

CYTOTOXIC ACTIVITY OF THE FLAVONOID COMPOUNDS ISOLATED FROM *Tetrameles nudiflora* LEAVES

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Abstract

Tetrameles nudiflora is one of the endemic plants in Wallace region, especially in Mekongga Forest, Southeast Sulawesi, Indonesia. As part of our project in the field of natural drug discovery based on local bioresources, this study aimed to isolate secondary metabolite compounds from the ethyl acetate fractions of the leaves of *T. nudiflora* and evaluate their cytotoxic activities against breast carcinoma cancer lines, MCF-7. The methods used were various chromatography techniques such as vacuum column chromatography, gravitation column chromatography, thin layer chromatography, preparative thin layer chromatography, and re-crystallization using two different organic solvents. In the present study, two flavonoid compounds, kaempferol (1) and quercetin (2) were identified. The structures of 1-2 were determined using serial spectroscopic techniques, including mass spectrometry, one- and two-dimensional nuclear magnetic resonance. Moreover, using 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide (MTT) assay method, both compounds showed *in vitro* cytotoxic activity IC₅₀ of 34.162 and 133.148 µg mL⁻¹, respectively against MCF-7 cell lines.

Key words : *Tetrameles nudiflora*, flavonoid, kaempferol, quercetin, breast carcinoma, MCF-7 cancer cell lines

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Introduction

Indonesia, as one of the rich countries in the world for natural product resources, has already used many kind of plants and herbs species as traditional medicines. Many diseases such as cancer have been reported to be cured by traditional medicine system in Indonesia (WHO, 2014). The traditional medicine may offer minor side effect because it is free of synthetic chemicals. Nevertheless, further investigation is still required to determine the efficacy of the traditional medicinal plants in treating and curing cancer (Kamuhabwa *et al.*, 2000; Ngoua-Meye-Misso *et al.*, 2019). More than 3,000 traditional medicinal plants have been already identified in Indonesia (Zuhud, 2009). Among them, 2,518 species have been utilized, although only 262 species were used in traditional medicine system (Hariana, 2013; Buss, A. D *et al.*, 2010). *Tetrameles nudiflora* is one of the members of Tetramelaceae family, Cucurbitales order, Angiosperms clade, and Plantae kingdom (Hanno *et al.*, 2011). This

plant is commonly found in Australia, Bangladesh, Bhutan, China, India, Laos, Malaysia, Myanmar, Sri Lanka, Thailand, Vietnam and Indonesia. In Indonesia, this plant is known as *binong* or *winong* and widely spread from Sumatra, Java, Sulawesi, Nusa Tenggara, and Papua (Priyadi *et al.*, 2010; Sosef *et al.*, 1998). Tetramelaceae is one of the tribes of flowering plants consisting of only two species, *Octomeles sumatrana* and *Tetrameles nudiflora*. *T. nudiflora* is a large tree with yellow flowers 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 (Priyadi *et al.*, 2010). In Indonesia, these trees are widely distributed across Sumatra, Java, Sulawesi, Nusa Tenggara (Southeast Islands) and Papua (Banjarnahor *et al.*, 2015). *T. nudiflora* was reported to have a potential biological inhibitory activity against α -glucosidase as antidiabetic (Minarti *et al.*, 2018). Kerala Forrest Research Institute also demonstrated that decoction from the bark of

this plant has been used as a laxative, diuretic agent, and anti-rheumatic (Sasidharan *et al.*, 1999). Moreover, the plant is also used to treat rheumatism, edema (Priyadi *et al.*, 2010) and itching (Doley *et al.*, 2014).

However, there are only few studies on secondary metabolites compounds of this plant, especially those from the leaves. Yet, our previous study indicated that *T. nudiflora* contains 20-epibryonolic acid and stigmasterol (Darmawan *et al.* 2018; Salahudin *et al.*, 2013). It led us to isolate, purify, and identify the further secondary metabolite compounds from the leaves of *T. nudiflora* and explore their biological activities. Therefore, the aim of this study was to explore the chemical constituents of the leaves of *T. nudiflora* using chromatography methods, to determine its chemical structure based on serial spectroscopic data, and to determine its potential as an anticancer against MCF-7 cell lines.

Materials and Methods

Extraction and Isolation

T. nudiflora leaves were collected from the Mekongga Forest, District of Kolaka, Southeast Sulawesi, Indonesia. The plant was determined in Herbarium Bogoriense, Research Centre for Biology, Indonesian Institute of Sciences (LIPI), Cibinong-Bogor, Indonesia.

