

CYTOTOXIC LACTONE-TYPE DITERPENOIDS AND TRITERPENOID FROM *Vitex pubescens* Vahl

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ABSTRACT

The stem bark of *V. pubescens* Vahl has been used as a traditional medicine in Indonesia. Isolation of the stem bark of *V. pubescens* Vahl with some column chromatography techniques led to obtaining the lactone diterpenoids, neoandrographolide (1), andrographolide (2), 14-deoxyandrographolide (3), and a pentacyclic triterpenoid compound, betulinic acid (4). Neoandrographolide (1) was reported in this plant for the first time. Cytotoxic activity of compounds 1-4 showed inactivity against HeLa (MTT assay).

Keywords: Cytotoxic, *V. pubescens* Vahl, 14-Deoxyandrographolide, Andrographolide, Neoandrographolide, Betulinic Acid.

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INTRODUCTION

Vitex genus consists of 300 species distributed in the tropics and sub tropics¹. This genus is rich in organic compounds, some of which are flavonoid, terpenoid, ecdysteroid, and iridoid glucoside². Many of them have interesting biological activities, such as anticancer³⁻⁵, antiinflammation^{6,7}, antioxidant⁸, antimicrobial⁹, antitrypanosomal¹⁰, antilarvaside¹¹, and antituberculosis¹².

V. pubescens Vahl is one of the *Vitex* genus which grows in the Asian territorial, such as Indonesia, Malaysia, India, Sri Lanka, and the Philippines. In Indonesia, it is mainly found in Sumatra, Kalimantan, and Java island¹. *V. pubescens* Vahl is used for traditional treatment for many diseases, its leaves and stem bark are used for back pain, cut, indigestion, fever, scorpion sting, increase appetite, dysentery, antiinflammation, cancer and rhinitis, and also used to increase stamina¹³. Ecdysteroid: pinnasterone, 20-hydroxyecdysone, turkesterone¹⁵, iridoid glucoside: pinnatoside and flavonoid: viscioside, apigenin and luteolin¹⁶ have been isolated from *V. pubescens* Vahl stem bark. Meanwhile, β -sitosterol, stigmasterol, 5-hydroxy-3, 7, 4'-trimethoxyflavone, 5-hydroxy-7,4'-dimethoxy-flavone, and 5-hydroxy-3,3',4',7-tetramethoxyflavone¹⁷ were also found in *Vitex pinnata* which grows in Malaysia.

Diterpenoid is the main secondary metabolites in *Vitex*. Lactone-type diterpenoid which has furan ring from C13 to C16 is considered as a specific constituent in this genus¹⁸. Diterpenoid compounds have interesting and diverse biological activities, such as 6-acetoxy-9-hydroxy-13[14]-labdan-16,15-olide, vitexilactone, rotundi-furan, vitetrifolin D, and vitetrifolin E¹⁹ which are active as anticancer agents, 9-hydroxy-13(14)-labden-15,16-olide and isoambreinolide which have antituberculosis²⁰ property and five diterpenoid compounds from *Vitex trifolia*, 4,4,8,10-tetramethyl-trans-decalin skeleton, vitexifolin E, vitexifolin F, vitexilactone, and 6-acetoxy-9-hydroxy-13(14)-labdan-16,15-olide which have good activity as antitrypanosomal¹⁰.

As a part of our research on Indonesian herbal medicine potencial as cytotoxic agents, in this paper will be reported the isolation and characterization of neoandrographolide (1) and its activity against cervix cancer cells (HeLa). The anticancer assay was also carried out on the three other compounds (2-4).

EXPERIMENTAL

Plant Material

The stem bark of *V. pubescens* Vahl was taken from the environment of the University of Riau, Pekanbaru, Indonesia. The plant was identified at the Andalas University Herbarium (ANDA), Biology Department, Andalas University.

Extraction and Isolation

V. pubescens Vahl stem bark (9.58 kg) was dried, ground, and macerated with methanol. Maceration process was carried out for three days with three repetitions. The extract was evaporated to yield methanol crude extract (532.2 g). The methanol extract was fractionated with n-hexane, dichloromethane, and ethyl acetate (EtOAc) solvent, respectively to get n-hexane extract (60.04 g), dichloromethane extract (57.82 g), ethyl acetate extract (227.79 g), and methanol extract (186.55 g).

Dichloromethane extract (57.82 g) was separated by using vacuum liquid chromatography (VLC) with solvents of n-hexane 100%, n-hexane:ethyl acetate (20%, 40%, 60%, 80%) and ethyl acetate 100%, to yield fraction A, B, C, D, E, and F.

