20-Epibryonolic Acid from *Tetrameles nudiflora* Leaves

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Abstract
20-epibryonolic acid (3), a triterpenoid compound, along with two sterol compounds of β-sitosterol (1) and stigmasterol (2), were isolated from the ethyl acetate fraction of methanolic extracts of Tetrameles nudiflora leaves. The chemical structures of the compounds were elucidated using various spectroscopic methods. 20-epibryonolic acid was found to exhibit anticancer activity against P-388 murine leukemia cells with an IC_{50} value of 57.93 μg/mL.

Keywords: *Tetrameles nudiflora,* 20-epibryonolic acid, β-sitosterol, stigmasterol, P-388 murine leukemia cell lines.

Introduction

Indonesia is well known as among the mega-biodiversity countries in the world. The Wallace regions (i.e. Sulawesi, Nusa Tenggara (Southeast islands) and Maluku (Moluccas)) have a diverse biodiversity, especially for plants, some of which are endemic. Our continuous research for bioactive compounds and the discovery of material drug candidates from this biodiversity among the Wallace regions has led us to explore and collect *Tetrameles nudiflora* R. Br.

T. nudiflora R. Br. (Datiscaceae), known in Indonesia as mara, winong, or binong, is a large tree that grows up to 50 m high and has a wide distribution in India, Sri Lanka, Myanmar, South China, Thailand, Indochina, Malaysia, North Australia, Laos and Vietnam[1]. In Indonesia, these trees are widely distributed across Sumatra, Java, Sulawesi, Nusa Tenggara (Southeast Islands) and Papua[2]. According to Priyadi et al[1], in Indonesia, the wood of this tree is used for temporary construction, paneling, partitioning, cheap planking, weatherboards, mouldings, packing crates, tea chests, matchboxes.

Moreover, the bark has several traditional uses as a laxative, diuretic and to treat rheumatism, oedema[1] and itching[3], which have not been scientifically proven through scientific publication. Leishmanicidal activity[4] and toxicological evaluation[2] are the only studies that discussed the potency of this plant.

Previous chemical content investigation indicates that *T. nudiflora* was reported to contain kaempferol, quercetin 3-*O*-mono- and diglycosides[5]. The aim of this study is to explore the chemical constituents of the leaves of *T. nudiflora* using chromatography methods, to determine its chemical structure based on spectroscopic data and to determine its potential as an anticancer against P-388 murine leukemia cell lines.

Material and Methods

General experimental procedure: Liquid-liquid fractionation was conducted using a glass separation funnel. Column chromatography was carried out using E. Merck Kieselgel 60 (0.063-0.200 mm). FT-NMR spectra were recorded on JEOL JNM-ECA 500, Fisher Scientific was used for melting point analysis and ESI-QTOF-MS was measured on Biosystem Mariner Biospectrometry.

Plant material: *Tetrameles nudiflora* leaves were collected from the Mekongga forest, Kolaka District, Southeast Sulawesi, Indonesia, on March 2012 and were identified at Herbarium Bogoriense, Research Center for Biology, Indonesian Institute of Sciences, Indonesia.

Extraction and isolation: 1.28 kg of dried and powdered *T. nudiflora* leaves were macerated successively with *n*-hexane and methanol to obtain 14.9 g of *n*-hexane extract and 185.5 g of methanol extract. Methanol extract was further fractionated with *n*-hexane, ethyl acetate, *n*-butanol and water. Ethyl acetate fraction was subjected to silica gel column chromatography, eluted with gradient solvent system of *n*-heksana-etil asetat-metanol and led to the isolation of *β*-sitosterol (1)[4,5] and stigmasterol (2)[5]. From the 30 sub-fractions collected, sub-fraction 8 (SF-8) was further purified by re-crystallization using chloroform-methanol to obtain a pure compound (Compound 3).

Cytotoxic activity: Cytotoxic activity assay was conducted using an MTT assay[5,6]. P-388 murine leukemia cells were seeded into 96-well culture dishes at an initial density of approximately $3x10^4$ cells cm-3. They were incubated in a humidified CO_2 incubator for 24 h. Various concentrations of samples dissolved in DMSO were added. Six desirable sample concentrations were prepared using PBS (phosphoric buffer solution, pH = 7.30-7.65), except for the control. Assay was terminated after 48 h of incubation by adding MTT reagent [3-(4,5-dimethyazol-2-yl)-2,5-diphenyl tetrazolium bromide] and the incubation continued for the next 4 h.

The MTT-stop solution containing sodium dodecyl sulphate (SDS) was then added followed by 24 h of incubation. Optical density was measured using a microplate reader at 550 nm. IC_{50} value was obtained from the plotted graph of percentage live cells compared to control (receiving only PBS and DMSO) against tested samples in various concentrations (μg/mL).