T. nudiflora leaves were sorted from unwanted materials, washed with water and dried using blower oven cabinet at 50 °C. Dried leaves were subjected to grinder (powder machine) to obtain dried and powdered leaves. After that, 1.28 kg of dried and powdered of *T. nudiflora* leaves were macerated in 10 L of methanol and evaporated using rotary evaporator to yield 185.30 g of methanol crude extract. Methanol soluble fraction (100 g) was subjected to column chromatography over silica gel and then was eluted with a gradient solvent system of n-hexane:EtOAc to obtain 30 fractions, namely F1-F30. Fraction 6 (F6) was further applied on Sephadex chromatography using methanol :dichloromethane (1:1) as eluent to give 10 sub-fractions (SF1-SF10). Compound **1** (15 mg) and **2** (20 mg) were isolated from sub-fractions 6.4 and 6.6 (SF 6.4 and SF-6.6, respectively).

General and chemical structure analysis

Chemical structure identification of compound **1** and **2** were performed using liquid

chromatography-mass spectroscopy (LC-MS) and one- and two-dimensional (1D- and 2D) - nuclear magnetic resonance (NMR). The 1D- and 2D-NMR spectra were recorded by JEOL JNM-ECA 500 spectrometer with CD₃OD as a solvent and TMS as an internal standard. LC-ESI-MS were measured by the Mariner Biospectrometry-Finnigan instrument with methanol (Merck) as a solvent. Column chromatography was performed using silica gel (200-300 mesh, Kieselgel 60, E. Merck) as stationary phase.

Cytotoxic assay

The cytotoxic assay was conducted according to the method described (Fajriah *et al.*, 2017; Megawati *et al.*, 2016; Harneti *et al.*, 2012). Firstly, MCF7 cells were incubated in the 96-well plates at an initial cell density of approximately 3×10^4 cells cm⁻³ for 24 h. Various sample concentrations (6.25, 12.5, 25, 50, 100 and 200 µg mL⁻¹) were then added. Samples were dissolved in dimethyl sulfoxide (DMSO) at the required concentration. Subsequent six desirable concentrations were prepared using phosphoric buffer solution (PBS) pH 7.30-7.65. Control wells received only DMSO. After 48 h incubation, assay was terminated by adding the dye reagent 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), also named as thiazol blue. The incubation was then continued for another 4 h, in which the MTT-stop solution containing SDS (sodium dodecyl sulphate) was added and another 24 h of incubation was conducted. Finally, optical density was read using a microplate reader at 550 nm. IC₅₀ values were taken from the plotted graph of percentage live cells compared to the control (%) (received only PBS and DMSO), versus the tested concentration of the samples (µm). The IC₅₀ value is the concentration required for 50% growth inhibition. Each assay and analysis were run in triplicate and averaged.

Results

The compound **1** was isolated as a yellowish-brown crystal. The LC-ESI-MS [M+H]⁺ at *m/z* 287 and the ¹H-NMR (500 MHz in CD₃OD) data showed that the compound **1** has six aromatic protons at δ_H 6.18, 6.39, 6.90 (2H) and 8.09 (2H).

Table 1. ^1H - and ^{13}C -NMR of compound **1** and Kaempferol

No.	Compound 1		Kaempferol (Xiao <i>et al.</i> , 2006)	
	δ_{H} ppm (ΣH , mult. J Hz)	δ_{C} ppm	δ_{H} ppm (ΣH , mult. J Hz)	δ_{C} ppm
2	-	148.1	-	146.9
3	-	137.2	-	136.5
4	-	177.6	-	176.5
4a	-	104.5	-	103.9
5	-	158.4	-	157.7
6	6.18 (1H, <i>d</i> , <i>J</i> = 1.95)	94.6	6.27 (1H, <i>d</i> , <i>J</i> = 2.0)	98.9
7	-	166.0	-	165.0
8	6.39 (1H, <i>d</i> , <i>J</i> = 1.95)	99.6	6.54 (1H, <i>d</i> , <i>J</i> = 2.0)	94.3
8a	-	162.6	-	160.1
1'	-	123.8	-	123.1
2'/6'	8.09 (2H, <i>d</i> , <i>J</i> = 9.08)	130.8 (2C)	8.16 (2H, <i>d</i> , <i>J</i> = 8.8)	130.3
3'/5'	6.91 (2H, <i>d</i> , <i>J</i> = 9.08)	116.4 (2C)	7.03 (2H, <i>d</i> , <i>J</i> = 8.8)	116.1
4'	-	160.7	-	161.9

The compound **2** was isolated as a yellowish-brown crystal. The LC-ESI-MS $[\text{M}+\text{H}]^+$ at m/z 303.04 and the ^1H -NMR (500 MHz, in CD_3OD) data showed that the compound **2** has five aromatic protons at δ_{H} 6.18 (1H, *d*, *J* = 1.95 Hz, H-9), 6.39 (1H, *d*, *J* = 1.95 Hz, H-1), 6.88 (1H, *d*, *J* = 8.43 Hz, H-5'), 7.63 (1H, *dd*, *J* = 1.95 & 8.43 Hz, H-6'), and 7.73 (1H, *d*, *J* = 1.95 Hz, H-2').

