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Non-Phenolic Compounds from the Stem Bark of Red Mangrove (*Rhizophora stylosa*) and Evaluation of Their Cytotoxicity and Larvacidal Activity

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Abstract
Non-phenolic compounds namely lupeol and β-sitosterol had been separated from the stem bark of Indonesian red mangrove (*Rhizophora stylosa*). The lupeol obtained a colorless powder with a melting point of 210-212 ^{o}C, whereas β-sitosterol obtained as a colorless powder with a melting point of 136-137 ° C. Isolates were separated using chromatographic techniques, purified by recrystallization, and their structures determined by spectroscopic methods (UV, IR, and MS) and compared with literature data. They showed cytotoxicity against *Artemia salina* and larvacidal acitivity against *Aedes aegypti* larvae.
Keywords: *Rhizophora stylosa*, lupeol, β-sitosterol, *Artemia salina*, *Aedes aegypti*

1. Introduction

Red mangrove (*Rhizhopora stylosa*) is one of the mangrove species in the Rhizophora family that grows widely in coastal areas in Indonesia. Red mangrove a tree with a height of 10 m, cylindrical stem, outer stem bark grayish brown to blackish brown. The plant is found in diverse habitats, namely in tidal areas, mud, sand, and rocks. Red mangrove has been widely used by people as a traditional medicine to stop the bleeding, diarrhea, fever, malaria, and colds (Noor et al, 1999; Akyar, 2010). It had been reported the existence of non phenolic secondary metabolites in triterpenoids and steroids groups from *R. stylosa*. Pentacyclic triterpenoids that had antioxidant activity, namely 3β-O-(*E*)-coumaroyl-15α-hydroxy-β-amirin, 15α-hydroxy-β-amirin, 3β-taraxerol, 3β-taraxerol format, taraxerol 3β-acetate, 3β-O-(*E*)-coumaroyl-taraxerol, and 3β-O-(*Z*)-coumaroyl-taraxerol had been separated from the stems of *R. stylosa* (Li et al, 2008). From the leaves of *R. stylosa* had been isolated non-phenolic compounds of cereaborin, cis-cereaborin, taraxerol, β-sitosterol, and β-deucasterol (Yang et al, 2008). In the course of our studies, a phytosteroid of β-sitosterol (**1**) and triterpenoid of lupeol (**2**) had been isolated from the stem bark of *R. stylosa*. In this paper, we reported the isolation and structure determination of those isolates and evaluation of their cytotoxicity aginst *Artemia salina* Leach and larvacidal activity against *Aedes aegypti.*

2. Materials and Methods

2.1 General Experimental Procedures

Melting point was measured by Fisher John melting point apparatus and was uncorrected. UV spectra were recorded on Shimadzu Pharmaspec UV-1700 spectrophotometer. IR spectrum in KBr film was determined by Buck Scientific-500 spectrophotometer. Mass spectrum (MS) was recorded on Shimadzu QP-5000 spectrometer using electron impact (EI) ion mode EI. Kieselgel 60 GF-254 (Merck) and silica gel G 60 63-200 μm (Merck) were used for vacuum liquid chromatography (VLC) and flash chromatography (FC), respectively. Precoated silica gel 60 F-254 (Merck) 0.25 mm, 20 x 20 cm was used for thin layer chromatography (TLC) and spots were detected by spraying with the sulphuric acid solution 5% (v/v) in ethanol followed by heating. Toxicity of isolates against *Artemia salina* L and *Aedes aegypti* larvaes were evaluated using Brine shrimp lethality test (BSLT) and WHO standart method modified.

2.2 Plant Materials

The stem bark of *R. stylosa* were collected from the coastal of Tambak Osowilangun distric, Surabaya, Indonesia in January 2013. A voucher spesimen was identified and deposited at the herbarium of the Purwodadi Botanical Garden, East Java, Indonesia. Furthermore, the samples was cleaned of dirt, small cut, and dried at room temperature. The dry samples were milled to obtain the powders ready to be extracted.

