Triterpenoids from Stem Bark of *Dysoxylum excelsum* and Their Cytotoxic Activity against MCF-7 Breast Cancer Cells

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Abstract: *The diversity of structures of triterpenoids provides a variety of interesting pharmacological effects, one of them being anticancer. Sources of triterpenoids are found in* Dysoxylum excelsum*, it is still rarely studied, and it potentially contains triterpenoids with cytotoxic activity. Therefore, the research aimed to isolate the triterpenoids from the stem bark of* D. excelsum *and determine their cytotoxic efficacy against the breast cancer cells of MCF-7. The dried stem bark of* D. excelsum *was macerated using methanol and then fractionated with* n*-hexane and ethyl acetate to obtain the corresponding fractions. Two triterpenoids, 3α,7α-dihydroxyapotirucalla-14,24-Z-dien-26-oic acid (**1**) and masticadienonic acid or 3-oxo-tirucalla-7,24-Z-dien-26-oic acid (**2**) were isolated in the first place from the* Dysoxylum *genus. Compounds **1** and **2** were assayed for their cytotoxic potency anti- MCF-7 using the MTT assay and gave moderate IC_{50} values of 60.87 and 72.86 μM, respectively. Based on their analogs, partial structure modification of the compounds might increase the activity; thus, they can be further utilized as better lead compounds for anticancer drugs candidate.*

Keywords: Dysoxylum excelsum*; triterpenoids; Meliaceae; cytotoxic; MCF-7 breast cancer*

■ INTRODUCTION

Of the known Meliaceae family, around 200 species of the *Dysoxylum* genus are distributed widely in India, China, Australia, and Southeast Asia, including Indonesia [1-2]. Diverse compound groups of this genus, for instance, alkaloids [3-4], limonoids [5-7], steroids [8], sesquiterpenoids [9-11], diterpenoids [12-14], triterpenoids [15-19], and triterpenoid glycosides [20] mostly have been elucidated. Furthermore, numerous bioactive compounds such as central nervous system depressants, anti-respiratory syncytial virus, antifeeding, antitumor triterpenoid glycosides [20], anti-inflammatory [3,17,21-22], antirheumatic, and cardiac-active alkaloids [5], cytotoxic [23-28], antimicrobial [2,18-29], and antibacterial [30-31] have been isolated from *Dysoxylum* plants.

D. excelsum Blume is a plant with a tall tree distributed in Asia, yet it has been barely investigated on its chemical constituents and cytotoxic activity [32]. In the previous finding, Zainuddin et al. [33] isolated a tirucallane skeleton-type of triterpenoid, masticadienolic acid, from a similar natural source as we reported here. A potent cytotoxic property against the breast cancer cells of MCF-7 was exhibited by masticadienolic acid (IC_{50} 3.5 μm). To obtain prospective lead compound models as cancer drug candidates, exploration of other cytotoxic compounds in this species needs to be done. Perhaps, it might further inspire the

development of synthetic cancer drugs based on their structure-activity relationship (SAR).

In this article, we reported the isolation and structural elucidation of two triterpenoids, 3α,7α-dihydroxyapotirucalla-14,24-*Z*-dien-26-oic acid (**1**) and masticadienonic acid or 3-oxo-tirucalla-7,24-*Z*-dien-26-oic acid (**2**) which have been reported for the first-ever in the *D. excelsum*. Furthermore, compounds **1**-**2** were *in vitro* assayed cytotoxic potential against the breast cancer cells of MCF-7 using a microtetrazolium (MTT) assay.

■ EXPERIMENTAL SECTION

Materials

The stem bark of *D. excelsum* was obtained from Bogor Botanical Garden, Bogor, West Java, Indonesia, in June 2016. Its taxonomy was determined in the Laboratory of Bogoriense Herbarium, Indonesia (collect. No III. F. 67).

