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Steroids from The Stem Bark of *Dysoxylum nutans* (Meliaceae) and Their Cytotoxic Effect Against MCF-7 Breast Cancer Cell Lines

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Abstract

Three steroids, 3*α*-hydroxystigmast-5(6), 22-diene-7-one (**1**), stigmasterol **(2)** and 3-hydroxy-7*β*-methoxystigmast-5(6)-ene (**3**), were isolated from the stem bark of *Dysoxylum nutans*. The chemical structures were identified by spectroscopic data, which includes IR, 1D-NMR, 2D-NMR, and HR-TOFMS as well as by comparing previously reported spectral data. Compounds **1-3** were tested for cytotoxic effect against MCF-7 breast cancer cell lines and compound **1** showed the strongest cytotoxic activity with an IC_{50} value of 20.13 ± 0.06 µM.

Keywords: Cytotoxic activity, *Dysoxylum nutans*, MCF-7 breast cancer cells, Meliaceae, stigmastane-type steroids.

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INTRODUCTION

The genus *Dysoxylum* belongs to the Meliaceae family, which consists over 80 species (Hu *et al*., 2014a), that are widely distributed in India, China, Malaysia, Indonesia, Australia, and New Zealand (Luo *et al*., 2002; Cao *et al*., 2013). In addition, it is rich in limonoids (Zhou *et al*., 2015; Han *et al*., 2015), tirucallane-type triterpenoids (Hu *et al*., 2014a; Luo *et al*., 2002; Huang *et al*., 2011), lanostane-type triterpenoids (Jiang *et al*., 2015; Zou *et al*., 2017; Tang *et al*., 2012), dammarane-type triterpenoids (Cao *et al*., 2013; Yan *et al*., 2014a), and steroids (Yan *et al*., 2014a; Wah *et al*., 2013, Govindachari *et al*., 1999).

Previous investigation reported that compounds isolated from the genus Dysoxylum exhibit diverse biological activities, which includes antitumor (Cao *et al*., 2013), antimicrobial (Gopalakrishnan *et al*., 2015), antibacterial (Hu *et al*., 2014b), antiparasitic (Lakhsmi *et al*., 2007), post-coital contraceptive (Das *et al*., 2013), and cytotoxic (Han *et al*., 2015; Ragasa *et al*., 2014; Kurimoto *et al*., 2011; Zhang *et al*., 2010; Ismail *et al*., 2009; Farabi *et al*., 2017).

As part of our investigation for anticancer substances from Indonesian *Dysoxylum* plants, methanol extract from dysoxyl nutans showed strong cytotoxic activity against MCF-7 breast cancer cell lines *in vitro*. *D. nutans, which is a high* plant and widely distributed in South East Asia (Luo *et al*., 2002; Cao *et al*., 2013). The plant is used in Indonesian for traditional medicine for fevers, infected wounds and skin diseases (Heyne, 1982). Although secondary

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metabolites of other *Dysoxylum* species have already been investigated, the phytochemical investigation of *D. nutans* has not yet been reported. The isolation, structure determination and cytotoxic effect of these isolated compounds are described.

MATERIAL AND METHODS

General Experimental Procedure

Melting points were measured using an IA9000 electrothermal melting point apparatus (Bibby Scientific Limited, Staffordshire, UK). The optical rotations were recorded on a Perkin-Elmer 341 polarimeter (Waltham, MA, USA). The UV spectra was obtained using a TECAN Infinite M200 pro, with methanol (Switzerland). The IR was recorded on a SHIMADZU IR Prestige-21 in KBr (Kyoto, Japan). Mass spectra were measured using a Water QTOF HR-MS XEV^{otm} mass spectrometer (Waters, Milford, MA, USA). The NMR data were recorded on Bruker 600 MHz (Billerica, MA, USA) and JEOL ECZ-600 spectrometer (Kyoto, Japan) at 800 MHz for ^{1}H and 150 MHz using tetramethylsilane as an internal standard. Column chromatography was conducted on silica gel 60 (70-230 mesh and 230-400 Mesh) (Merck, Darmstadt, Germany). TLC plates were precoated with silica gel GF_{254} (Merck, Darmstadt, Germany 0.25 mm) and evidence was obtained by spraying with 10% sulphuric acid in ethanol, followed by heating.