2.3 Extraction and Isolation

The stem bark dried powdered of *R. stylosa* (3.5 kg) was exhaustively extracted by maceration using *n*-hexane solvents at room temperature. Evaporation of *n*-hexane extract in vacuo revealed 12.0 g blackish-green residue. A portion of 6.0 g of the extract was separated by VLC using silica gel Merck G 60 F-254 as stationary phase

and solvents of increasing polarity (*n*-hexane, a mixture of *n*-hexane-ethyl acetate, and the ethyl acetate) produced 130 fractions (15 mL each). The results of the separation was monitored by thin layer chromatography using *n*-hexane-ethyl acetate = 9: 1 as eluen. Removal of the solvent under reduced pressure of the combined fractions of 40-48 gave the brown solid (1.09 g). It was rechromatographed by FC with *n*-hexane-ethyl acetate (97 : 3) as eluen, obtained 92 fractions (10 mL each). The combined fractions of 54-92 were recrystallized in a methanol, yielded lupeol (**1**) (74.9 mg). While the combined fractions of 55-64 from VLC (300 mg) were recrystallized in methanol produced β-sitosterol (**2**) (136 mg).

Lupeol (**1**) was obtained as a colorless powder from methanol with a melting point of 210-212 °C and a positive test with Liebermann-Burchard reagent (violet color). Measurement of the ultraviolet spectrum in *n*-hexane gave absorption peaks at a wavelength of 209.5 nm. Infrared spectrum of **1** prepared by KBr technique gave absorption bands at wavenumbers of 3306 (OH), 2947, 2872 (alkyl CH steching), 1640 (C = C), 1465, 1379 (CH alkyl bending), and 1037 (C-O). The results of the analysis by gas chromatography proved that **1** had a high degree of purity because it showed one peak at a retention time of 33.365 minutes. The mass spectrum (EIMS) of **1** showed peaks at the region m / z (relative intensity,%): 426 (6), 218 (100), 207 (13), 203 (31), 189 (31), 175 (13) , 161 (19), 147 (25), 122 956), 107 (56), 95 (63), 81 (94), 69 (69), 41 (81).

β-Sitosterol (**2**) obtained as a colorless powder from methanol with a melting point of 136-137 ° C and a positive test with Liebermann-Burchard reagent (blue color). Measurement of the ultraviolet spectrum in *n*-hexane gave absorption peaks at a wavelength of 208.3 nm. Infrared spectrum of **2** prepared by the KBr technique gave absorption bands at wavenumbers of 3343 (OH), 2944 (alkyl C-H stretching), 1646 (C = C), 1457, 1374 (alkyl C-H bending), and 1045 (C-O). Chromatogram resulted from gas chromatography analysis of **2**, showed one peak at a retention time of 38.745 minutes. The mass spectrum (EIMS) of **2** showed peaks at the region m/z (relative intensity,%): 414 (21), 396 (12), 381 (9), 354 (3), 329 (18), 303 (15), 273 (12), 255 (12), 231 (12), 213 (18), 199 (9), 185 (6), 173 (9), 159 (18), 145 (30), 133 (21), 119 (21), 107 (39), 81 (36), 57 (42), 43 (100).

2.4 Cytotoxicity Assay against Artemia salina Leach Larvae

Isolate (10.0 mg) was dissolved in 2 mL of chloroform to obtain a stock solution of 5000 µg/ mL. Furthermore, as many as 10, 25, 50, 75, and 100 mL of solution were inserted into a different vials. After the solvent evaporates, into each vial was added 10 µL of DMSO (Merck) followed by 10 larvae of *Artemia salina* and sea water up to 5 mL volume. After 24 hours of incubation, the number of dead *Artemia. salina* larvae were counted. The cytotoxicity of isolates expressed by LC_{50} was obtained using probit analysis in SPSS program (Mc Laughlin et al, 1991).

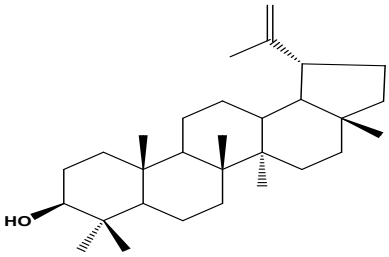
2.5 Larvacidal Activity Assay against Aedes aegypti Larvae

The third-instars larvae of *Aedes aegypti* were obtained from the Entomology Laboratory of Tropical Disease Institute, Surabaya, Indonesia. Larvacidal activity of isolate were conducted using WHO standart method modified (WHO, 2005). Preparing the aqueous solution of isolate with various concentrations of 100, 250, 500, 750, and 1000 µg/mL. Dimethylsulphoxide (DMSO) (Merck) was added to increase the solubility of isolate in dechlorinated water. A portion of 50 mL of each solution was put into a petri disk with 9 cm diameter, followed by adding 10 third-instars larvae of *Aedes aegypti*. The larvae mortality were observed after 24 hours. Its LC_{50} value was obtained using probit analysis in SPSS program.