Fraction E (16.278 g) was separated by column chromatography (silica 70-230 mesh) using eluent of n-hexane and ethyl acetate with an increase in polarity to obtain 13 fractions (fraction E₁-E₁₃). Fraction E₁₁ (1.125 mg) was purified by flash column chromatography (silica 230-400 mesh) using eluent of hexane-ethyl acetate (1:1, 4:6, 3:7, 2:8, 1:9) and ethyl acetate 100% to yield 12 fractions (fraction A_{11.1}-A_{11.12}). Fraction A_{11.11} (1.105) was purified with column chromatography (silica 230-400 mesh) with eluent DCM:EtOAc (1:1, 3:7, 0:10) to afford compound **1** (16 mg) and compound **2** (36 mg). The isolation process of compound **3** and **4** were fully explained in previous publication²¹⁻²³.

Cytotoxic Activity Assay

HeLa cells were cultured in RPMI 1640 media containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin antibiotic and incubated in a CO₂ 5% incubator at 37°C for 24 hours. After 24 hours of growth, HeLa cells (1 x 10⁴ cell/mL, 100 μL) were distributed on 96-well plate, and 100 μL of the tested compound (dissolved in DMSO) was added to the well in various concentration. The mixture of HeLa cells and the tested compound was incubated for 24 hours with CO₂ 5% at 37°C. After 24 hours of incubation, 10 μL of MTT was added and allowed to stand for 4 hours. The living cells will react to MTT which produces a purple color of formazan. The MTT reaction was stopped with stopper reagent (HCl 10% in SDS), and incubated overnight at room temperature. The absorbance was then measured with ELISA reader at λ 595 nm.

Spectral Data

Neoandrographolide (1). M.p. 170-171 °C; UV (MeOH) λ_{max} nm: 204; IR ν_{max} cm⁻¹: 3369, 2927, 2868, 1746, 1070; ¹H NMR (500 MHz, CD₃OD) δ ppm: 0.71 (3H, s, H-20); 1.03 (3H, s, H-18); 0.96 (1H, td, H-3); 1.1 (1H, td, H-3); 1.27 (1H, dd, H-5); 1.78 (1H, m, H-1), 1.94 (1H, m, H-1), 1.66 (1H, m, H-9); 1.46 (1H, m, H-2); 1.65 (1H, m, H-2); 1.37 (1H, m, H-6); 1.86 (1H, m, H-6); 2.0 (1H, m, H-7); 2.42 (1H, m, H-7); 1.62 (1H, m, H-11); 1.80 (1H, m, H-11); 2.1 (1H, m, H-12); 2.38 (1H, m, H-12); 3.21 (1H, d, J=9.1 Hz, H-19); 4.09 (1H, d, J=9.7 Hz, H-19); 4.82 (2H, d J=1.95, H-15); 4.63 (1H, s, H-17), 4.86 (1H, s, H-17), 7.34 (1H, t, J=1.95, H-14); 3.16 (1H, H-2'); 3.24 (1H, H-5'); 3.30 (1H, H-4'); 3.32 (1H, H-3'); 3.69 (1H, H-6'); 3.85 (1H, H-6'); 4.18 (1H, H-1'); ¹³C NMR (125 MHz, CD₃OD) δ ppm: 177.09 (C-16), 149.42 (C-8), 147.71 (C-14), 134.91 (C-13), 107.36 (C-17), 105.16 (C-1'), 78.33 (C-3'), 77.78 (C-5'), 75.36 (C-2'), 73.54 (C-19), 72.17 (C-15), 71.78 (C-4'), 62.84 (C-6'), 57.94 (C-9), 57.78 (C-5), 40.74 (C-4), 40.31 (C-1), 39.77 (C-7), 39.47 (C-10), 37.28 (C-3), 28.41 (C-18), 25.72 (C-6), 25.54 (C-12), 23.05 (C-11), 20.15 (C-2), and 15.93 (C-20). HMQC and HMBC: see Table-1.

RESULTS AND DISCUSSION

Extraction of dried stem bark of *V. pubescens* Vahl with methanol produced crude extract (532.2 g). It was then fractionated with n-hexane, dichloromethane, and ethyl acetate solvent, respectively. Dichloromethane fraction (57 g) was separated with VLC to yield 6 main fractions (A-F). Purification of

fraction A, C, and E produced compound 1-4 which were identified as neoandrographolide, andrographolide, 14-deoxyandrographolide, and betulinic acid. Structure determination of compound 2-4 has been described in the previous studies²¹⁻²².