Plant Material

The stem bark of *D. nutans* was obtained in Bogor Botanical Garden, West Java Province, Indonesia in August 2017. The plant specimen was deposited at Herbarium with collection number, III. F. 98.

Extraction and Isolation

The dried grounded stem bark (900.0 g) was extracted using methanol exhaustively (10 L) at room temperature for 5 days. Removal of the solvent on a rotary evaporator gives an extract of concentrated methanol (111.6 g). The concentrated methanol extract was first suspended in water and sequentially separated using *n*-hexane and ethyl acetate, and directly evaporated to give *n*-hexane (20.5 g) and ethyl acetate (10.5 g), respectively. The *n*-hexane soluble fraction (20.0 g) was fractionated by vacuum liquid chromatography (VLC) on silica gel using a gradient *n*-hexane-ethyl acetate to give 8 fractions (A–H). Fraction E (3.9 g) was separated by column chromatography on silica gel using 3% mixtures of *n*-hexane-ethyl acetate as eluting solvents (100:0–70:30) to give 8 sub-fractions (E1-E8). Sub-fraction E5 (1.1 g) was further separated by column chromatography on silica gel, with *n*-hexane- ethyl acetate (2% stepwise) as solvent system to give 7 sub-fractions (E5a-E5g). Similarly, sub-fraction E5e (0.1 g) was separated by column chromatography on silica gel, with 1% mixtures of *n*-hexane- ethyl acetate as a solvent (100:0-80:20) to give (13.0 mg). Sub-fraction E5f was separated by column chromatography on silica gel, with *n*-hexane: ethyl acetate (8:1) as a solvent to give **2** (3.0 mg).

The ethyl acetate extract (10.5 g) was separated by vacuum liquid chromatography with 10% mixture of *n*-hexane-ethyl acetate-methanol (10:0-7:3) as a solvent to give 4 fractions (A-D). Fraction D (4.6 g) was separated by column chromatographed on silica gel with chloroform-ethyl acetat (9:1) as a solvent system to give **3** (2.0 mg).

Bioassays of Cytotoxic Activity (Skehan *et al.*, 1990)

MCF-7 cells were grown in 96-well plates with initial cell densities of approximately 3 x 10^4 cm^{-3}. After 24 hours of incubation for cell growth, various concentrations of the sample were added. Furthermore, the sample was first dissolved in DMSO at the required concentration. The next six desired concentrations were prepared using PBS (phosphorus buffer solution, pH = 7.30 - 7.65). The control wells only accept DMSO, and the test was stopped after an incubation period of 48 hours by adding PretoBlueTM Cell Viability Reagent and the incubation was further continued for 1-2 hours until the color change is observed. Optical density was read using a micro plate reader at 570 nm. IC_{50} values were taken from cell charts of the percentage life plotted compared to the control (%), and the concentration of the tested compounds (μM). An IC_{50} value is the concentration needed to inhibit 50% growth. Each test and analysis was carried out in triplicate and average.

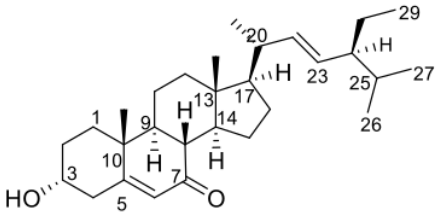
3. RESULTS AND DISCUSSION

The concentrated methanol extract from dried stem bark of *D. nutans* was extracted with *n*-hexane and ethyl acetate. The *n*-hexane extract was separated by vacuum-liquid chromatography (VLC) on silica gel 60 by

gradient elution. The VLC fraction was separated by column chromatography on silica gel to give compounds **1-2**. The ethyl acetate was prepared as described for compounds **1-2** and give compound **3** (Figure 1).