Table 2. ^1H - and ^{13}C -NMR of the compound **2** and quercetin

No.	Compound 2		Quercetin (Xiao <i>et al.</i> , 2006)	
	δ_{H} ppm (ΣH , mult. J Hz)	δ_{C} ppm	δ_{H} ppm (ΣH , mult. J Hz)	δ_{C} ppm
1	-	-	-	-
2	148.0	-	155.3	-
3	137.3	-	133.0	-
4	177.6	-	177.6	-
5	104.6	-	103.7	-
6	158.3	-	160.8	-
7	94.5	6.39 (<i>d</i> , 1.95)	93.4	6.53(<i>d</i>)
8	165.7	-	163.4	-
9	99.3	6.18 (<i>d</i> , 1.95)	98.4	6.27 (<i>d</i>)
10	162.6	-	156.1	-
1'	124.2	-	121.0	-
2'	116.0	7.73 (<i>d</i> , 1.95)	115.7	7.70 (<i>dd</i>)
3'	146.3	-	144.6	-
4'	148.9	-	148.2	-
5'	116.3	6.88 (<i>d</i> , 8.43)	115.2	6.99(<i>d</i>)
6'	121.7	7.63 (<i>dd</i> , 1.95 & 8.43)	122.1	7.81(<i>d</i>)

Examination of the cytotoxicity activity of the compound **1** and **2** against breast carcinoma (MCF-7) cells lines showing that kaempferol and quercetin compounds were active as anticancer. The IC_{50} values of those compounds were 34.162 and 133.148 $\mu\text{g mL}^{-1}$, respectively.

Discussion

The constant coupling values, it can be seen that δ_{H} 6.18 was correlated with δ_{H} 6.39 in *meta* position. Meanwhile, δ_{H} 6.90 (2H) showed an *ortho* correlation with δ_{H} 8.09 (2H). Presence of the AB pattern of aromatic ring proton system of the A ring (the first aromatic ring in flavonoid chemical structure) was shown by two proton signals at δ_{H} 6.18 and 6.39 (Fig. 1a). Moreover, the A_2B_2 pattern seen in the B ring suggesting that the B ring has two symmetric proton peaks at δ_{H} 6.90 and 8.09, with an *ortho* correlation (Fig. 1b). The ^{13}C -NMR (125 MHz, in CD_3OD) spectrum showed the presence of six artificial methyl (CH) appearing at δ_{C} 94.6, 99.5, 116.4 (2C), and 130.8 (2C), with eight quaternary carbon at δ_{C} 104.5, 123.8, 137.2, 148.1, 158.4, 160.7, 162.6, and 166.0, and a carbonyl group at δ_{C} 177.5.

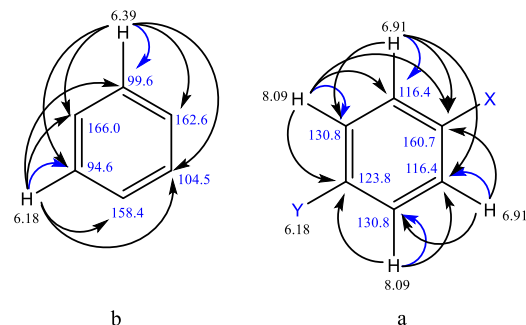


Figure 1. Pattern of proton system of aromatic ring at the A and B rings of compound **1**

Based on ^1H -, ^{13}C -NMR and LC-ESI-MS data, supported by HMQC and HMBC analysis (Fig. 2), the LC-ESI-MS data and the comparison with appropriate reference (Table 1), it can be concluded that compound **1** is kaempferol (Xiao *et al.*, 2006) (Fig. 3).

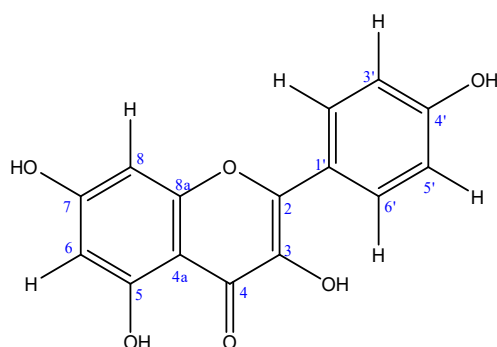
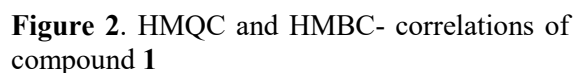


Figure 3. Chemical structure of kaempferol (Xiao *et al.*, 2006)

The coupling constant data of ^1H -NMR signals of the compound **2** showed that the proton signal at δ_{H} 6.18 correlated with the proton signal at δ_{H} 6.39 in *meta* position. A proton signal at δ_{H} 7.63 correlated to δ_{H} 7.73 and 6.88 in *ortho* and *meta* positions, respectively (Fig. 4).