3. Results and Discussion

*3.1 Lupeol (**1**)*

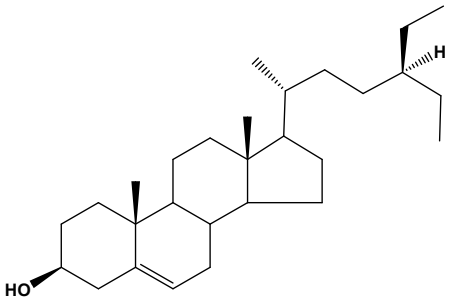
The violet color resulted from Liebermann-Burchard test showed that compound **1** belonged to the triterpene (Robinson, 1991). The UV spectrum exhibited maxima at 209,5 nm, indicating no conjugated double bond. Sharp absorption band at 2947 cm^{-1} region in the infrared spectrum, supported the presence of hydrocarbon skeleton at triterpene compounds. The EIMS spectrum of compound **1** showed a molecular ion of m/z 426, corresponding to the molecular formula $C_{30}H_{50}O$ (DBE = 6). The mass spectrum of **1** had resembled with those of lupeol in the literature data (Ogihara, 2000; Haque et al, 2006). Fragment ions at m/z 189 (M-$C_{16}H_{29}O$), 207 (M-$C_{16}H_{26}$), 218 (M-$C_{14}H_{24}O$) were characteristic for the triterpene series of lupane. Fragmentation patterns at the mass spectra supported that compound **1** was lupeol. From the above results, compound **1** was identified as lupeol. It is the first time reported from *R. stylosa*.



Lupeol **(1)**

3.2 β-Sitosterol (2)

The emergence of the blue color resulted from Liebermann-Burchard test exhibited that **2** was a steroid compound (Robinson, 1991). The existence of hydrocarbon skeleton of steroid was showed by sharp absorption band at 2944 cm^{-1} on the infrared spectrum. The EIMS spectrum of **2** showed a molecular ion of m/z 414, corresponding to the molecular formula of $C_{29}H_{50}O$ (DBE = 5). Fragment ion m/z 255 ($M-C_{10}H_{21}-H_2O$) was characteristic for steroid β-sitosterol, resulting from the loss of the side chain and a water molecule (Kolak et al, 2005). The existence of the OH group was supported by the absorption band at 3343 cm^{-1} on the infrared spectrum and fragment ions at m/z 396 ($M-H_2O$) on the mass spectrum. The mass spectrum of 2 had a high similarity with β-sitosterol in literature data (Kolak, et al., 2005). Fragmentation patterns in the mass spectra supported that compound **2** was β-sitosterol. From the above results, compound **2** could be identified as β-sitosterol. The presence of β-sitosterol had been reported from the leaves of *R. stylosa* (Yang et al, 2008).



β-Sitosterol **(2)**

3.3 Cytotoxicity Isolates against Artemia salina Leach

Compound lupeol and β-sitosterol were evaluated their cytotoxicity against *Artemia salina* Leach using Brine shrimp lethality test. Cytotoxicity values were expressed as LC_{50} determined by probit analysis using SPSS program. Results of these tests were descibed in Table 1.

Table 1. Cytotoxicity of Lupeol and β-Sitosterol against *Artemia salina* Leach

Isolate Concentration (µg/mL)	Average of Percentage mortality		LC_{50} (µg/mL)	
	Lupeol	β-sitosterol	Lupeol	β-sitosterol
10	17	20	81.796	89.167
25	27	27		
50	33	30		
75	47	37		
100	57	57		

Based on the BSLT known that lupeol and β-sitosterol had LC_{50} values of 81.796 and 89.167 µg/mL, respectively. Both isolates could be categorized as a toxic substance because their LC_{50} values were less than 200 µg/mL (Meyer et al, 1982). Thus the two compounds had potency to be developed as anticancer agent.