3*α*-hydroxystigmast-5(6),22-diene-7-one (1)

White crystal; m.p. 138-140 °C; $[\alpha]^{28.4}_{D}$ - 0.67° (*c* 0.3, $CHCl_3$); IR (KBr) ν_{max} 3438, 2926, 1736, 1462, 1040 cm^{-1}; NMR ($CDCl_3$, 600 MHz for ^{1}H-NMR and 150 MHz for ^{13}C-NMR) see Table 1; HR-TOFMS *m/z* 449.3553 $[M+Na]^+$ (Calcd. for $C_{29}H_{46}O_2$, *m/z* 426.3558).



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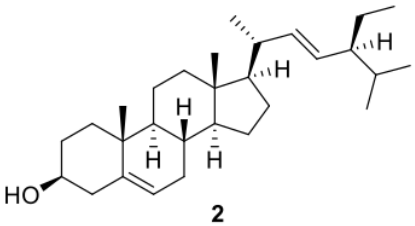

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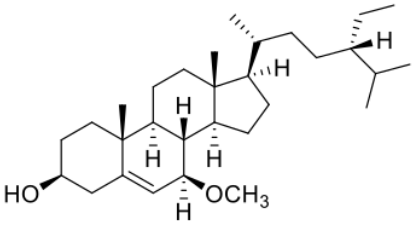

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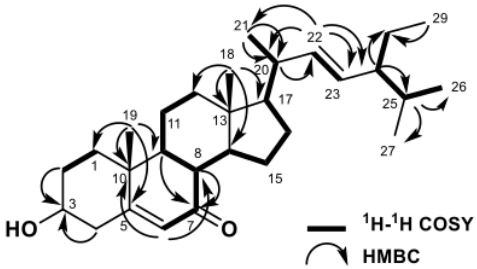
Figure 1. Structures of Compounds **1**-**3**.

Compound **1** was obtained as a white crystal with m.p. 138-140 °C and $[\alpha]^{D}_{28.4}$ - 0,67° (*c* 0.3; $CHCl_3$). Its molecular composition was determined as $C_{29}H_{46}O_2$ by HR-TOFMS spectrum *m/z* 449.3553 $[M+Na]^+$ along with IR data (Table 1), which indicates seven degrees of unsaturation. The UV spectrum shows no conjugated double bonds with maximum absorption above 200 nm. The IR spectrum showed absorption band corresponding to the hydroxyl (3423 cm^{-1}), aliphatic (2926 cm^{-1}), carbonyl (1736 cm^{-1}), olefinic (1468 cm^{-1}), and C-O bond from alcohol (1040 cm^{-1}). The ^{1}H-NMR spectrum showed the presence of 6 methyl groups, which consists of 2 protons resonating at δ_H 0.55 (Me-18) and 1.05 (Me-19) as *singlet*, 3 methyl at δ_H 0.71 (3H, *d*, *J* =3.6 Hz, Me-21), 0.70 (3H, *d*, *J* =6.5 Hz, Me-26), 0.88 (3H, *d*, *J* =6.5 Hz, Me-27) as *doublet* and one at δ_H 0.90 (3H, *d*, *J* = 3.6, Me-29), as *triplet*. Three olefinic protons at δ_H 5.55 (1H, *d*, *J* = 1.6, H-6), 5.11 (1H, *dd*, *J* =15.2 Hz, H-22) and 5.39 (1H, *dd*, *J* = 8.6, 15.2 Hz, H-23) as well as an oxymethine proton at δ_H 3.54 (*br.s*, H-7) were also observed in the ^{1}H-NMR spectrum. The ^{13}C-NMR together with DEPT spectra showed twenty nine carbon signals, which includes six methyls, eight methylenes, eight methines (including one oxygenated sp^3 carbons at δ_C 70.5), three sp^2 methines (δ_C 126.1, 138.0, 129.4), two sp^3 quaternary carbons, one sp^2 quaternary carbons (δ_C 165.7) and 1 carbonyl at δ_C 202.3. These unsaturation were calculated for eight out of the total seven degrees of unsaturation. All four degrees of unsaturation were consistent with the structure of tetracyclic stigmastane with additional carbonyl and olefin groups (Huang *et al*., 2009; Yan *et al*, 2014b).