Instrumentation

A Perkin-Elmer spectrum-100 FTIR was employed to record the IR spectra on a KBr Disk. The high-resolution mass was analyzed with a QTOF MS of Waters Xevo. Comprehensive NMR spectra, together with ^{1}H-^{1}H COSY, HSQC, HMBC, and ROESY experiments, were measured on a 500 MHz NMR spectrometer of Agilent with a DD2 console system using a TMS internal standard. Column chromatography matrix was using silica gel 60 with the mesh size of 70–230 and 230–400 (Merck) and chromatorex ODS (Fuji Silysia Chemical, Japan), while a TLC plate was based on aluminium silica gel-coated with fluorescent indicator F_{254} (Merck, 0.25 mm. To visualize the TLC spots, a staining agent of 10% ethanolic H_2SO_4 (v/v) was sprayed and subsequently heated and irradiated under UV/Vis light at 254 and 365 nm.

Procedure

Preparation of extracts

The air-dried stem bark of *D. excelsum* (2.7 kg) was ground and entirely macerated with MeOH (5 L) at ambient temperature (± 25 °C) for 3 days. The filtrated methanolic extract was then concentrated under a rotary vacuum at a temperature of ± 40 °C. After evaporation of the solvent at vacuo, the crude methanolic extract (300 g) was suspended in water (400 mL) and then successively partitioned with *n*-hexane (3 × 100 mL) and EtOAc (3 × 100 mL) to yield the corresponding extract of 16.2 and 102.7 g, respectively.

Isolation of triterpenoids

The EtOAc fraction (102.7 g) was separated on a vacuum-liquid chromatography (VLC) loaded with dry silica gel 60 (230–400 mesh) and eluted gradient using *n*-hexane and EtOAc (10:0–0:10, v/v) to result in 10 fractions (A-J). Fraction C (6.37 g) was then purified on a column chromatography (silica gel 70–230 mesh, eluent *n*-hexane:EtOAc (10:0–8:2, v/v)) to obtain 16 fractions (C1-C16). Further purification was employed for the subfraction of C14 (362.8 mg) using a silica gel 60 column chromatography (eluent *n*-hexane:EtOAc (10:0–5:5, v/v) to yield **2** (109.9 mg). On the other hand, fraction E (15.15 g) was separated on a VLC column packed with dry silica gel 60 (eluent *n*-hexane:EtOAc (10:0–3:7, v/v) to afford 9 fractions (E1-E9). Extra purification of the E8 fraction (127.0 mg) was accomplished on silica gel 60 column chromatography with eluent of *n*-hexane:EtOAc (10:0–0:10, v/v) to give 12 subfractions (E8a-E8l). Finally, the compound **1** (12.9 mg) was obtained from the subfraction of E8f (36.6 mg), after additional purification on ODS column chromatography (eluent MeOH:MeCN:H_2O (7:2:1, v/v/v).

Determination of cytotoxic activity using MTT assay

Initially, the MCF-7 breast cancer cells were attached and cultivated into 96-well plates at 3×10^4 cells cm^{-3} density and then incubated for 24 h. Afterwards, various concentrations of samples dissolved in DMSO were added to the well plates. Later, six desirable concentrations were prepared at buffered pH of 7.30–7.65 (phosphoric buffer solution). The well plates of negative control contained only DMSO, and positive control of doxorubicin was also prepared. Once a 48-h incubation period was finished, the assay was quenched by MTT reagent [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide]. An MTT-quencher dissolved in SDS (sodium dodecyl sulfate) was then dropped to the plates for further 2–4 h incubation, followed by final incubation for another 24 h. Upon

scanning of a microplate reader at 450 nm, the optical density of the samples was obtained. Based on the linear regression of the corrected percentage live of cells (%) versus the tested concentration of compounds (μg/mL), the IC_{50} values were computed. Each assay was conducted three times, and their analysis results were reported as averaged IC_{50} values with relative standard deviation.