3.4 Larvacidal activity of Isolates against Aedes aegypti Larvae

Compound lupeol and β-sitosterol were evaluated their larvacidal activity against the third-instars larvae of *Aedes aegypti*. Larvacidal activity values expressed as LC_{50} was determined by probit analysis using SPSS program. Results of these assay were summarized in Table 2.

Table 2. Larvacidal Activity of Lupeol and β-Sitosterol against *Aedes aegypti* Larvae

Isolates Concentration (μg/mL)	Average of percentage mortality		LC_{50} value (μg/mL)	
	Lupeol	β-sitosterol	Lupeol	β-sitosterol
100	23,3	13,3	380,74	540,34
250	40,0	26,7		
500	66,7	60,0		
750	76,7	66,7		
1000	90,0	76,7		

Based on the test results found that lupeol and β-sitosterol had LC_{50} value of 380.74 and 540.34 μg/ mL, respectively. According to the APEA and the ERDC (Swan et al., 1994), a compound that had LC_{50} value between 100-1000 μg/mL was considered to have moderate toxicity. Thus both isolated compounds had moderate larvacidal activity against *Aedes aegypti* larvae. However lupeol is more effective in terms larvacidal activity compared to β-sitosterol.

4. Conclusions

Non-phenolic compounds namely lupeol and β-sitosterol had been separated from the *n*-hexane extract of the stem bark of red mangrove (*Rhizophora stylosa*). Both isolates showed cytotoxicity against *Artemia salina* and larvacidal activity against *Aedes aegypti* larvae, so that they could be developed as anticancer agent and larvacidal agent.

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References

Akyar. (2010). *Inhibitory test and tlc bioauthography analysis of root and fruit extract of mangrove (Rhizophora stylosa Griff.) against vibrio harveyi*. Makasar: Hasanudin University.

Haque, M.E., Shekhar, H.U., Mohamad, A.U., Rahman, H., Islam, A.M., Hossain, M.S. (2006). Triterpenoids from the stem bark of avicennia officinalis. *J. Pharm. Sci.* 5, 1, 53-57.

Kolak , U., Topcu, G., Birteksoz, S., Otuk, G., Ulubelen, A. (2005). Terpenoids and steroids from the roots of *salvia blepharochlaena*. *Turk. J. Chem.* 29, 177-186.

Li, D.L., Li, X.M., Wang, B.G. (2008). Pentacyclic triterpenoids from the mangrove plant *(Rhizophora stylosa)*. *Natural Product Research*. 22, 9, 808-813.

Mc Laughlin, J.L., Chang, C-J, Smith, D.L. (1991). *The Unesco regional workshop on the bioassay of natural product with special emphasis on anticancer agent.* UM Malaysia.

Meyer, B.N., Ferrigni, N.R., Putnam, J.E., Jacobsen, L.B., Nicholas, D.E., Mc Laughlin, J.L. (1982). Brine shrimp: a convenient general biossay for active plant constituent. *Planta Med*. 45, 31-32.

Noor, Y.R., Khazali, M., Suryadiputra, I.N.N. (1999). *Guide in introducing the Indonesian mangroves*. Bogor : PHKA/WI-IP.

Ogihara, K., Nishizato, S., Ishigaki, M., Higa, M., Yogi., S. (2000). Triterpenoids and phytosteroids from the leaves of *cynanchum liukiuense*. *Bull. Fac. Sci*. 70, 83-92.

Robinson, T. (1991). *The Organic Constituents of Higher Plants*. (6th ed). North Amherst, MA : Cordus Press.

Swan, J. M., Neff, J. M., Young, P. C. (1994). Environmental implications of offshore oil and gas development in Australia - the findings of an independent scientific review. Sydney: Australian Petroleum Exploration Association.

World Health Organization. (2005). *Guidelines for laboratory and field testing of mosquito larvacides.* [Online] Available: http://whqlibdoc.who.int/hq (20 March 2014).

Yang., X.H., Li, H.B., Chen, H., Li, P., Ye, B.P. (2008). Chemical constituents in the leaves of *Rhizophora stylosa* l and their biological activities. *Yao Xue Xue Bao*. 43, 9, 974-978.

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