1

Figure 2. Selected HMBC and ^{1}H-^{1}H COSY correlations for **1**.

A detailed comparison of the NMR data of 1 with those of 3-hydroxystigmast-4,22-diene-7-one, isolated from *Hedyotis diffusa* (Cayme & Ragasa, 2004), exhibited that the structures of the two compounds are very similar. The detail structure of **1** was supported from the ^{1}H-^{1}H COSY and HMBC experiments (Figure 2). The ^{1}H-^{1}H COSY spectrum of compound **1** showed correlations in H_1-H_2, H_6-H_7-H_8-H_9-H_{11}-H_{12}, H_{14}-H_{15}-H_{16}-H_{17}-H_{20}-H_{22}-H_{23}-

H_{24}-H_{25}-H_{26}, supporting the presence of stigmastane structure in compound **1**. In the HMBC spectrum, the correlation of methyl protons to their neighboring carbons can influence the six methyls at C-10, C-13, C-20, C-25 (2 ×), and C-29, respectively. The HMBC cross peak of the methylene protons at H-2 (δ_H 1.48 and 1.80) and H-4 (δ_H 2.10 and 2.13) on an oxygenated carbon at δ_C 70.5 (C-3), indicated the hydroxyl group is located at C-3. Correlation from methine proton δ_H 1.91 (H-8) and 1.20 (H-9) as well as an olefinic proton at δ_H 5.55 to δ_C 202.3 (C-7) were used to assign a carbonyl group is located at C-7.

Table 1. NMR data for **1** (600 MHz for ^{1}H and 150 MHz for ^{13}C in $CDCl_3$).

No	1				3-hydroxystigmasta-4,22-dien-7-one (Cayme & Ragasa, 2004)	
	^{13}C-NMR	^{1}H-NMR	HMBC	COSY	^{13}C-NMR	^{1}H-NMR
	δ_C/ppm	δ_C/Mult (J/Hz)			δ_C/ppm	δ_C/Mult (J/Hz)
1	38.5	1.00 *m*		2	36.9	1.90 *m*
		1.89 *m*				1.22 *m*
2	31.1	1.48 *m*	3	1	31.8	1.54 *m*
		1.80 *m*				1.57 *m*
3	70.5	3.54 *brs*			72.9	3.26 *brs*
4	45.4	2.10 *d* (2.34)	3		41.5	2.33 *d* (2.35)
		2.13 *d* (2.34)				2.30 *d* (2.35)
5	165.7	-			166.5	-
6	126.1	5.55 d (1.56)	8,9,10	7	126.8	5.86 *d* (1.58)
7	202.3	-		6,8	200.8	-
8	40.2	1.91 *m*	7	7,9,14	45.7	1.53 *m*
9	49.9	1.20 *m*	7	8,11	50.3	1.37 *m*
10	36.0	-			38.9	-
11	26.0	2.19 *m*		9,12	21.3	1.61 *m*
		0.99 *m*				1.61 *m*
12	39.0	1.83 *dt* (13.03 & 2.28)		11	39.8	2.05 *dt* (13.03 & 2.28)
		0.93 *m*				1.24 *m*
13	43.2	-			43.6	-
14	54.7	0.96 *m*		8,15	50.4	1.53 *m*
15	26.3	1.02 *m*		14,16	25.4	1.81 *m*
		2.18 *m*				1.16 *m*
16	40.2	1.85 *m*		15,17	25.6	1.82 *m*
		1.36 *m*				1.36 *m*
17	49.9	1.20 *m*		16,20	56.5	1.27 *m*
18	11.9	0.55 *s*	12,13,14,17		12.5	1.02 *s*
19	17.3	1.05 *s*	1,5,9,10		19.8	1.32 *s*
20	39.7	0.97 *m*		17,21	36.3	2.13 *m*
21	21.2	0.71 *d* (3,6)	17,20,22	20	21.4	0.98 *d* (3.6)
22	138.0	5.11 *dd* (8.7 & 15.12)	20,21,23,24	23	138.8	5.48 *dd* (8.7 & 15.12)
23	129.4	4.89 *dd* (8.7 & 15.12)		22	129.9	5.06 *dd* (8.7 & 15.12)
24	50.3	2.36 *m*	25,28	25,28	52.3	1.87 *m*
25	33.9	0.89 *m*	26,27	24,26	26.5	1.60 *m*
26	19.7	0.70 *d* (6.48)		25	21.1	0.92 *d* (6.48)
27	19.0	0.88 *d* (6.48)			19.5	0.91 *d* (6.48)
28	21.2	1.85 *m*		24	34.6	0.90 *m*
		0.63 *m*			36.6	1.71 *m*
29	21.0	0.90 *t* (3.60)			21.5	0.89 *t* (3.60)

