compound 1 as a white powder 70.6 mg. KLT Test to compounds 1 with three each eluent system generates different Rf values and showed only one spot, which means that this compound was pure.

Toxicity Test

Brine shrimp lethality test (BST) was conducted to the extracts and Compound 1 using *A. salina* brine shrimp. One mg samples in Eppendorf tubes were diluted with 100 μ L of DMSO and 150 μ L aquabidest. The solution (200 μ L) was taken and diluted with 600 μ L aquabidest to make a solution with the concentration of 1000 μ g/ mL. Further dilution was done in microplate with varied concentrations of the sample and a volume of 100 μ L. Around 5 to 10 *A. salina* brine shrimps with the age of 48 hours was taken randomly, included in microplate containing the samples and were then incubated for 24 hours. Furthermore, the LC₅₀ value was determined from mortality percentage to logarithm dose.

Antibacterial test

Test bacteria used in this study comes from a pure culture laboratory of Microbiology, Faculty

of Pharmacy Hasanuddin University, Indonesia. The test bacteria consist of gram-positive bacteria (*B. subtilis* and *S. aureus*) and gram-negative bacteria (*P. aeruginosa*, *E. coli* and *S. thypi*). The bacteria was rejuvenated in TSA medium in slanted tubes for 2 x 24 hours at 25 °C. Colonies were grown in agar slant taken one loop, then homogenized with 9 ml of saline solution, and incubated at 10 cm petri dish containing *Mueller Hinton Agar* (MHA) medium. Test was performed with an anti-bacterial agar diffusion method using 6 mm diameter paper discs and were deposited on the surface of MHA medium. Furthermore extract and isolate the compound 1 dropped as much as 20 μ L and incubated for 2 x 24 hours at 25 °C. The testing activity of extracts and isolated compounds is done by measuring the zone of inhibition of bacterial culture using a slide rule in mm.

Antifungal test

Cultures fungal test derived from laboratory of Microbiology, Faculty of Pharmacy Hasanuddin University (*C. albicans*, *M. furfur* and *A. niger*) were grown in YPD medium. Antifungal test performed by the agar diffusion method using sterile 6 mm diameter paper discs and were deposited on the surface of YPD medium, and incubated for 3 x 24 hours at a temperature of 25 °C. Testing of antifungal activity of extracts and isolate compound same as the antibacterial test.

RESULTS AND DISCUSSION

Compound 1 formed white crystalline (70.6 mg) with melting point of 284 – 285 °C and positive triterpenoids with LB reagent. Infrared spectrum showed a sharp peak at 3449 cm^{-1} indicating the presence of hydroxyl groups (OH) : 1737 cm^{-1} showing (ester C = O), 1261 cm^{-1} showing (acetic), and a peak at 802 cm^{-1} are typical for double bond at position C-12 and C-13 in a penta cyclic triterpenes. Data ¹H-NMR (CDCl₃, 500 MHz), δ_{H} (ppm), ¹³C (CDCl₃, 125 MHz) δ_{C} (ppm), COSY, HMQC and HMBC, as shown in Table 1.

