Chemical Constituents of Indonesian *Micromelum minutum* Leaves and Their Cytotoxicity Against MCF-7 and 4T1 Breast Cancer Cells

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Abstract. *Micromelum minutum* is used widely in traditional folk medicine. Although this species has been investigated extensively and several bioactive compounds have been isolated, little work has been done on Indonesian *M. minutum*. This research aimed to study the chemical constituents and biological activities of *M. minutum* cultivated in Bantimurung Bulusaraung National Park, South Sulawesi, Indonesia. The isolated compounds were assessed for their cytotoxicity towards MCF-7 and 4T1 cell lines by MTT method. The dried ground leaves of *M. minutum* were sequentially macerated with n-hexane, ethyl acetate, and methanol. The n-hexane and ethyl acetate extracts contained a flavonoid 5,7-dihydroxy-3,4',8-trimethoxyflavone (**1**) which inhibited MCF-7 and 4T1 cell viability by 50% at concentrations of 369±8 and 227±5 µM, respectively. Further separation of the ethyl acetate extract by column chromatography yielded acetyldihydromicromelin A (**2**) and a mixture of dihydromicromelin A (**3**) and dihydromicromelin B (**4**), which were not active toward MCF-7 and 4T1 cells.

Keywords: *5,7-dihydroxy-3,4',8-trimethoxyflavone; breast cancer cell line; cytotoxic activity; dihydromicromelin derivatives; Micromelum minutum.*

1 Introduction

Micromelum minutum is a shrub or small tree under the Rutaceae family and widely used in several traditional medicines. The leaves are used to cure fever and headache, scabies, leprous spots, ringworm and are also considered to be emmenagogue, while the boiled roots are applied to treat ague [1].

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M. minutum is a species with four varieties [3], of which three are widespread in Indonesia, i.e. var. *minutum*, var. *tomentosum* and var. *villosum* [4]. Previous phytochemical studies on *M. minutum* revealed the presence of 6-prenylcoumarins [5]-[8], 8-prenylcoumarins [8]-[13], tetracyclic coumarins [14]][15], capnolactone derivatives coumarins [16]-[19], oxazole [20], carbazole [20],[21], and pyranoquinolin alkaloids [9], polyoxygenated flavonoids [8],[22],[23], dihydrocinnamic acid derivatives [14],[24], and triterpenes [17]. It is well-known that *M. minutum* contains coumarins as a main component, but there are many variations of chemical constituents in *M. minutum*, depending on the location of the plant. Some coumarins isolated from *M. minutum* have potent anticancer properties, i.e. 8-hydroxyisocapnolactone-2′,3′-diol and 2′,3′-epoxyisocapnolactone [25]-[27]; klauslacton E, minutin A, and minutin B [19]; 7-demethylmurralonginol isovalerate, murralonginol, murralonginol isovalerate, microminutin, and murrangatin [11].

Although *M. minutum* from several countries has been extensively surveyed with the isolation of some bioactive compounds, *M. minutum* growing in Indonesia has not been investigated phytochemically or pharmacologically. Our preliminary study showed that the leaf extracts of *M. minutum* collected in Bantimurung Bulusaraung National Park, South Sulawesi, Indonesia have cytotoxic activity toward breast cancer cell lines. In this study, we report the isolation of 5,7-dihydroxy-3,4',8-trimethoxyflavone (**1**), acetyldihydromicromelin A (**2**), and a mixture of dihydromicromelin A (**3**) and dihydromicromelin B (**4**); and their cytotoxicity towards breast cancer cell lines of MCF-7 and 4T1.

2 Materials and Methods

2.1 Materials

M. minutum leaves were collected from Bantimurung Bulusaraung National Park, Makassar, South of Sulawesi in March 2017 and determined at the Department of Pharmaceutical Biology, Faculty of Pharmacy, Universitas Gadjah Mada (UGM).