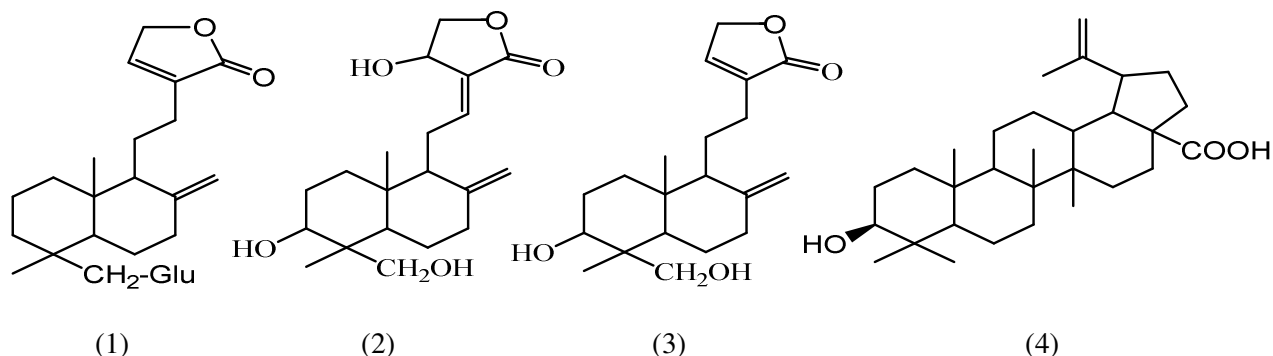
Compound 1 forms a white solid with a melting point of 170-171°C. The IR spectra showed absorption for hydroxyl group, carbonyl group, and C-H aliphatic at ν_{maks} 3369, 1746 and 2927 cm^{-1} , respectively. ^1H NMR (Table 1) spectrum indicated several characteristics of the Compound 1. There was the presence of a vinyl group (δ_{H} 4.63 and 4.86 ppm, H-17), and two oxygenated methylenes (δ_{H} 4.82 ppm, H-15; 3.21, and 4.09 ppm, H-19). Two olefin bonds at C-14 and C-17 provided chemical shift at δ_{H} 4.63-7.34 ppm. The proton at C-14 showed at higher frequency (δ_{H} 7.34 ppm) due to conjugation effect of the carbonyl group. ^1H -NMR spectrum exhibited some signals suitable for glucosyl group, were identified as one oxidized methylene carbon (δ_{H} 3.69 and 3.85 ppm, H-6'), five oxidized methinecarbones at δ_{H} 4.18 ppm (H-1'), 3.16 ppm (H-2'), 3.32 ppm (H-3'), 3.30 ppm (H-4'), and 3.24 ppm (H-5'). ^{13}C NMR and DEPT data analysis (Table 1) revealed some signals for 26 carbon atoms. Two methyl carbons displayed at δ_{C} 15.93 ppm (C-20) and 28.41 ppm (C-18). The presence of glucosyl group was marked by several signals at δ_{C} 105.16 ppm (H-1'), 75.36 (H-2'), 78.33 (H-3'), 71.78 (H-4'), 77.83 ppm (H-5'), and 62.48 ppm (H-6'). Moreover, there were also found the signals for C-carbonyl (δ 177.06 ppm, C-16), two oxidized methylene carbons (δ 72.17, C-15 and 73.54 ppm, C-19). The presence of olefin methine carbons was marked by signals at δ 147.71 ppm (C-14) and one hybridized methylene carbon sp^2 at 107.36 ppm (C-17). Spectra analysis of ^1H - ^1H COSY, HMQC, and HMBC supported compound 1 as neoandrographolide. The data of compound 1 was confirmed by the previous research²³.