■ RESULTS AND DISCUSSION

Compound **1** was isolated as a white crystal. The molecular formula calculated by HRTOFMS ($[M+Na]^+$) was found as $C_{30}H_{48}O_4$ with *m/z* 495.3459, calcd. 495.3450. Based on the MS result supported by NMR data (Table 1), the unsaturation degree of compound **1** was confirmed at seven. The IR spectrum revealed the moieties of hydroxyl at 3445 cm^{-1}, an α,β-unsaturated carbonyl at 1692 cm^{-1}, and olefinic carbon at 1640 cm^{-1}. The ^{1}H-NMR spectrum (Table 1) discovered methyl signals as six singlets at δ_H/ppm 0.82, 0.89, 0.90, 1.04, 1.06, and 1.86, and one doublet at δ_H/ppm 0.95 (3H, d, *J* = 6.5 Hz, H-21). Two olefinic methines at δ_H/ppm 5.42 (1H, dd, *J* = 1.5, 3.6 Hz, H-15) and 5.95 (1H, t, *J* = 7.4 Hz, H-24), as well as two oxymethine signals at δ_H/ppm 3.32 (1H, t, *J* = 3.0 Hz, H-3) and 3.87 (1H, t, *J* = 3.1 Hz, H-7), were also assigned. In addition, the ^{13}C-NMR data showed 30 varied chemical shifts of carbon (Table 1). Being supported by the HSQC data, the carbon signals were representing seven methyls at δ_C/ppm 15.8 (C-19), 18.9 (C-18), 19.0 (C-21), 21.1 (C-27), 22.6 (C-29), 28.3 (C-30), and 28.8 (C-28), eight methylenes at δ_C/ppm 17.3 (C-11), 24.9 (C-6), 26.3 (C-2), 26.9 (C-23), 33.5 (C-1), 35.3 (C-12), 35.6 (C-16), and 36.1 (C-22), eight methines at δ_C/ppm 34.4 (C-20), 40.8 (C-5), 42.6 (C-9), 61.3 (C-17), 73.2 (C-7), 75.9 (C-3), 120.0 (C-15), and 143.6 (C-24), six quarternary carbons at δ_C/ppm 37.7 (C-4), 38.4 (C-10), 45.0 (C-8), 47.6 (C-13), 128.0 (C-25), 162.8 (C-14), and one carbonyl at δ_C/ppm 169.4 (C-26). Therefore, the ^{1}H- and ^{13}C-NMR data implied the presence of a triterpenoid tetracyclic skeleton in **1** (Fig. 1). To prove the existence of the skeleton (A, B, C, and D) in structure **1**, spectra of ^{1}H–^{1}H COSY and HMBC were recorded (Fig. 2). The correlations between H_1-H_2-H_3, H_5-H_6-H_7, H_9-H_{11}-H_{12}, H_{15}-H_{16}-H_{17}-H_{20}, and H_{21}-H_{20}-H_{22}-H_{23} were observed in the ^{1}H–^{1}H COSY spectrum of **1**, thus supporting the occurrence of triterpenoid tetracyclic structure in **1**. Additionally, the HMBC correlations of the methyl protons to their nearby carbons supported the interpretation of proton signals of the six singlets of methyl and one of secondary methyl. All anticipated correlations (CH_3-18 → C-13, 12, 14, 17; CH_3-19 → C-10, 1, 5, 9; and CH_3-30 → C-8, 7, 9, 14) revealed strong cross-peaks. An olefinic moiety was discovered by the HMBC correlations of H-16 to C-14 at δ_C/ppm 162.8 and C-15 at δ_C/ppm 120.0, and H-15 to C-13 at δ_C/ppm 47.6, C-16 at δ_C/ppm 35.6, and C-17 at δ_C/ppm 61.3. Similarly, the appearance of α,β-unsaturated carboxylic acid was also revealed by HMBC correlation signals of H-24 to C-27 at δ_C/ppm 21.1, C-22 at δ_C/ppm 36.1, and H-27 to C-24 at δ_C/ppm 143.6, C-25 at δ_C/ppm 128.0, C-26 at δ_C/ppm 169.4. Other correlations from CH_3-28, CH_3-29 to C-3 at δ_C/ppm 75.9, and from CH_3-30 to C-7 at δ_C/ppm 73.2, implied that hydroxy groups were located at C-3 and C-7, respectively. Furthermore, the presence of a side-chain structure of **1** was confirmed by the HMBC cross-peaks of CH_3-21 to C-17 at δ_C/ppm 61.3, C-20 at δ_C/ppm 34.4, and C-22 at δ_C/ppm 36.1. To resolve the relative stereochemical configuration of **1**, the analysis of coupling constants and the rotating-frame nuclear overhauser effect spectroscopy (ROESY) experiment were conducted (Fig. 3). The coupling constants of H-3 at δ_H/ppm 3.32 (t, *J* = 3.0 Hz) and H-7 at δ_H/ppm 3.87 (t, *J* = 3.1 Hz) proposed their orientation in an equatorial position and *β*-oriented [34-35]. Moreover, based on the ROESY spectrum, the correlations of H-3/CH_3-19/CH_3-29 and H-7/CH_3-30 further verified the *α*-configuration of the hydroxy moieties at C-3 and C-7. In addition, the ROESY correlations of CH_3-28/H-5 directed the *α*-configuration of CH_3-28. Correlations of H-9/CH_3-18 and CH_3-18/H-20 indicated the *α*-orientation of H-20, and the correlations of H-17/H-12*β* and H-12*β*/CH_3-21 designated *β*-orientation of CH_3-21 which was in agreement with those of apotirucallane-type triterpenoids. The configuration of the *Z* double bond among C-24 at δ_C/ppm 143.6 and C-25 at δ_C/ppm 128.0 was resolved by the ROESY correlation between H-24 at δ_H/ppm 5.95 (t, *J* = 7.4 Hz) and CH_3-27 at δ_H/ppm 1.86 (s) [2]. Overall, the structure of compound **1** had an