2.2 General Experimental Procedures

Melting points were measured on a Büchi Melting Point B-540 and are uncorrected. The purity and mass spectra were analyzed on an LC-MS/MS (Waters Xevo TQD, column Acquity UPLC BEH C-18 1.7 µm). The FT-IR spectra were obtained within KBr pellet using a Spectrum 100 Perkin Elmer Precisely. The UV spectra were recorded on a UV-Vis spectrometer (Hitachi U-2900, SpectraCuvet QS-104 ES Quartz Glass). ^{1}H-NMR (500 MHz), ^{13}C-NMR (125 MHz), DEPT 135°, COSY, HMBC and HSQC spectra were obtained on

an NMR JEOL JNM-ECZ500R. Chemical shifts are displayed in δ (ppm) with tetramethylsilane (TMS) as internal standard. Vacuum liquid chromatography was conducted on silica gel Merck 60 PF254 Art No.1.07747, while column chromatography was done using 60 Art No. 1.07734.1000. The solvents used to eluate the columns were analytical-grade and were made of different mixtures of n-hexane-chloroform and chloroform-methanol unless otherwise specified. Thin layer chromatography (TLC) was carried out by using Merck aluminium sheet pre-coated silica gel 60 F_{254}. A cytotoxic assay was performed by using the MTT method.

2.3 Maceration and Isolation

Dried ground leaves (5.5 kg) were macerated successively with n-hexane, ethyl acetate and methanol to give crude extracts of 145.9 g, 337.0 g, and 272.2 g, respectively, after solvent evaporation. The n-hexane extract (20 g) was fractionated using vacuum liquid chromatography on silica gel eluted with different mixtures of n-hexane-chloroform-methanol yielded 9 fractions (F1-F9). Fraction 6 (3.1 g) was further purified using gravity column chromatography to provide 9 fractions (F1-6.F). Fraction F6.F showed single yellow spots and was identified as **1** (18.2 mg). Compound **1** was isolated from ethyl acetate extract as the major constituent of the leaves. Separation of this extract by using vacuum liquid chromatography yielded 9 fractions (G1-9). Further purification of G5 and G6 resulted in the isolation of **1** (4.23 g). Fraction G4 was subjected to column chromatography and yielded 81 fractions. Fractions 28-42 were combined (294 mg) and was rechromatographed by eluting with n-hexane:chloroform 7:3 v/v to give 211 fractions. Purification of combined fractions 45-50 and 178-201 afforded **2** (39.2 mg) and a mixture of **3** and **4** (22.2 mg), respectively.

5,7-dihydroxy-3,4',8-trimethoxyflavone (**1**): yellow needles with m.p. 176-177°C. UV (MeOH) λ_{max} nm (log ε): 273 nm (4.36), 322 nm (4.18), 360 nm (4.08). MS ESI+ m/z: 345.45 $[M+H]^+$. FT-IR (KBr disc) cm^{-1}: 3308, 3081, 2942, 1647, 1611, 1029. ^{1}H-NMR (500 MHz, $CDCl_3$) and ^{13}C-NMR (125 MHz, $CDCl_3$) (see Table 1).

Acetyldihydromicromelin A (**2**): white needles with m.p. 173.3-173.7°C. UV (MeOH) λ_{max} nm (log ε): 322 nm (4.11), 293 (sh), 250 (sh), 220 nm (4.22). MS ESI+ m/z: 333.30 $[M+H]^+$. FT-IR (KBr disc) cm^{-1}: FT-IR (KBr disc) cm^{-1}: 3077, 2947, 2976, 1745 (br), 1622, 1217. ^{1}H-NMR (500 MHz, $CDCl_3$) and ^{13}C-NMR (125 MHz, $CDCl_3$) (see Table 2).

Dihydromicromelin A (**3**) and dihydromicromelin B (**4**): epimers mixture, yellowish white needles with m.p. 169.2-169.9°C, UV (MeOH) λ_{max} nm (log ε):

323 nm (4.00), 293 (sh), 250 nm (sh), MS ESI+ m/z: 291.34 $[M+H]^+$, FT-IR (KBr disc) cm^{-1}: 3401, 3072, 2906, 2931, 1731, 1621, ^{1}H-NMR (500 MHz, $CDCl_3$) and ^{13}C-NMR (125 MHz, $CDCl_3$) (see Table 2).