Table 1
NMR spectroscopy data of compounds 1

No	H-NMR, δ ppm (multiplicitas, J dlm Hz)	C – NMR δ ppm	δ_c (lit)	COSY H \leftrightarrow H	HMQC H \leftrightarrow C	HMBC H \leftrightarrow C
1	1.76 (t, $J = 3.25; 7.8; 13.6$) 1.23 (1H, m)	38.24	38.0	2	C-1	C2
2	1.80 (1H, m)	23.70	23.5	1 & 3	C-2	
3	4.50 (t, $J = 9.1$)	81.10	80.9	2	C-3	C1, C2, C23, C24, C31
4	-	37.87	37.6			
5	1.30 (1H, m)	55.46	55.2	6		
6	1.52 (1H, m) 1.28 (1H, m)	18.35	18.1	5 & 7	C-6	C23; C24
7	1.56 (1H, m) 1.31 (1H, m)	32.69	32.4	6	C-7	
8	-	39.45	39.2			
9	1.43 (1H, dd, $J = 3.2$ & 9.75)	47.73	47.5	11	C-9	
10	--	37.17	36.9			
11	1.97 (1H, ddd, $J = 4.5; 9.1; 13.6$)	23.10	22.8	9 & 12	C-11	C12, C13
12	5.25 (1H, t, $J = 3.9$)	122.74	122.5	11	C-12	C9, C14
13	-	143.78	143.6			
14	-	41.73	41.5			
15	1.38 (1H, m) 1.13 (1H, m)	27.84	27.6	16	C-15	
16	1.61 (1H, m) 1.36 (1H, m)	23.57	23.3	15	C-16	C28
17	-	46.72	46.5			
18	2.86 (1H, dd, $J = 4.55; 13.65$)	41.10	40.8	19	C-18	C12, C13, C14
19	1.24 (1H, m)	46.0	45.8	18	C-19	
20	-	30.85	30.6			
21	1.31 (1H, m)	32.62	33.7	22	C-21	
22	2.02 (1H, m) 1.77 (1H, m)	33.96	32.4	21	C-22	C28
23	0.85 (3H, s)	28.22	28.0		C-23	C3, C5
24	0.86 (3H, s)	16.84	16.6		C-24	C6; C3
25	0.93 (3 H, s)	15.57	15.3		C-25	C1; C5; C9; C10
26	0.74 (3 H, s)	17.35	17.1		C-26	C8, C14
27	1.12 (3 H, s)	26.08	25.8		C-27	C8, C13, C14
28	-	183.96	184.3			
29	0.90 (3 H, s)	32.84	33.0		C-29	C19, C20
30	0.94 (3 H, s)	23.51	23.5		C-30	C19, C20
31	-	171.25	171.1			
32	2.04 (3 H, s)	22.06	21.3		C-32	C31

$^1\text{H-NMR}$ spectrum of compounds 1 showed the typical signals including; 7 seven singlet signal with multiplicity at δ_{H} 0.85 (H-23); 0.86 (H-24); 0.93 (H-25); 0.74 (H-26); 1.12 (H-27); 0.90 (H-29) and 0.94 ppm (H-30) for the methyl protons attached to the quaternary carbon. At δ_{H} 5.25 ppm (1H, t, $J = 3.9$ Hz, H-12) showed a vinyl proton coupling with methylene protons δ_{H} 1.97 ppm (1H, ddd, $J = 4.5; 9.1; 13.6$ Hz, H-11), at δ_{H} 1.80 ppm (1H, m, H-2) coupling with metinoksi protons (protons are substituted acetyl group), appeared at δ_{H} 4.5 ppm (1H, t, $J = 9.1$ Hz, H-3), other signals at δ_{H} 2.04 ppm area is the methyl protons (3H, s, H-32) attached to the carbonyl carbon ($\text{CH}_3\text{-C=O}$). $^{13}\text{C-NMR}$ spectrum showed 32 carbon signals consisting of 8 methyl carbon, 10 methylene carbon, 5 metin carbon and 9 quaternary carbon. A ring that has two quaternary carbon C-4 and C-10 respectively shown in δ_{C} 37.87 and 47.73 ppm, a tertiary carbon C-5 at δ_{C} 55.46 ppm, two secondary carbon is C-1 and C-2 are shown in δ_{C} 38.24 and 23.70 ppm. In addition to the A ring of carbon oxy identified the C-3 at δ_{C} 81.10 ppm. In the B ring there are two quaternary carbon C-8 and C-10 are shown in δ_{C} 39.45 and 47.73 ppm, ie two tertiary carbon C-5 and C-9 at δ_{C} 55.46 and 37.17 ppm, two secondary carbon is C-6 and C-7 on δ_{C} 18.35 and 32.69 ppm. In the C rings there are comprising a quaternary carbon signal for the alkene carbon atoms C-13 at δ_{C} 143.78 ppm, then the C-8 and C-14 at δ_{C} 39.45 and 41.73 ppm. Two tertiary carbon is C-9 at δ_{C} 37.17 ppm and C-12 at δ_{C} 122.74 ppm were identified as alkene carbon atoms. In the D ring that there are three quaternary carbon is C-13, C-14 and C-17 respectively shown in δ_{C} 143.78, 41.73 and 46.72 ppm, a tertiary carbon C-18 at δ_{C} 41.10 ppm, two

