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Cover Page Footnote

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Identification and Bioactivity Studies of Flavonoid Compounds from *Macaranga hispida* (Blume) Mull.Arg

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Abstract

Two flavonoid compounds, 5,7,3',4'-tetrahydroxy-6-geranylflavonol (1) and kaempferol 7-O-β-glucose (2) have been isolated from the leaves of *Macaranga hispida* (Blume), Mull.Arg. Isolation and purification were conducted by chromatography methods and chemical structure characterization was carried out by spectroscopic methods. The 5,7,3',4'-tetrahydrxyi-6-geranyl flavonol (1) and kaempferol 7-O-glucose (2) had moderate cytotoxic activity against *murine leukemia* P-388 cell lines with IC_{50} value of 0.22 and 101.5 µg/mL, respectively. The IC_{50} for antioxidant activities of (1) and (2) were 2.83 and 13.95 µg/mL, respectively. The LC_{50} of (1) and (2) from BSLT were 350 and >1000 µg/mL, respectively.

Abstrak

Identifikasi dan Studi Bioaktifitas Senyawa Flavonoid dari *Macaranga hispida* (Blume) Mull.Arg. Dua senyawa flavonoid, 5,7,3',4'-tetrahidroksi-6-geranilflavonol (**1**) dan kaemferol 7-O-β-glukosa (**2**) telah diisolasi dari daun *Macaranga hispida (Blume) Mull. Arg*. Isolasi dan pemurnian dilakukan dengan metode kromatografi dan karakterisasi struktur kimia dilakukan dengan metode spektroskopi. 5,7,3', 4'-tetrahidroksi-6-geranil flavonol (**1**) kaemferol 7-O-glukosa (**2**) memiliki aktivitas sitotoksik sel murine leukemia P-388 dengan nilai IC_{50} masing-masing 0,22 dan 101,5 µg/mL. Aktivitas antioksidan **(1)** dan **(2)** dinyatakan dengan nilai IC_{50} masing-masing sebesar 2,83 dan 13,95 µg/mL. Nilai LC_{50} dengan uji BSLT (**1**) dan (**2**) masing-masing 350 dan >1000 µg/mL.

Keywords: anti cancer murine leukemia P-388 cell lines, antioxidant activity, BSLT, kaempferol 7-O-glucose, cytotoxicity, Macaranga hispida (Blume) Mull. Arg, 5,7,3',4'-tetrahydroxy-6-geranyl flavonol

Introduction

Macaranga hispida (Blume) Mull. Arg, known locally as Mahang (Indonesia) is one of approximately 300 species of the *Macaranga* (Euphorbiaceae) genus, It was widespread growth, from Africa and Madagascar, to the Asian tropic region, North Australia and the Pacific Islands [1]. As part of our study about natural products, drug discovery, including exploration of potential bioactivity of plants originating from Indonesia, the chemical content of the ethyl acetate and butanol fraction obtained from partitioning methanol extract of *M. hispida* was explored using the column chromatography method.

A Previous study reported that flavonoid and terpenoid are the major components of the *Macaranga* genus [2-9]. Apigenin, isolated from *Portulaca. oleracea* L and the bioactive compound also had potential antibacterial activity indicating that it can be used for development of antibacterial drugs for the treatment of diseases associated with these pathogenic bacteria [10]. Meanwhile, flavonoid glycoside compounds have been found in abundance in plants (flowers, fruits, vegetables, bean and tea) [11-13].

However, there are no publication established about chemical constituents of *M. hispida* plant. In this paper, we describe the structure elucidation and cytotoxic properties of flavonoid compounds isolated from the methanol extract of the leaves of *M. hispida*.

Materials and Methods

General. The ^{1}H- and ^{13}C-NMR spectra were recorded with a JEOL JNM-ECA 500 spectrometer instrument with CD_3OD as a solvent and TMS as an internal

standard. LCMS were measured with the Mariner Biospectrometry-Finnigan instrument with methanol p.a for analysis (Merck) as a solvent. The Column chromatography was carried out with silica gel (200 - 300 mesh, Kieselgel 60, E. Merck) for isolation.