2.4 Cytotoxic Activity

MCF-7 and 4T1 breast cancer cell lines were obtained from the collection of the Cancer Chemoprevention Research Center, Faculty of Pharmacy UGM, gifted by Prof. Masashi Kawaichi, Nara Institute of Science and Technology (NAIST), Japan, and originally purchased from American Type Culture Collection (ATCC). The cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) complete media (Gibco). The cells were cultured at 37 °C in a 5% CO_2 atmosphere in the complete medium containing penicillin 150 U/mL and streptomycin 150 µg/mL (Gibco, Invitrogen USA), fungizone (Amphotericin B) 1.25 µg/mL (Gibco), and fetal bovine serum (FBS) (Sigma-Aldrich, USA) (20% for MCF-7 cells [28] and 10% for 4T1 cells [29].

Approximately10^4 MCF-7 cells/well and $2.3x10^3$ 4T1 cells/well were cultured in 96-well plates and then incubated in a 5% CO2 incubator (Heraeus) at 37 °C for 24 h. Following incubation, the medium was removed, the cells were washed with phosphate-buffered saline (PBS) and then isolate solutions in a serial concentration series were added in triplicate. The isolates were diluted with dimethyl sulfoxide (DMSO) (Merck) to make stock solutions. The final concentration of DMSO should not exceed 6%. After a 24-h incubation, the culture medium was removed and the cells were washed with PBS for each well. Subsequently, 100 µL of MTT (0.5 mg/mL) (Sigma) was added to each well. The incubation was continued for ±3 h at 37 °C until formazan was formed. The reaction was terminated by adding 100 µL of stopper solution (10% SDS in 0.01 N HCl) to dissolve the formazan crystals and the cells were incubated overnight at room temperature and protected from light. The absorbance was measured by using an ELISA reader at 595 nm and then converted to a percentage of viable cells. The IC_{50} value was calculated as previously described in [30]0,[31].

3 Results

The dried ground leaves of *M. minutum* were gradually macerated with n-hexane, ethyl acetate and methanol to give hexane (145.9 g), ethyl acetate (337.0 g) and methanol (272.2 g) extracts after evaporation of the solvents. Further separation of the hexane extract by column chromatography yielded methoxy flavone **1**, while separation of the ethyl acetate extract yielded **1** as the major constituent of the leaves and three micromelin derivative coumarins, **2** and a mixture of epimers **3** and **4**. The structure of these compounds were

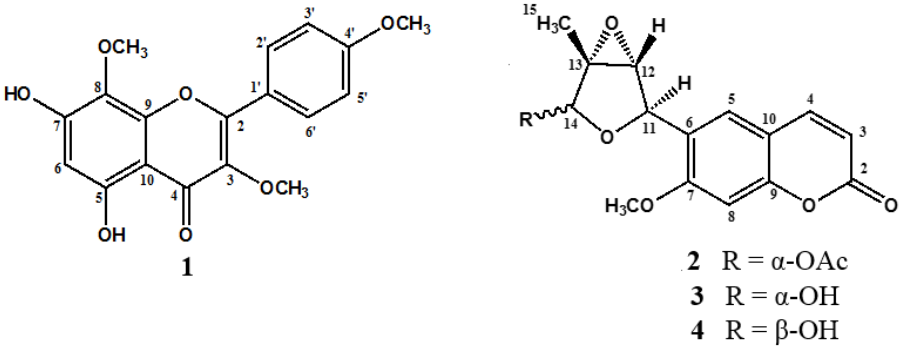

Figure 1 Figure 1 Chemical constituents of Indonesian *M. minutum*. 5,7-dihydroxy-3,4',8-trimethoxyflavone (**1**), acetyldihydromicromelin A (**2**), dihydromicromelin A (14α-OH) (**3**), and dihydromicromelin B (14β-OH) (**4**).

characterized by NMR examination (^{1}H- and ^{13}C-NMR, DEPT, HSQC, COSY, and HMBC) experiments and by comparing their spectral data with published values as 5,7-dihydroxy-3,4',8-trimethoxyflavone (**1**), acetyldihydromicromelin A (**2**), dihydromicromelin A (**3**) and dihydromicromelin B (**4**), respectively (Figure 1).