secondary carbon is C-15 and C-16 at δ_C 27.84 and 23.57 ppm. In the E ring, there are two quaternary carbon is C-17 and C-20 at δ_C 46.72 and 30.85 ppm, a tertiary carbon C-18 at δ_C 41.10 ppm, three secondary carbon is C-19, C-21 and C-22 respectively shown in δ_C 46.0, 36.62 and 33.96 ppm. Another signal that appears at δ_C 183.96 ppm showed the carbonyl carbon of a carboxylic acid (C-28). These signals indicate a compound olean-12-en-substituted acetyl group at C-3. Position functional groups or relationship bonding in the molecular structure of compound 1 can be proved by the existence of long-range correlation $^1\text{H} \rightarrow ^{13}\text{C}$ HMBC spectrum as listed in Table 1. Correlation distance protons at δ_H 4.5 ppm (H-3) with a quaternary carbon at δ_C 171.25 ppm (C-31) which reinforces that the acetyl group attached to the C-3, methylene carbon at 38.24 ppm (C-1), methyl carbon at 28.22 ppm (C-23) and the methyl carbon at 16.84 ppm (C-24). Correlation distance of protons at δ_H 5.25 ppm (H-12) with the carbon at δ_C 37.17 metin ppm (C-9) and the quaternary carbon at δ_C 41.73 ppm (C-14) indicates that the C-12 and C-13 an alkene carbon at the C rings. Besides some signals of methyl groups are proton at δ_H 0.85 ppm (Me-23) with the carbon at δ_C 81.10 ppm (C-3) and δ_C 55.46 ppm (C-5), the proton at δ_H 0.86 ppm (Me-24) with the carbon at δ_C 18.35 ppm (C-6) and δ_C 81.10 ppm (C-3), the proton at δ_H 0.93 ppm (Me-25) with the carbon at δ_C 55.46 ppm (C-5) and δ_C 38.24 ppm (C-1), the proton at δ_H 0.74 ppm (Me-26) with carbon δ_C 39.45 ppm (C-8) and δ_C 41.73 ppm (C-14), the proton at δ_H 1.12 ppm (Me-27) with the carbon at δ_C 39.45 ppm (C-8), δ_C 143.78 ppm (C-13) and δ_C 41.73 ppm (C-14), the proton at δ_H 0.90 ppm (Me-29) with the carbon at δ_C 46.0 ppm (C-19) and δ_C 30.85 ppm (C-20), the proton at δ_H 0.94 ppm (Me-30) with the carbon at δ_C 46.0 ppm (C-19) and δ_C 30.85 ppm (C-20), which identified that the methyl groups attached to the quaternary carbon atoms as shown in Figure 1 in compound 1. Based on data: Spectra of H-NMR, C-NMR, COSY, HMQC and HMBC shown in Table 1, it can be concluded that the isolate compound 1 is 3-acetyl-12-oleanen-28-oic acid. The above spectroscopic data are similar to the spectroscopic data of compounds that have been reported previously (Ogihara et al., 1997)¹¹ so that further ensure that compound 1 is 3-acetyl-12-oleanen-28-oic acid.

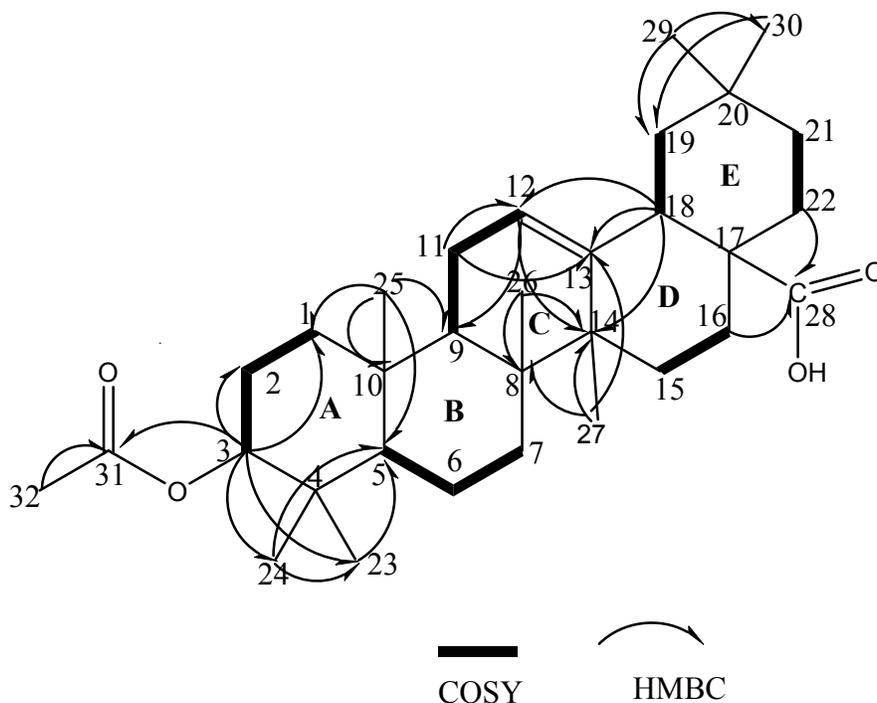


Figure 1
Compounds 1 is 3-acetyl-12-oleanen-28-oic acid.