apotirucallane skeleton. Instead of the configuration of CH_3-21, most of the NMR data of **1** resembled previously reported chisopatens C, an apoeuphane triterpenoid [36]. Consequently, compound **1** was determined as 3*α*,7*α*-dihydroxyapotirucalla-14,24-*Z*-dien-26-oic acid (Fig. 1), which was isolated from this genus for the first ever. The configuration of the tirucallane-type and euphane-type triterpenoids is distinguished by H-21. The *β*-configuration is tirucallane-type, whereas the *α* one is euphane-type [37].

Table 1. ^{1}H and ^{13}C-NMR data of **1** in $(CD_3)_2CO$ and **2** in $CDCl_3$ (δ in ppm, 500 MHz for ^{1}H, and 125 MHz for ^{13}C)[a]

Position	**1**		**2**	
	δ_C	δ_H (ΣH, mult., *J*/Hz)	δ_C	δ_H (ΣH, mult., *J*/Hz)
1	33.5	1.26 (1H, dt, 3.0, 12.6)	38.7	1.46 (1H, td, 4.8, 14.3)
		1.49 (1H, td, 3.4, 10.5)		1.99 (1H, dt, 2.9, 13.5)
2	26.3	1.50 (1H, m)	35.1	2.73 (1H, dt, 3.6, 14.3)
		1.93 (1H, m)		2.75 (1H, td, 5.4, 14.5)
3	75.9	3.32 (1H, t, 3.0)	217.1	-
4	37.7	-	48.0	-
5	40.8	2.14 (1H, dd, 2.6, 12.8)	52.5	1.72 (1H, t, 8.7)
6	24.9	1.63 (1H, td, 3.5, 10.1)	24.5	2.09 (1H, m)
		1.72 (1H, dt, 2.6, 13.0)		2.16 (1H, m)
7	73.2	3.87 (1H, t, 3.1)	118.0	5.30 (1H, dd, 3.3, 6.6)
8	45.0	-	146.1	-
9	42.6	2.07 (1H, m)	48.6	2.30 (1H, m)
10	38.4	-	35.2	-
11	17.3	1.48 (1H, m)	18.5	1.56 (2H, m)
		1.69 (1H, m)		
12	35.3	1.48 (1H, m)	33.8	1.66 (1H, m)
		1.96 (1H, m)		1.81 (1H, m)
13	47.6	-	43.7	-
14	162.8	-	51.3	-
15	120.0	5.42 (1H, dd, 1.5, 3.6)	34.2	1.49 (2H, m)
16	35.6	2.02 (1H, m)	28.4	1.29 (1H, m)
		2.27 (1H, m)		1.95 (1H, m)
17	61.3	1.46 (1H, m)	53.0	1.50 (1H, m)
18	18.9	1.04 (3H, s)	22.1	0.81 (3H, s)
19	15.8	0.90 (3H, s)	12.9	1.00 (3H, s)
20	34.4	1.63 (1H, m)	36.2	1.41 (1H, m)
21	19.0	0.95 (3H, d, 6.5)	18.4	0.89 (3H, d, 6.4)
22	36.1	1.19 (1H, m)	35.8	1.16 (1H, m)
		1.56 (1H, m)		1.55 (1H, m)
23	26.9	2.43 (1H, m)	27.1	2.45 (1H, m)
		2.51 (1H, m)		2.50 (1H, m)
24	143.6	5.95 (1H, t, 7.4)	147.3	6.08 (1H, t, 7.4)
25	128.0	-	126.0	-
26	169.4	-	173.0	-
27	21.1	1.86 (3H, s)	20.7	1.92 (3H, s)
28	28.8	0.89 (3H, s)	21.8	1.11 (3H, s)
29	22.6	0.82 (3H, s)	24.7	1.05 (3H, s)
30	28.3	1.06 (3H, s)	27.6	1.01 (3H, s)