3.1 5,7-dihydroxy-3,4',8-trimethoxyflavone (1)

The ESI+ mode mass spectrum showed a [M+H] peak at m/z 345.45 consistent with $C_{18}H_{16}O_{7.}$ This compound is suggested to be a flavonoid since it gave a positive phenolic test with $FeCl_3$ (blackish brown) and the yellow color became more intense when treated with ammonia. The UV spectrum gave absorbtion maxima at 273 (band II), 322 (band IA) and 360 nm (band IB), typical for a flavone binding, and auxochrome (OH or OCH_3) at carbon 3 (ring C) and 4 (ring B) as in kaempferol, kaempferol-4′-metil ester, herbasetin-8-metil ester, etc. [32]. The flavone without auxochrome at C3 as asasetin gave only one absorbtion maximum of band I. The presence of a hydroxyl group at position 7 of ring A was confirmed by the 9.5 nm bathochromic shifting of band II (5-20 nm) [33]. The IR spectrum showed a broad strong band at 3308 cm^{-1}, indicating the occurrence of hydroxyl groups, while the strong band at 1647 cm^{-1} is characteristic of the conjugated ketone carbonyl group. The benzene moiety was represented by bands at 3081 (aromatic C-H) and 1611 cm^{-1} (-C=C-). The very downfield signal (s, 1H) at 12.4 ppm is specific for OH group at C5 of the flavone skeleton. One-proton singlet signal at 6.39 was assigned to H6 of the ring A, while the pair doublet at 8.10 and 7.10 (each 2H, J = 9 Hz) belongs to H2′/H6′ and H3′/H5′ of the ring B, respectively. This is strongly supported by a cross peak between the H2′/H6′ and H3′/H5′ signals in the COSY spectrum. Three singlet signals each with integration of 3H were assigned to methoxy

groups at positions 8 (ring A), 3 (ring C) and 4′ (ring B), respectively. The existence of three aromatic methine protons and three methoxy groups was also

Table 1 NMR spectral data of 1.

C/H	δC	δH	HMBC $^2J - ^3J$	δC*	δH#
2	155.9			155.0	
3	138.9			137.9	
4	179.1			178.1	
5	155.1			155.9	
6	98.5	6.39 (1H, s)	C-10, 8, 7, 5	98.8	6.41
7	157.7			156.9	
8	126.8			127.5	
9	148.1			148.6	
10	105.8			104.1	
1′	122.9			122.3	
2′ & 6′	130.2	8.10 (2H, d, 9)	C-2′, 6′ 2, 4′	129.8	8.11 (2H, d, 10.5)
3′ & 5′	114.4	7.00 (2H, d, 9)	C-3′, 5′, 1′	114.3	7.04 (2H, d, 10.5)
4′	161.9			161.3	
3-OCH_3	60.4	3.84 (3H, s)	C-3	59.7	3.84
8-OCH_3	62.1	3.97 (3H, s)	C-8	60.9	3.98
4′-OCH_3	55.6	3.88 (3H, s)	C-4′	55.4	3.88
5-OH		12.40 (1H, s)	C-6, 7, 10		12.44

*Horie *et al.* (1998) [34]; #Pandey *et al.* (1984) [35]

observed in the DEPT spectrum. The HMBC spectrum showed cross correlation between 5-OH and C6, C7 and C10; H-6 with C5, C7, C8 and C10; H2′/H6′ with C2′ and C4′; H3′/H5′ with C1′; 3-OMe, 8-OMe and 4′-OMe and C3, C8 and C4′, respectively. Based on the spectral analysis and by comparing the proton and carbon spectral data with the literature [34]4,[35], constituent **1** was characterized as 5,7-dihydroxy-3,4',8-trimethoxyflavone (Figure 1).

3.2 Acetyldihydromicromelin A (2)

The ESI+ spectrum exhibited a [M+H] peak at m/z 333.33 suitable to the $C_{17}H_{16}O_7$ molecular formula. This compound gave blue fluorescence under 366 nm UV light, which turned to intense yellow when treated with ammonia, suggesting a coumarin. The UV spectrum gave absorbtion maxima at 322, 293 (sh), 250 (sh) and 220 nm characteristic for a coumarin oxygenated at position 7 of the benzopyrone ring [16],[17]. The presence of a broad band with strong intensity centered at 1735 cm^{-1} suggests the existence of δ-lactone carbonyl functionality overlapping with the ester carbonyl group of the side chain of the coumarin nucleus. A pair of doublet in the ^{1}H-NMR spectrum (each 1H, J = 10 Hz) at δ 6.30 and 7.60 strongly indicate a coumarin unsubstituted in the pyrone ring. These signals belong to H-3 and H-4, respectively. This was strengthened by the cross peak between the H-3 and H-4 signals in the COSY spectrum. Two