Plant material. The leaves of *Macaranga hispida* were collected from the Mekongga Forest, District of Kolaka, Southeast Sulawesi, Indonesia in March 2012. The plant was verified at Herbarium Bogoriense, Research Centre for Biology, Indonesian Institute of Sciences, Bogor, Indonesia.

Extraction and isolation. Powdered dried leaves of *M. hispida* (2.15 kg) were macerated in methanol. The methanol extract was evaporated to obtain 220 g of crude methanol extract. The methanol extract was partitioned with *n*-hexane, ethyl acetate (EtOAc), and butanol (BuOH), successively. The EtOAc soluble fraction (20 g) was chromatographed over a silica gel column and eluted successively with a gradient solvent system of *n*-hexane:EtOAc to obtain nine fractions (F1-F9). Compound **1** (40 mg) was crystallized from fraction 5 (F5) after it was evaporated and re-diluted with EtOAc and further purified with $CHCl_3$, acetone and MeOH to obtained a pure compound.

Compound 1: yellow powder, ESI-MS m/z 438.500 $[M+H]^+$, NMR (JEOL JNM-ECA 500) δ_H (500 MHz, in CD3OD): 6.50 (1H, *s*, H-8), 7.79 (1H, *d*, *J*=1.97, H-3'), 7.69 (1H, *dd*, *J*=8.48 and 1.97 Hz, H5'), 6.99 (1H, *d*, *J*=8.48, H-6'), 3.35 (1H, *d*, *J*=8.48 Hz, H-1"), 5.30 (1H, *t*, *J*=7.25Hz, H-2"), 1.82 (1H, *s* H-4"), 1.95 (1H, *dd*, *J*=7.25 Hz,H-5"), 2.08 (1H, *dd*, *J*=7.25 Hz, H-6"), 5.09 (1H, *t*, *J*=7.25 Hz, H-7"), 1.54 (1H, *s*, H-9"), 1.59 (1H, *s* H-10"). δ_C (125 MHz, in CD3OD): 146.30 (C-2), 137.28 (C-3), 177.39 (C-4), 104.45 (C-4a), 159.20 (C-5), 112.32 (C-6), 163.57 (C-7), 93.69 (C-8), 156.3 (C-8a), 124.45 (C-1'), 116.15 (C2'), 147.7 (C-3'), 148.8 (C-4'), 116.32 (C-5'), 121.69 (C-6'), 22.3 (C-1"), 123.8 (C-2"), 135.74 (C-3"), 16.38 (C-4"), 41.01 (C-5"), 27.92 (C-6"), 125.51 (C-7"), 132.18 (C-8"), 17.83 (C-9"), 25.95 (C-10").

The butanol soluble fraction (20 g) was chromatographed over a silica gel column and eluted successively with a gradient solvent system of *n*-hexane:EtOAc to obtained five fractions (F1-F5). Compound **2** (20 mg) was purified from F4 by column chromathography with $CHCl_3$, acetone and MeOH eluents to obtained a pure compound.

Compound 2: yellow powder, ESI-MS m/z 448.386 $[M+H]^+$, NMR (JEOL JNM-ECA 500) δ_H (500 MHz, in CD_3OD): 6.2 (1H, *d*, J=1.95, H-6), 6.4 (1H, *d*, *J*=1.95, H-8), 8.08 (2H, *d*, *J*=9.08, H-2'/H-6'), 6.88 (2H, *d*, *J*=9.08, H-3'/H-5'), 5.15 (1H, *d*, *J*=7.79, H-1"), 3.78 (1H, *d*, *J*=7,79, H-2"), 3.83 (1H, *d*, *J*=1,95, H-3"), 3.54 (1H, *dd*, *J*=5.84, H-4"), 3.44 (1H, *d*, *J*=5.84, H-5"), 3.63; 3.51 (2H, *dd*, *J*=5.84, H-6"). δ_C (125 MHz, in CD_3OD): 159.2 (C-2), 136.68(C-3), 179.8 (C-4), 105.8 (C-4a), 163.2 (C-5), 100 (C-6), 166.1 (C-7), 94.8 (C-8), 158.6 (C-8a), 122.8 (C-1'), 132.4 (C2'/C6'), 116.3 (C-3'/C-5'), 161.7 (C-4'), 105.1 (C-1"), 73.2 (C-2"), 70.2 (C-3"), 75 (C-4"), 77.4 (C-5"), 62 (C-6").