The stereochemistry of 1 was identified by a NOESY experiment (Figure 3), in which the NOESY correlations between Me-19 and H-3 indicated that the C-3 hydroxyl group is α-oriented. Similar to the NOESY observations, the cross peak between Me-18 and H-20, indicated that Me-21 was *α*-oriented. Furthermore, the NOESY cross peak, which was also observed between Me-21 / H-17, showed that the side chain at C-17 was β-oriented. In addition, the correlation between H-24 and H-7, indicated that an ethyl chain was *β*-oriented. Therefore, the structure of compound **1** was determined to be 3*α*-hydroxystigmast-5(6),22-diene-7-one.

The known compounds stigmasterol (**2**) (Ragasa *et al*., 2014) and 3-hydroxystigmast-7*β*-metoxy-5(6)-en (**3**) (Pettit *et al*., 2000) were identified by comparison with spectroscopic data with reported value. The presence of three steroids suggested that *Dysoxylum* genus can produce the steroid as one of the chemical markers.

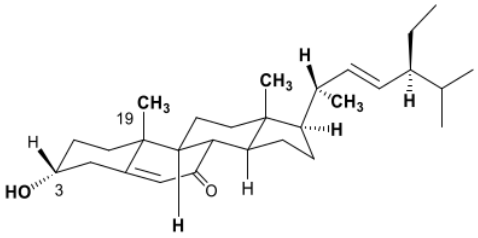


Figure 3. Selected NOESY correlations for **1**.

The cytotoxic effect of the three isolated compounds **1**-**3** were conducted against MCF-7 breast cancer cells according to a modified method previously described (Skehan *et al*., 1990), using Cisplatin as a positive control, IC_{50} 3.20 mg/mL (Supratman *et al*., 2019; Hadisaputri *et al*., 2012). Furthermore, compound **1**-**3**, showed cytotoxic activity with IC_{50} values of 20.13±0.06, 100.28±0.06 and 26.35±0.04 *μ*M respectively. The presence of carbonyl or methoxy group at the C-7 position increases the cytotoxic activity, replacing 7-OH on compound **1** with 7-OMe on compound **3** slightly reduces reactivity (Simon *et al*., 1998).

4. CONCLUSIONS

Three steroids, 3α-Hydroxystigmast-5 (6), 22-Dien-7-en (1), as well as two well-known steroids, Stigmasterol (2) and 3-Hydroxy-7β-methoxystigmast-5 (6) -one (3) was isolated from the stem back of *D*. *nutans*. Compound **1** showed the strongest cytotoxic activity with an IC_{50} value of of 20.13 ± 0.06 μM.

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