Table-1: ^1H and ^{13}C NMR Data of Compound 1

No C	δ_{H} (ppm), integrity, multiplicity, J	δ_{C} (ppm)	DEPT	δ_{C} (ppm)*	HMBC
1	1,94 (m); 1,78 (m)	40,31	CH_2	40,23	C4, C6 C8, C9
2	1,65 (m); 1,46 (m)	20,15	CH_2	20,26	C1, C4
3	1,1 (td, $J=3,25$; 13 Hz); 0,96 (td, $J=3,9$; 13,6 Hz)	37,28	CH_2	37,21	C2, C5, C18, C19
4	-	40,74	C	40,66	
5	1,27 (dd, $J=2$; 13 Hz)	57,78	CH	57,71	C6, C9, C10, C18, C19, C20
6	1,86 (dt); 1,37 (td)	25,72	CH_2	25,44	C5, C7, C8, C10
7	2,42 (m) ; 2(m)	39,77	CH_2	39,68	C5, C6, C8, C17
8	-	149,32	C	127,2	
9	1,66 (m)	57,94	CH	57,86	C4, C5, C8, C11, C12, C17, C20
10	-	39,47	C	39,37	
11	1,8 (m); 1,62(m)	23,05	CH_2	22,97	C8, C9, C12, C17, C20
12	2,38 (m); 2,1 (m)	25,54	CH_2	25,64	C10, C11, C13, C14, C16
13	-	134,91	C	134,83	
14	7,34 (t, $J=1,95$ Hz)	147,71	CH	147,59	C12, C13, C15, C16
15	4,82 (d, $J=1,95$ Hz)	72,17	CH_2	72,06	C13, C14, C16
16	-	177,09	C	176,89	
17	4,86 (s); 4,63 (s)	107,36	CH_2	107,25	C7, C8, C9
18	1,03 (s)	28,41	CH_3	28,31	C2, C3, C5, C10, C19
19	4,09 (d, $J=9,7$ Hz); 3,21 (d=9,1 Hz)	73,54	CH_2	73,46	C3, C10, C18, C1'
20	0,71 (s)	15,93	CH_3	15,84	C1, C5
1'	4,18 (d, 7,8 Hz))	105,16	CH	105,07	C19
2'	3,16 (t, $J=9,1$ Hz)	75,36	CH	75,28	C1', C3', C4',
3'	3,32 (t, $J=8$ Hz)	78,33	CH	78,25	C4',
4'	3,30 (t, $J=9,05$ Hz)	71,78	CH	71,72	C6', C5'
5'	3,24 (m)	77,78	CH	77,74	4'
6'	3,85 (dd, $J=2$; 11,7 Hz) 3,69 (dd, $J=5,2$; 11,7 Hz)	62,84	CH_2	62,77	C4', C5'

* Ref²³

Study of the literature showed neoandrographolide and 14-deoxyandrographolide were found for the first time in *Vitex* genus. Andrographolide was previously found in *V. limonifolia*²⁴, and betulinic acid was also isolated from *V. trifolia*²⁵ and *V. negundo*²⁶.



Cytotoxic activity against cervix cancer cells (HeLa) was treated to 1-4. The percentage viability of isolated compounds at 25 and 50 $\mu\text{g/mL}$ is displayed at Picture 1. Andrographolide (2) gave good inhibition where the viability of HeLa cells reached 72.7% at 50 $\mu\text{g/mL}$. Compound 1, 3, and 4 had no effect at this concentration. IC_{50} value showed no potential activity of the tested compounds due to the low value of IC_{50} ($\text{IC}_{50} < 30 \mu\text{g/mL}$)²⁷.

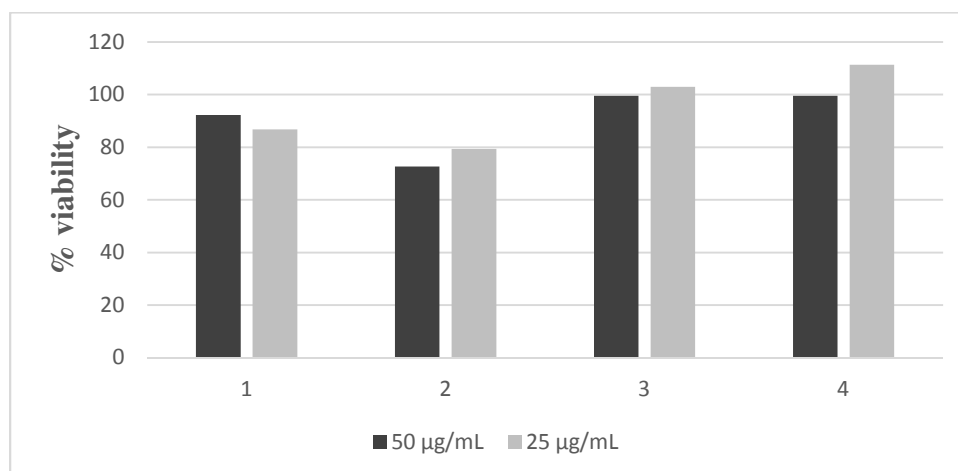


Fig.-1: Antiproliferative effects of isolated compounds of *V. pubescens* Vahl stem barks on cervix cancer cells (HeLa)

CONCLUSION

Three lactone diterpenoid compounds, neoandrographolide (1), andrographolide (2), 14-deoxyandrographolide (3), and a triterpenoid compound, betulinic acid (4), had been isolated from the stem bark of *V. pubescens* Vahl. Compound 1-4 showed no antiproliferative effect on HeLa cells with $\text{IC}_{50} > 100 \mu\text{g/mL}$.

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