Antioxidant activity [14]. Antioxidant analysis was conducted using "DPPH free scavenging activity" method with a slight modification. Various concentrations of the *M. hispida* extract/fractions in 0.8 ml methanol were mixed with 0.2 mL of methanolic solution containing 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals, resulting a final concentration of the DPPH of 0.2 mM and sample concentrations up to 100 µg/mL. The mixture was shaken vigorously and left to stand for 30 min at room temperature, the absorbance was then measured using a spectrophotometer at 515 nm. The percentage of inhibition (free radical scavenging activity) was calculated by the equation: [1 - (B/A)] x 100%; whereas A is absorbance in the absence of a sample and B is absorbance in the presence of a sample. The IC_{50} value denoted the concentration of the sample required to scavenge 50% of the DPPH free radicals.

BSLT activity [15,16]. Brine shrimp eggs (*Artemia salina*, Sanders TM Great Salt Lake, Brine Shrimp Company L.C., U.S.A.) were hatched in sea water. Two unequal compartments in the plastic chamber with several holes in the divider were used for hatching. The eggs were sprinkled into the larger compartment which was darkened, while the smaller compartment was illuminated.

After a 48 hours incubation period at room temperature, nauplii (larvae) were collected by pipette from the illuminated side, whereas their shells were left in another side.

Before adding 100 µL of sea water. Serial dilutions were made in the wells of 96-well microplates (Nunc, Denmark) in triplicate in 100 µL sea water. Control wells with DMSO were included in each experiment. A suspension of nauplii containing 10 to15 organisms (100 µL) was added to each well. The plates were covered and incubated at room temperature for 24 hours. Plates were then examined under the binocular steromicroscope and the numbers of dead (non-motile) nauplii in each well were counted. One hundred microliters of methanol were then added to each well to immobilize the nauplii and after 15 minutes the total numbers of brine shrimp in each well were counted. Analysis of the data was performed by probit analysis on a Finney computer program to determine the lethal concentration to half of the test organisms (LC_{50}).

Cytotoxic activity. Compounds 1 and 2 were tested *in vitro* for its cytotoxicity against the P388 cell line. The P388 cells growth inhibition by compound 1 and 2 were

analyzed by the 3-(4,5-dimethylthyazole-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay [17]. MCF-7 cells were seeded in 96 well plates and incubated with MTT (5 mg/mL) for 4 h. Cells were further solubilized by adding 100 µL of DMSO. Absorbance was read at 570 nm. Cell viability of treated cells was expressed as the amount of dye reduction compared to untreated control cells. The wells that contained only medium and 10 mL of MTT were used as blanks for the plate reader.

Result and Disscussion

Structure elucidation of isolated flavonoids. Compound **1** UV light absorption (MeOH): 260 sh, 370 sh. IR: *v*cm^{-1}/ max KBr: 3606 (O–H), 1716 (C=O), 1554, 1446 (aromatic), 1317, 1290, 2964, 2918 (isoprenyl) and 1128, 1091.

Based on the ^{1}H-NMR data, compound **1** had four aromatic protons with two aromatic systems, The ABX system was at δ 7.69 (*dd*, *J*=8,48 and 1,97 Hz), 6,99 (*d*, *J*=8,48 Hz) and 7,79 (1H, *dd*, *J*=1,97 Hz) and had a singlet proton at δ 6.40 in ring A. Two olefinic proton triplet signals at δ 5.09 and 5.30 indicated a typical isoprenyl group, with three methyl singlets at δ 1.82, 1.54 and 1.59, and three methylene signals at δ 3.35, 1.95 and 2.08. ^{13}C-NMR spectrum data showed 25 carbon atoms which consisted of four aromatic methine (*d*) signals at δ 93.9, 121.69, 116.32 and 118.5. Twelve quaternary carbon atoms at δ 146.30, 137.28, 104.45, 159.20, 112.32, 163.57, 156.3, 124.45, 147.7, 148.8, 135.74, 132.18 and 1 carbonyl atom at δ 177.39. Two typical isoprenyl groups correlated with only three methyl signals which indicated that the moiety was geranyl.