singlet signals at δ 7.59 and 6.84 were assigned to H-5 and H-8, respectively. The singlet (3H) signal at δ 3.96 belongs to a -OCH_3 at C-7. All of the above analysis confirms the side chain located at position 6. Three singlet (1H) signals at δ 5.43, 3.86 and 3.86 together with two singlet (3H) signals at δ 1.52 and 2.18 belong to the oxygenated prenyl unit of the side chain as in acetyldihydromicromeline, previously reported as the constituent of Indian *M. minutum* [8]. These proton resonances were allocated to H-11, H-12, H-14, H_3-15 and H_3-17, respectively. The presence of ten positive signals in the DEPT spectrum and the HMBC correlations (H3 with C2, 10; H4 with C2, 5, 9; H5 with C4, 7, 9, 11; H8 with C6, 7, 9, 10; C11 with C5, 6, 12, 13; H12 with C11, H14 with C13; H15 with C13; H17 with C16 and 7-OCH_3 with C7) strongly support this structure. From the data analysis and by comparison to proton and carbon spectral data from a previous report [8], constituent **2** was determined as acetyldihydromicromeline A (Figure 1).

3.3 Dihydromicromelin A (3) and Dihydromicromelin B (4)

Compound **3** and **4** were suggested to be coumarins since the isolate containing them also gave intense yellow fluorescence when treated with ammonia. The presence of an [M+H] ion peak at m/z 291.34 (42 MU less than that of **2**) in the ESI+ mass spectrum indicated the molecular formula $C_{15}H_{14}O_6$. The UV spectrum exhibited absorbtion maxima at 323, 293 (sh) and 250 (sh) nm typical for a coumarin oxygenated at position 7 of the benzopyrone ring as **2**. The IR spectrum was close to that of **2**; the only differences were the lack of a sharp and strong ester carbonyl signal at 1731 cm^{-1} and the appearance of a new broad strong vibrational band at 3401 cm^{-1} for a hydroxyl group. These data indicate the lack of an acetyl group in **2**. This was strongly supported by the loss of methyl (δ 21.29) and carbonyl (δ 169.43) signals in the ^{13}C-NMR spectrum, the absence of methyl protons at δ 2.18 in the ^{1}H-NMR spectrum, and the appearance of a new signal at δ 3.10, which was assigned to the OH group. The signal patterns in the NMR spectra were similar to those of **2**, but each signal seemed to be split into two [36]. The ^{1}H-NMR data corresponded to the published value [8]. Thus, the isolate contains a mixture of epimers dihydromicromelin A (14α-OH) and dihydromicromelin B (14β-OH) (Figure 1).

3.4 Cytotoxic Activity

The constituents of Indonesian *M. minutum* leaves have various cytotoxic effects towards MCF-7 and 4T1 cell lines. Based on the curve of cell viability and concentration, compound **1** reduced the viability of MCF-7 and 4T1 cancer cells along with an increased concentration (dose-dependent effect). The

flavone, compound **1,** was more active than the coumarin compounds (**2-4**) (Table 3).

Table 2 NMR spectral data of **2**, **3**, and **4** [$CDCl_3$, 500 MHz (^{1}H-NMR), 125 MHz (^{13}C-NMR)].