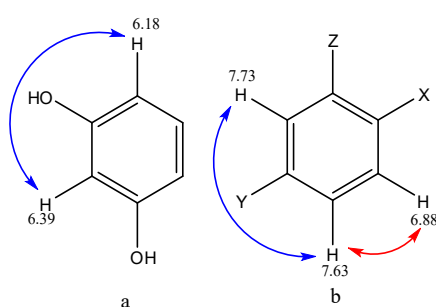


Figure 4. The proton pattern of the A and B rings of the compound **2**

Two proton peaks at 6.18 ppm (1H, *d*, *J* = 1.95 Hz) and 6.39 ppm (1H, *d*, *J* = 1.95 Hz) in the first ring (the A ring) showed the AB pattern of aromatic ring proton system (Fig. 4a), and the second ring (the B ring) showed the AXB pattern, indicated that the second aromatic ring

has three-substituted protons at δ_{H} 7.63 (1H, *dd*, $J = 1.95$ & 8.43 Hz), 6.88 ppm (1H, *d*, $J = 8.43$ Hz,), and 7.73 ppm (1H, *d*, $J = 1.95$ Hz) (Fig. 4b). ^{13}C -NMR (125 MHz, in CD_3OD) data showed the presence of five artificial methyl (CH) groups appearing at δ_{C} 94.5 (C-6), 99.3 (C-8), 116.0 (C-2'), 116.3 (C-5'), and 121.7 (C-6'), with 10 different signals of quaternary carbon at δ_{C} 104.6 (C-4a), 124.2 (C-1'), 137.3 (C-3), 146.3 (C-3'), 148.0 (C-2), 148.9 (C-4'), 158.3 (C-5), 162.6 (C-8a), 165.7 (C-7), and 177.6 (C=O, C-4). In addition, the chemical shift at δ_{C} 177.5 ppm indicated the presence of a carbonyl group. Based on the NMR data above (^1H - and ^{13}C -NMR), supported by HMQC and HMBC data (Fig. 5), the LC-ESI-MS data, and the comparison with appropriate reference (Table 2), it can be concluded that compound **2** is quercetin (Xiao *et al.*, 2006) (Fig. 6).

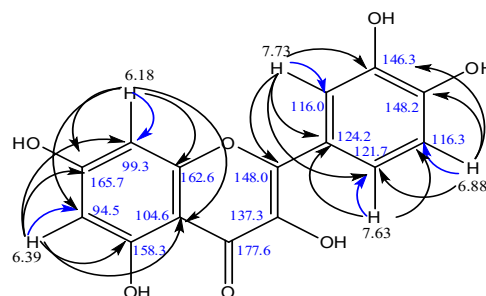


Figure 5. HMQC and HMBC correlations of compound **2**

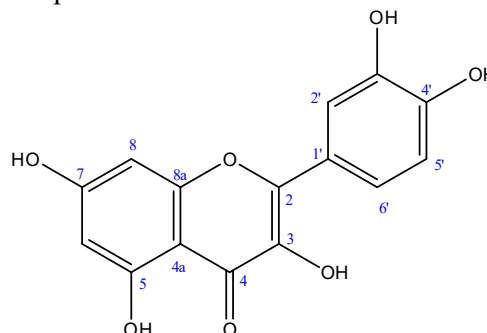


Figure 6. The chemical structure of quercetin (Xiao *et al.*, 2006)

The cytotoxicity activity of the compound **1** (kaempferol) and **2** (quercetin) against breast carcinoma (MCF-7) cells, a kaempferol can cause apoptosis in MCF-7 cells through the introduction of the intrinsic caspase cascade and through the down-regulation of the Polo-like-kinase-1 (PLK-1), so that kaempferol has higher activity than quercetin (Stapel *et al.*, 2013).

Conclusion

Isolation and purification of kaempferol and quercetin from ethyl acetate fraction 6 (F-6) of leavers of *T. nudiflora* using column chromatography method have been done successfully. The chemical structure elucidation was determined based on the spectroscopic data analysis (1D-, 2D-NMR and LC-ESI-MS) together with the appropriate reference comparison. Kaempferol and quercetin compounds showed high cytotoxicity activity against breast carcinoma (MCF-7) cells line with IC₅₀ values of 34.162 and 133.148 µg mL⁻¹, respectively.

Acknowledgements

Authors are thankful to Prof. Dr. Sci. Muhammad Hanafi for his beneficial support, discussion and collaboration, as well as to Mrs. Puspa Dewi N. Lotulung, M.Eng for the mass spectroscopy measurement and interesting discussion. Moreover, we also would like to thank to Indonesian Institute of Sciences (LIPI) for funding our research project through LIPI Competitive Research Grant 2012-2014.

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