Table 2

Results of toxicity tests of extract and compound 1 of the bark *M. umbellate* (Houtt) Stapf var. *degrabrata* are against *A. Salina*.

No.	Extract Sample	Extract Weight (gr)	LC ₅₀ µg/ml
1	Hexane	36.21	407.38
2	Chloroform	24.06	460.79
3	Ethyl acetic	6.25	405.58
4	Methanol	393.58	408.79
5	Compounds 1	70.6 mg	361.93

Table 3

Test results of the antibacterial and antifungal activities of extracts and compounds 1 of the bark *M. umbellate* (Houtt) Stapf var. *Degrabrata*.

Extract/ Compounds	Zona of Inhibition (diameter in mm)						
	<i>B.subtilis</i>	<i>S. aureus</i>	<i>E. coli</i>	<i>S. thypi</i>	<i>C.albicans</i>	<i>M. furfur</i>	<i>A. niger</i>
Hexane	14.1	12.7	12.5	9.7	14.5	10.0	9.9
Chloroform	11.0	10.6	9.0	n.i	7.1	9.5	7.6
Ethyl acetic	13.7	16.7	12.0	9.7	10.6	8.1	16.7
Methanol	15.0	13.6	12.1	10.1	14.9	10.9	11.4
Compound 1	15.8	9.0	12.0	n.i	15.2	n.i	8.3

Description: (n.i) not inhibit

Bioactivity test results of n-hexane, chloroform, ethyl acetic, methanol and compound 1 from the plant to *A. salina* (Table 2) showed a low toxicity with LC₅₀ value of between 361.93 to 460 µg/mL, but the most active compound against *A. salina* is a compound 1 with the LC₅₀ value of 361.93 µg/mL. Extract or pure compound with the LC₅₀ value of greater than 100 µg/ml is considered to have low toxicity while the one with the LC₅₀ value of less than 100 µg /ml is considered as highly active category (high toxicity) against *A. salina*¹². Based on the bioactivity of antimicrobial test results as shown in Table 3, it is clear a concentration of 1,000 µg/mL of n-hexane, methanol and compound 1 it has the highest inhibition against the growth of bacteria *B. subtilis* with inhibition zones respectively were 14.1 mm, 15.0 mm and 15.8 mm and also against *C. albicans* with zones of inhibition: 14.5 mm, 14.9 mm and 15.2 mm. The ethyl acetic extracts showed growth inhibition against bacteria *S. aureus* and fungus *A. niger* with inhibition zone of 16,7 mm. Chloroform extracts showed weak inhibition against bacteria and fungi. At concentrations below

1,000 µg/mL inhibition exhibited by the extract and compound 1 against the growth of bacteria and fungi do not even show weaker inhibition or completely inactive. A compound is said to be as antimicrobial if the compound gives an average inhibition zone greater than 14 mm¹³. Based on the results of antimicrobial tests in Table 2, it can be concluded that the n-hexane, ethyl acetic, methanol and compound 1 from the bark of *M. umbellate* plants have potential as antimicrobial compounds and are able to inhibit the growth of bacteria and fungi with an average inhibition zone greater than 14 mm, especially against bacteria *B. subtilis* and *S. aureus* as soon as the fungus *C.albicans* and *A. niger*.

CONCLUSION

1. 3-acetyl-12-oleanen-28-oic acid which belonged to the oleanan have been isolated from the bark of n-hexane fraction of *M. umbellate* (Houtt) Stapf var. *degrabrata*.
2. Hexane, chloroform, ethyl acetic, methanol extract and compound 1 from the bark of the plant are toxic to *A. Salina* with LC₅₀

361.93 to 460 µg/ mL and the most active compound against *A. salina* is compounds 1 with LC₅₀ 361.93 µg/ mL.

3. The extract n-hexane, methanol and compound 1 at the concentration 1,000 µg/mL

showed the highest inhibition against the bacteria *B. subtilis* and *C. albicans*. and ethyl acetic extract showed the highest inhibition against *S. aureus* and *A. niger*, respectively showed inhibition zone greater than 14 mm.

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