Results and Discussion

Extraction and isolation: A two-step isolation process of the methanolic extract of *T. nudiflora* leaves using column chromatography led to the isolation of *β*-sitosterol (1) and

stigmasterol (2). A further purification step through chloroform-methanol re-crystallization process of sub-fraction 8 (SF-8) of the ethyl acetate fraction led to the isolation of another compound (compound 3).

Compound 3: White needles; mp 276-279 ^{0}C; ESI-QTOF-MS *m/z* 439 $[M-H_2O+H]^+$, 479 $[M + Na]^+$, 935 $[2M + Na]^+$. ^{1}H NMR (DMSO-d_6, 500 MHz) δ 4.29 (1H, *d*, *J* = 4.5 Hz, -OH), 2.97 (1H, *m*, H-3), 2.26 (1H, d, *J* = 15.5 Hz, H-18), 1.07 (3H, *s*, H-29), 0.96 (3H, *s*, H-28), 0.89 (3H, *s*, H-26), 0.86 (3H, *s*, H-23), 0.85 (3H, *s*, H-25), 0.82 (3H, *s*, H-27), 0.65 (3H, *s*, H-24). ^{13}C NMR (DMSO-d_6, 125 MHz) δ 180.15 (C-30), 134.27 (C-9), 133.83 (C-8), 77.31 (C-3), 50.72 (C-5), 44.69 (C-18), 41.91 (C-14), 39.83 (C-20), 38.91 (C-4), 37.64 (C-10), 37.20 (C-13), 37.21 (C-16), 35.21 (C-1), 34.49 (C-22), 33.06 (C-29), 31.55 (C-17), 31.08 (C-28), 30.51 (C-19), 30.34 (C-21), 29.92 (C-12), 28.68 (C-23), 28.17 (C-7), 27.66 (-2), 25.05 (C-15), 22.39 (C-26), 20.74 (C-11), 20.23 (C-25), 19.38 (C-6), 17.76 (C-27), 16.59 (C-24).

Compound 3, a triterpenoic acid, was recrystallized from chloroform-methanol as white needles. The mass fragmentation pattern of compound 3 was shown to be at *m/z* 439 $[M-H_2O+H]^+$, 479 $[M+Na]^+$ and 935 $[2M+Na]^+$. The ^{1}H-NMR spectrum showed a signal for eight tertiary methyl groups δ_H 1.07, 0.96, 0.89, 0.86, 0.85, 0.82 and 0.65. A hydroxyl proton signal at δ_H 4.29, a hydroxylated proton signal at δ_H 2.97 and a doublet signal for methine group at δ_H 2.26, were also present. A downfield shift of H-18 signal corresponds to its *cis* position toward carboxylic acid groups. It was supported by an HMBC cross signal between H-18 and C-30 ($^4J_{CH}$). ^{13}C- and DEPT NMR demonstrated thirty carbon peaks, eleven of which are methylene group (δ_C 37.21, 35.21, 34.49, 30.51, 30.34, 29.92, 28.17, 27.66, 25.05, 20.74, 19.38).

Cross signals in HMBC spectrum were also found between H-18 to C-20, H-29 to C-30, H-23 to C-3 and C-24, H-24 to C-3 and C-5, H-25(CH_3) to C-1 and C-9, H-26(CH_3) to C-8 and C-14, H-27 to C-12, H-28(CH_3) to C-18 and C-22 and H-29(CH_3) to C-21, as well as H-H COSY cross signal between H-3 and proton of hydroxyl group attached to C-3 (fig. 2).

A secondary hydroxylated carbon signal at δ_C 77.31 (C-3), a carbonyl carbon signal at δ_C 180.15 (C-30) and two olefinic carbon signals at δ_C 133.83 (C-8) and 134.27 (C-9), were also present. The presence of hydroxylated carbon group and carbonyl carbon, as well as the presence of two olefinic carbons, supported with NMR spectral data (HMQC, HMBC and H-H COSY), ESI-QTOF-MS *m/z* 439 $[M-H_2O+H]^+$, 479 $[M + Na]^+$, 935 $[2M + Na]^+$, as well ^{1}H- and ^{13}C-NMR spectral data comparison[7], indicated that compound **3** was 20-epibryonolic acid.

Cytotoxic activity: Anticancer activity of compound 3 using an MTT assay method against murine leukemia P-388 cell lines demonstrated low anticancer activity with an IC_{50} value 57.93 µg/mL.