[a] the assignments were based on correlation experiments of ^{1}H-^{1}H COSY, HSQC, and HMBC

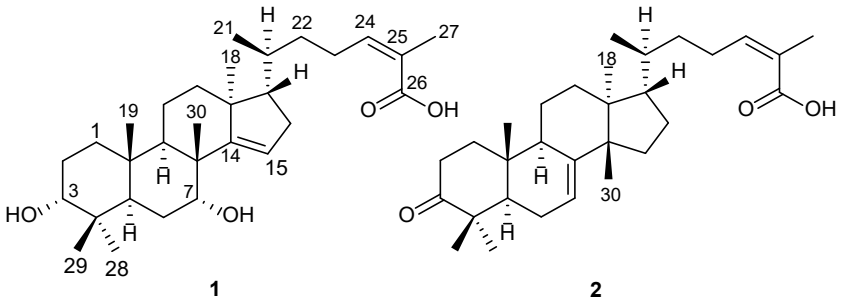

Fig 1. The structures of **1**-**2** isolated from *D. excelsum*

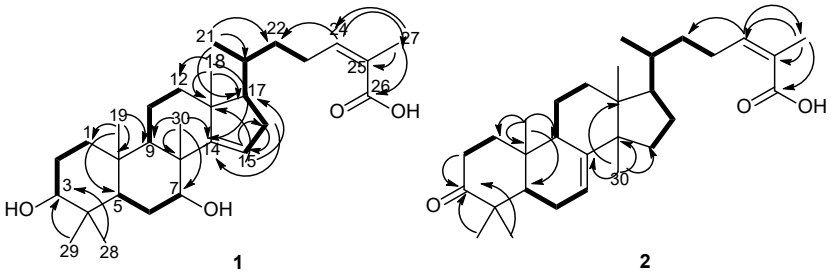

Fig 2. Key HMBC (⟶) correlations of **1**-**2**

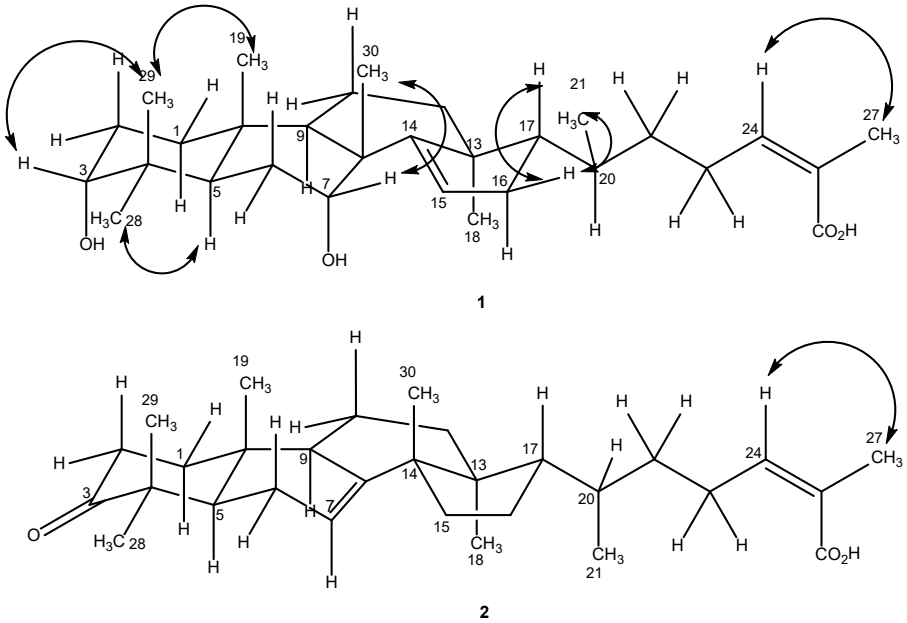

Fig 3. Key ROESY (⇢) correlations of **1**-**2**

Compound **2** was acquired as colorless needle-like crystals. The mass spectrum recorded by HRTOFMS ($[M+Na]^+$) showed the molecular formula as $C_{30}H_{46}O_3$ with *m/z* 477.3311, calcd. 477.3345. Being supported by NMR data (Table 1), the unsaturation of the compound was found by MS at eight degrees. Three main functional groups, i.e., a carbonyl ketone (1706 cm^{-1}), an α,β-unsaturated carbonyl (1678 cm^{-1}), and olefinic groups (1636 cm^{-1}) were revealed by the IR spectrum. The ^{1}H-NMR spectrum displayed nine proton signals consisting of six tertiary methyl moieties at δ_H/ppm 0.81, 1.00, 1.01, 1.05, 1.11, and 1.92, and one secondary methyl moiety at δ_H/ppm 0.89 (3H, d, *J* = 6.4 Hz, H-21), and two olefinic methines at δ_H/ppm 5.30 (1H, dd, *J* = 3.3, 6.6 Hz, H-7)

and 6.08 (1H, t, J = 7.4 Hz, H-24). On the otherside, the ^{13}C-NMR spectrometer identified 30 carbon signals (Table 1), consisted of seven methyls at δ_C/ppm 12.9 (C-19), 18.4 (C-21), 20.7 (C-27), 21.8 (C-28), 22.1 (C-18), 24.7 (C-29), and 27.6 (C-30), nine methylenes at δ_C/ppm 18.5 (C-11), 24.5 (C-6), 27.1 (C-23), 28.4 (C-16), 33.8 (C-12), 34.2 (C-15), 35.1 (C-2), 35.8 (C-22), and 38.7 (C-1), six methines at δ_C/ppm 36.2 (C-20), 48.6 (C-9), 52.5 (C-5), 53.0 (C-17), 118.0 (C-7), and 147.3 (C-24), six quarternary carbons at δ_C/ppm 35.2 (C-10), 43.7 (C-13), 48.0 (C-4), 51.3 (C-14), 126.0 (C-25), and 146.1 (C-8), and two carbonyl carbons at δ_C/ppm 173.0 (C-26), and 217.1 (C-3). The NMR evidence of compound 2 implied a tetracyclic triterpenoid skeleton (Fig. 1) emphasized by the ^{1}H–^{1}H COSY analysis (Fig. 2). The analysis revealed the correlations between H_1-H_2, H_5-H_6-H_7, H_9-H_{11}-H_{12}, H_{15}-H_{16}-H_{17}-H_{20}, and H_{21}-H_{20}-H_{22}-H_{23}-H_{24}. Based on the HMBC cross-peaks (Fig. 2), the occurrence of the carbonyl ketone at C-3 was supported by the correlation of H-28 at δ_H/ppm 1.11, H-29 at δ_H/ppm 1.05, and the methylene protons H-2 at δ_H/ppm 2.75 to the C-3 of carbonyl carbon at δ_C/ppm 217.1. The HMBC correlations of CH_3-19 at δ_H/ppm 1.00 to C-10 at δ_C/ppm 35.2, C-1 at δ_C/ppm 38.7, C-5 at δ_C/ppm 52.5, and C-9 at δ_C/ppm 48.6 verified the bonding of CH_3-19 to C-10, whereas the correlations between CH_3-30 at δ_H/ppm 1.01 and C-14 at δ_C/ppm 51.3, C-13 at δ_C/ppm 43.7, C-15 at δ_C/ppm 34.2, and C-8 at δ_C/ppm 146.1 confirmed the bonding of CH_3-30 to C-14. On the other side, the presence of HMBC correlation between CH_3-19 and C-9 at δ_C/ppm 48.6 confirmed another olefinic carbon at C-7/C-8 and olefinic of C-24/C-25. Overall, compound **2** has a similar α,β-unsaturated carboxylic acid group as confirmed in **1**. The relative configuration of **2** was further resolved by the ROESY experiment (Fig. 3). This evidence suggested that the structure of **2** has the skeleton-type of a tirucallane triterpenoid and resembles masticadienolic acid [33], and it was in agreement with masticadienonic acid [38]. Thus, compound **2** was determined as masticadienonic acid or 3-oxo-tirucalla-7,24-*Z*-dien-26-oic acid (Fig. 1).