C/H	**2**		**3**		**4**		ADMA		DMA	DMB
	δC	δH	δC	δH	δC	δH	δC	δH	δH	δH
2	160.84	-	160.97		161.13		159.30			
3	113.67	6.30 (1H, d, 10)	113.67	6.30 (1H, d, 9)	113.37	6.27 (1H, d, 9.5)	113.73	6.29 (d, 10)	6.33 (d, 10)	6.29 (d, 10)
4	143.33	7.60 (1H, d, 9.5)	143.40	7.66 (1H, d, 9.5)	143.77	7.66 (1H, d, 9.5)	143.20	7.60 (d, 10)	7.70 (d, 10)	7.70 (d, 10)
5	126.68	7.59 (1H, s)	125.98	7.41 (1H, s)	127.83	7.77 (1H, s)	126.76	7.50	7.46 (s)	
6	123.26	-	124.02		124.22		123.34			
7	159.23	-	159.34		159.34		160.81			
8	98.86	6.84 (1H, s)	99.12	6.84 (1H, s)	98.78	6.82 (1H, s)	98.92	6.84	6.88 (s)	6.87 (s)
9	155.79	-	155.87		155.74		155.85			
10	111.97	-	112.25		112.21		112.03			
11	77.95	5.43 (1H, s)	76.24	5.41 (1H, s)	77.07	5.39 (1H, s)	77.93	5.43	5.43 (s)	5.41 (s)
12	63.56	3.86 (1H, s)	66.56	3.78 (1H, s)	64.43	3.77 (1H, s)	63.63	3.83	3.79 (s)	3.78 (s)
13	64.42	-	65.91		65.44		64.44			
14	96.81	6.38 (1H, s)	98.48	5.41 (1H, s)	98.36	5.49 (1H, s)	96.89	6.83	5.41 (s)	5.52 (s)
15	12.49	1.52 (3H, s)	12.97	1.56 (3H, s)	12.76	1.58 (3H, s)	12.50	1.51	1.56 (s)	1.58 (s)
-OCH_3	56.16	-	56.15	3.95 (3H, s)	56.15	3.96 (3H, s)	56.17		3.97 (s)	3.98 (s)
-OH	-			3.10 (s)	-	3.46 (s)	-			
-OAc (16)	169.43						169.30			
(17)	21.29						21.23			

ADMA = acetyldihydromicromelin A; DMA = dihydromicromelin A; DMB = dihydromicromelin B; [$CDCl_3$, 270 MHz (^{1}H-NMR), 67.89 MHz (^{13}C-NMR)] [8]

Table 3 IC_{50} values of compounds isolated from Indonesian *M. minutum* leaves.

Sample	**IC_{50}** (µg/mL)	
	MCF-7 cells	**4T1 cells**
Compound 1	127 ± 2.78 (369 ± 8.08 µM)*	78 ± 1.66 (227 ± 4.82 µM)*
Compound 2	na	na
Compounds 3 and 4	na	na

na = not active (>500 µg/mL); *average ± SE

4 Discussion

The leaves of *M. minutum* from Bantimurung Bulusaraung National Park, South Sulawesi contained 5,7-dihydroxy-3,4',8'-trimethoxyflavone (**1**) as a major component along with acetyldihydromicromelin A (**2**) and a mixture of epimers dihydromicromelin A (**3**) and dihydromicromelin B (**4**). Polymethoxylated flavonoids are known to occur in this species as free [22],[23] or as a 7-O-ether with murrangatin [11], but its existence as a major component in the leaves of Indonesian *M. minutum* is an interesting finding. In contrast to the presence of a great deal of chemical variation in coumarins of *M. minutum*, polymethoxyflavones, i.e. 5,7-dihydroxy-3,4′,6,8-tetramethoxyflavone, (**1**), 5-hydroxy-3,4′,7,8-tetramethoxyflavone and 5-hydroxy-3,4′,6,7,8-pentamethoxy-

flavone are flavonoids so far reported as the constituents of this plant. The three coumarins (**2-4**) were previously isolated from the Assamese collection of *M. minutum* [8]. The flavonoid (**1**) was shown to have cytotoxic activity towards MCF-7 and T47D cell lines, but all the coumarins were found to be not active. Micromelin itself is also not active against MCF-7, but it exhibited cytotoxicity against the cholangiocarcinoma cell line, KKU-100, and oral cancer cell line, KB, with IC_{50} of 9.2 μg/mL and 31.5 μg/mL, respectively [11],[37].

5 Conclusion

Leaves of *M. minutum* from Bantimurung Bulusaraung National Park, South Sulawesi contained 5,7-dihydroxy-3,4′,8′-trimethoxyflavone as a major constituent along with acetyldihydromicromelin A and a mixture of epimers dihydromicromelin A and dihydromicromelin B. Compound 5,7-dihydroxy-3,4′,8′ -trimethoxyflavone had cytotoxic activity on MCF-7 and 4T1, while all the isolated coumarins were not active.

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