Based on this NMR data, (^{1}H- and ^{13}C-NMR) compound **1** is a geranylated flavonoid derivative. This was supported by the HMBC experiment, which showed the geranyl group was located at C-5, with the presence of long range coupling between the methylene group at δ 3.35 (H-1") which correlated with C-5 at δ 112.32 (Figure 1). From the 1D- and 2D-NMR data, confirmed by LCMS data (m/z 439.20 [M+H]$^{+}$), compound **1** was identified as the 5,7,3',4'-tetrahydroxy-6-geranyl flavonol (Figure 1).

Based on NMR data, supported by LC-MS data and reference comparisons (Figure 1.), Compound **1** was identified as the 5,7,3',4'-tetrahydroxy-6-geranyl flavonol [18]. This was the first report of 5,7,3',4'-tetrahydroxy-6-geranyl flavonol content in *Macaranga hispida*.

Coumpond **2** had UV light absorption of MeOH: 265 sh, 350 sh. IR: *v*cm–1/ max KBr: 3242 (O–H), 1772 (C=O), 1550, 1490 (aromatic), 3923 (glycoside) and 1128, 1091. The ^{1}H-NMR data showed six aromatic protons at 8.08 (*d*, 9.08) (2H) / δ_C 132.4 and 6.88 (*d*, 9.08) (2H) / δ_C 116/ δ_C 116.3, which indicated the presence of an A2B2 ring system. In addition, signal two singlets at δ_H δ 6.2 (*d*, 1.95)/ δ_C 100, 6.4 (*d*, 1.95) / δ_C 94.8, were shown in rings A and B. Based on this chemical shift data, compound **2** was predicted as a kaemferol derivative. The anomeric proton (H-1") at δ_H 5.15 ((*d*, *J*=7.79), which correlated to carbon at δ_C 105.1, indicated that the linkage between the sugar residue and aglycone (flavone) was O-glycosidic at C-7 of the aglycon oxyigen atom. The *O*-glycosidic linkage was confirmed by HMBC correlations between the anomeric proton at δ_H 5.14 with the proton atom at δ_H 6.4 (H-8) (Figure 2).

The ^{13}C-NMR data showed 25 carbon atoms consisting of six aromatic methine signal at δ 100, 94.8, 132.4(2C), and 116.3(2C). There were quaternary carbon atoms at δ 163.2, 159.2, 105.8, 163.2, 136.7, 158.6, 166.1, and one carbonyl atom at δ 179.8. The proton signal at δ 6.2 (^{1}H, *d*, *J*=1.95) and 6.4 (^{1}H, *d*, *J*=1.95) which have correlated in the meta position indicated that these two protons were located in the same aromatic ring. Proton aromatics (δ 8.08 (^{2}H, *d*, *J*=9.08) and 6.88 (^{2}H,*d*, *J*=9.08) which have a correlation in the ortho position indicates another aromatic ring.

The 1D- and 2D-NMR [19] confirmed by mass spectroscopic data (m/z 449 [M+H]$^{+}$), that compound **2** was elucidated as kaemferol 7–O-β-glucoside. It was isolated for the first time from *M. hispida* (Blume) Mull. Arg.