An MTT assay to evaluate the cytotoxic potency of compounds **1**-**2** anti-MCF-7 was represented as IC_{50} values. The value will express how many substances are required to inhibit 50% of the biological process, thus revealing the activity degree of antagonist agents in pharmacological research [39]. In addition, the standard inhibitor of cancer (doxorubicin) [2] was also tested as the reference under the same condition. All the compounds (**1**-**2**) gave the IC_{50} values of 60.87 and 72.86 µM (Table 2 and Table S1, S2), respectively, which were classified as moderate cytotoxic [40]. In general, the resulted IC_{50} was roughly higher by two times than the positive control. Compound **2** exhibited weaker cytotoxic activity than **1,** which might be caused by the appearance of a carbonyl ketone moiety at C-3 that decreased the cytotoxicity [41]. Compound **1** did not show considerably better cytotoxic activity than **2**, which was suggested due to the occurrence of the hydroxyl moieties at C-3 and C-7 and the loss of olefinic moiety at C-7/C-8 [42]. Such analog compounds were reported to have various cytotoxicity. Two tirucallanes, 3β,16β,21α,25-tetrahydroxy-20,24-cyclotirucalla-7(8)-ene and 16β,21α,25-trihydroxy-20,24-cyclotirucalla-7(8)-en-3-one, displayed significant cytotoxic potency anti-MCF-7 and other cell lines with IC_{50} values of 6.64–10.55 and 8.90–12.08 µM, respectively [2]. Furthermore, five apotirucallanes exhibited moderate cytotoxic activities against MCF-7 at an IC_{50} range of 78.7–85.8 µM [23]. The SAR analysis showed that the occurrence of an aromatic heterocyclic such as pyrrole in the side chain combined with 3α-OH substituents influenced mostly the potent cytotoxicity [40].

Table 2. IC_{50} values of compounds **1** and **2** against the breast cancer cells of MCF-7

Compound	IC_{50} (µM) ± Stdv
1	60.87 ± 8.93
2	72.86 ± 5.74

Positive control: doxorubicin, IC_{50} = 35.67 µM

■ CONCLUSION

Two triterpenoids had been isolated from the stem bark of *D. excelsum*, 3*α*,7*α*-dihydroxyapotirucalla-14,24-*Z*-dien-26-oic acid (**1**) and masticadienonic acid or 3-oxo-tirucalla-7,24-*Z*-dien-26-oic acid (**2**) whereas compound **1** was found for the first time in the genus. The IC_{50} values of the compounds (**1**-**2**) against the

breast cancer cell lines of MCF-7 showed moderate cytotoxicity and were roughly two times less potent than the positive control of Doxorubicin. Compound **1** exhibited a lower IC_{50} value (60.87 μM) than **2** (72.86 μM) which might be due to the occurrence of a hydroxyl moiety at C-3. Such partial structure modification of the isolated compound with a side chain of aromatic heterocyclic and a hydroxy moiety at C-3 might increase the activity significantly. Thus, it can be further utilized as a more potential lead compound for anticancer drug candidates.

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■ AUTHOR CONTRIBUTIONS

TM, EJ, D conducted the experiment, US, DHPH, and N conducted the structure elucidation, and SRM, JAA, RM, and AZ wrote and revised the manuscript. All authors agreed to the final version of this manuscript.

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