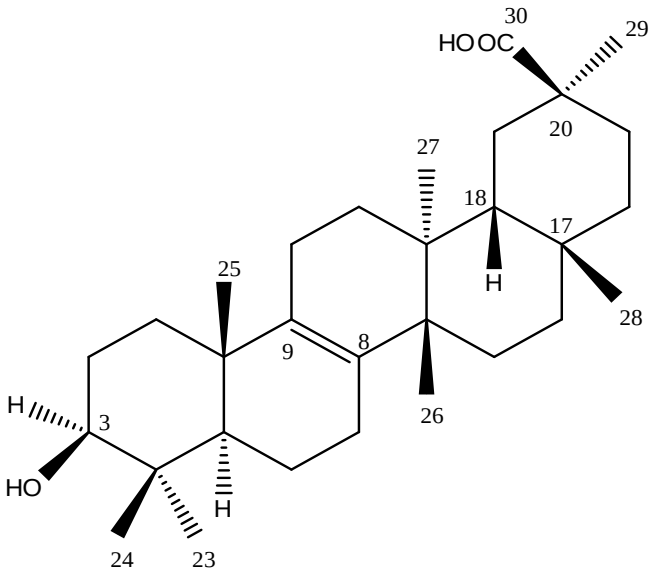

Figure 1: Chemical structure of 20-epibryonolic acid

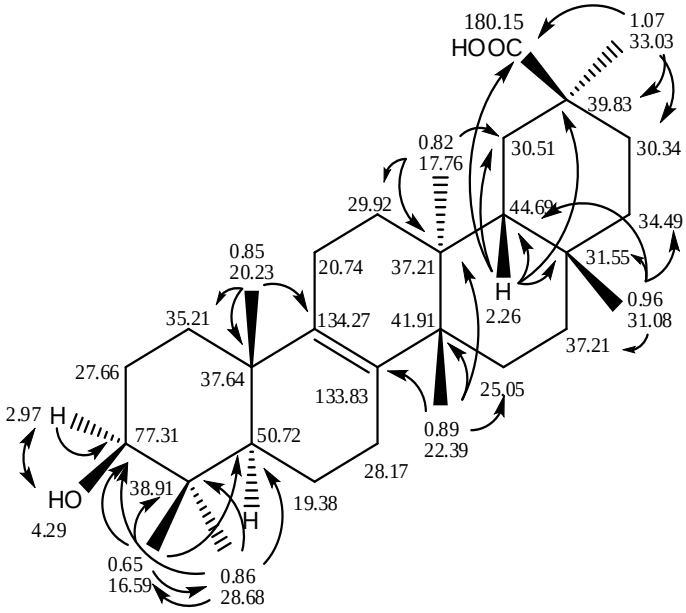

Figure 2: HMQC, HMBC and H-H COSY correlations

Conclusion

A triterpenoic acid, 20-epibryonolic acid (compound 3), along with two sterol compounds of β-sitosterol (1) and stigmasterol (2), were isolated from ethyl acetate fraction of methanolic extract of *Tetrameles nudiflora* leaves. The cytotoxicity activity against murine leukemia P-388 cell lines demonstrated potential activity as an anticancer with an IC_{50} value 57.93 µg/mL.

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Table 1
NMR Data of 20-epibryonolic acid and compound 3

S. N.	20-epibryonolic acid[7]		Compound 1	
	δH (ΣH, multp, *J* (Hz), ppm)	**δC (ppm)**	**δH (ΣH, multp, *J* (Hz), ppm)**	**δC (ppm)**
-OH	4.28 (1H, *br, d*, 4.8)		4.29 (1H, *d*, 4.5)	
1		34.74		35.21
2		27.17		27.66
3	2.99 (1H, *m*)	76.89	2.97 (1H, *m*)	77.31
4		38.5		38.91
5		50.27		50.72
6		18.89		19.38
7		27.67		28.17
8		133.41		133.83
9		133.89		134.27
10		37.16		37.64
11		20.23		20.74
12		29.43		29.92
13		36.75		37.20
14		41.42		41.91
15		24.58		25.05
16		36.76		37.21
17		31.06		31.55
18	2.28 (1H, *d*, 15)	44.24	2.26 (1H, *m*, 15.5)	44.69
19		30.02		30.51
20		39.44		39.83
21		29.85		30.34
22		34.02		34.49
23	0.89 (3H, *s*)	28.19	0.86 (3H, *s*)	28.68
24	0.68 (3H, *s*)	16.07	0.65 (3H, *s*)	16.59
25	0.88 (3H, *s*)	19.73	0.85 (3H, *s*)	20.23
26	0.92 (3H, *s*)	21.88	0.89 (3H, *s*)	22.39
27	0.85 (3H, *s*)	17.27	0.82 (3H, *s*)	17.76
28	0.99 (3H, *s*)	30.59	0.96 (3H, *s*)	31.08
29	1.10 (3H, *s*)	32.57	1.07 (3H, *s*)	33.06
30		179.8		180.15

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