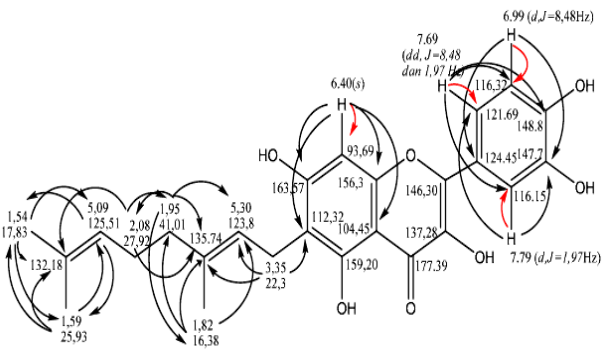

Figure 1. The Structure of Compounds 1

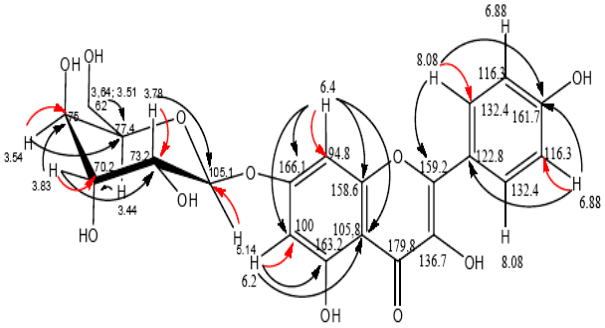

Figure. 2. The Structure of Compounds 2

Antioxidant activity. Table 1 showed that, quercetin (positive control), compounds **1** and **2** have IC_{50} values 4.39, 2.83 and 13.95 µg/ml, respectively. Due to similarities as the flavonoid, compound **1** has the highest antioxidant activity compared to quercetin (positive control) and compound **2**. Both compounds had IC_{50} values lower than 100 µg/mL. Thus they could be categorized as antioxidant active compounds.

BSLT activity. The BSLT test was conducted on compounds **1** and **2**. The toxicity test method was carried out because the cost was low and the results were be trustworthy. This test observed the mortality rate caused by the test compound. According to Meyer, active compounds will result in high mortality, and compounds with $LC_{50} \leq 1000$ µg/mL can be categorized as compounds that have potent toxicity. A Smaller LC_{50} value means the compound was to be increasingly active/toxic.

Table 2 showed no dead nauplii in the control groups, which means the sea water was in good condition. The obtained LC_{50} value for compound **1** was smaller than compound **2**, which means that compound **1** was more toxic than compound **2**.

Cytotoxic activity. Based on the results in Table 3 above, showed that compound **1** was very active as anticancer against P388 cancer cells with IC_{50} value 0.22 µg/mL, compared to compound **2** with IC_{50} value 101.5 µg/mL. the different between compound **1** and **2** are in the isoprenyl and sugar group. Based on the anticancer activity result (Table 3) indicated that isoprenyl substituent bound to A ring of flavonoid increased the activity (compound **1**), while sugar substituent decreased the anticancer activity (compound **2**).

Table 1. Results of the Antioxidant Activity Test (IC_{50}) of Compound 1 and 2

Sample	Conc (µg/mL)	% inhibition	IC_{50} (µg/mL)
Quercetin	20	96.601	4.39
	10	72.987	
	5	45.886	
	1	12.120	
Compound **1**	20	96.146	2.83
	10	95.059	
	5	80.237	
	1	10.968	
Compound **2**	100	94.107	13.95
	50	94.290	
	10	44.208	
	5	17.815	

Table 2. Results of the BSLT Activity Test (LC_{50}) of Compound 1 and 2

Sample	Conc (µg/mL)	Mortalities	LC_{50} (µg/mL)
Compound **1**	10	5,00	350
	100	21,87	
	500	48,27	
	1000	75,86	
Compound **2**	10	2,70	> 1000
	100	6,89	
	500	14,28	
	1000	30,76	

Table 3. Results of the Anti-Cancer Activity Test (LC_{50}) of Compound 1 and 2

Sample	IC_{50} (µg/mL) P388
Compound **1**	0.22
Compound **2**	101.5

Conclusions

After isolation, purification and characterization of the phenolic compound isolated from ethyl acetate and butanol fraction of the methanol extract of *M. hispida* leaves, we concluded that there are two compound obtained of i.e compound of the 5,7,3',4'-tetrahydroxy-6-geranyl flavonol **(1)** and kaempferol 7-*O*-*β*-glucose **(2)**. Compound **1** has a higher activity than the compound **2** in a row with a value of 2.83 µg/mL and 13.95 µg/mL, The BSLT analysis results obtained smaller LC_{50} for compound **1** (350 µg/mL) compared to compound **2,** which had a value greater than 1000 µg/mL, Therefore compound **1** was more toxic than compounds **2**. P388 for anticancer activity of compounds **1** and **2** respectively, 0.22 dan 101.5 µg/mL.

Acknowledgments

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