Phenolic Constituents from the Wood of *Morus australis* with Cytotoxic Activity

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A new methylated flavonol, 5,7,2′,4′-tetrahydroxy-3-methoxyflavone (**1**), had been isolated from the methanol extract of the wood of *Morus australis*, along with nine known compounds, kuwanon C (**2**), morusin (**3**), morachalcone A (**4**), oxyresveratrol (**5**), 4′-(2-methyl-2-buten-4-yl)oxyresveratrol (**6**), moracins M (**7**) and C (**8**), alboctalol (**9**), and macrourin B (**10**). The structures of these compounds were determined based on spectral evidence, including UV, IR, NMR, and mass spectra. Cytotoxic properties of compounds **1**–**10** were evaluated against murine leukemia P-388 cells. The prenylated stilbene **6** and 2-arylbenzofuran **8**, and morusin (**3**) were found to have strong cytotoxic effects with IC_{50} values of 6.9, 8.7, and 10.1 μM, respectively.

Key words: Morus australis, Murine Leukemia P-388 Cells, 5,7,2′,4′-Tetrahydroxy-3-methoxyflavone

Introduction

Morus is a small genus consisting of about 13 species. It is one of the economically important genera of the family Moraceae due to its leaves which serve as food for silkworms (Nomura, 1988). This genus contains a variety of phenolic compounds including isoprenylated flavonoids, stilbenes, 2-arylbenzofurans, coumarins, chromones, xanthones and a variety of Diels-Alder adducts (Nomura, 1988; Nomura and Hano, 1994). Some of these compounds exhibit interesting biological properties such as antiphlogistic, anti-inflammatory, diuretic, hypotensive effects, and some are also known as phytoalexins. In previous papers, we had reported two stilbene dimers, andalasins A and B, from a native Indonesian *Morus*, *M. macroura* (Syah *et al*., 2000, 2004). As part of our work in search of new cytotoxic compounds (Sahidin *et al*., 2005; Usman *et al*., 2006; Hakim *et al*., 2006; Syah *et al*., 2006; Muhtadi *et al*., 2006), we had examined another species of *Morus*, *M. australis* Poir., growing in Indonesia. Previous phytochemical investigations on this plant had revealed the presence of 2-arylbenzofuran, flavanone, and flavone derivatives, as well as Diels-Alder adducts (Nomura and Fukai, 1982; Nomura *et al.*, 1983; Ko *et al.*, 1997, 1999; Shi *et al.*, 2001). This paper reports the isolation of ten phenolic compounds, including a new flavon-3-ol derivative, 5,7,2′,4′-tetrahydroxy-3-methoxyflavone (**1**), from the methanol extract of the wood of this plant, as well as their cytotoxic properties against murine leukemia P-388 cells.

Results and Discussion

The powdered and dried wood of *M. australis* was macerated with methanol at room temperature, and a dark-brown residue was obtained after solvent evaporation. A portion of the extract was fractionated using vacuum liquid chromatography on silica gel to give nine major fractions A–I. According to TLC analysis, most of the components were contained in fraction E, and purification of this fraction yielded 5,7,2′,4′-tetrahydroxy-3-methoxyflavone (**1**) (Fig. 1), along with four known phenolic compounds morachalcone A (**4**) (Hano *et al*., 1989), oxyresveratrol (**5**) (Nomura, 1988), 4′-(2-methyl-2-buten-4-yl)oxyresveratrol (**6**) (No-

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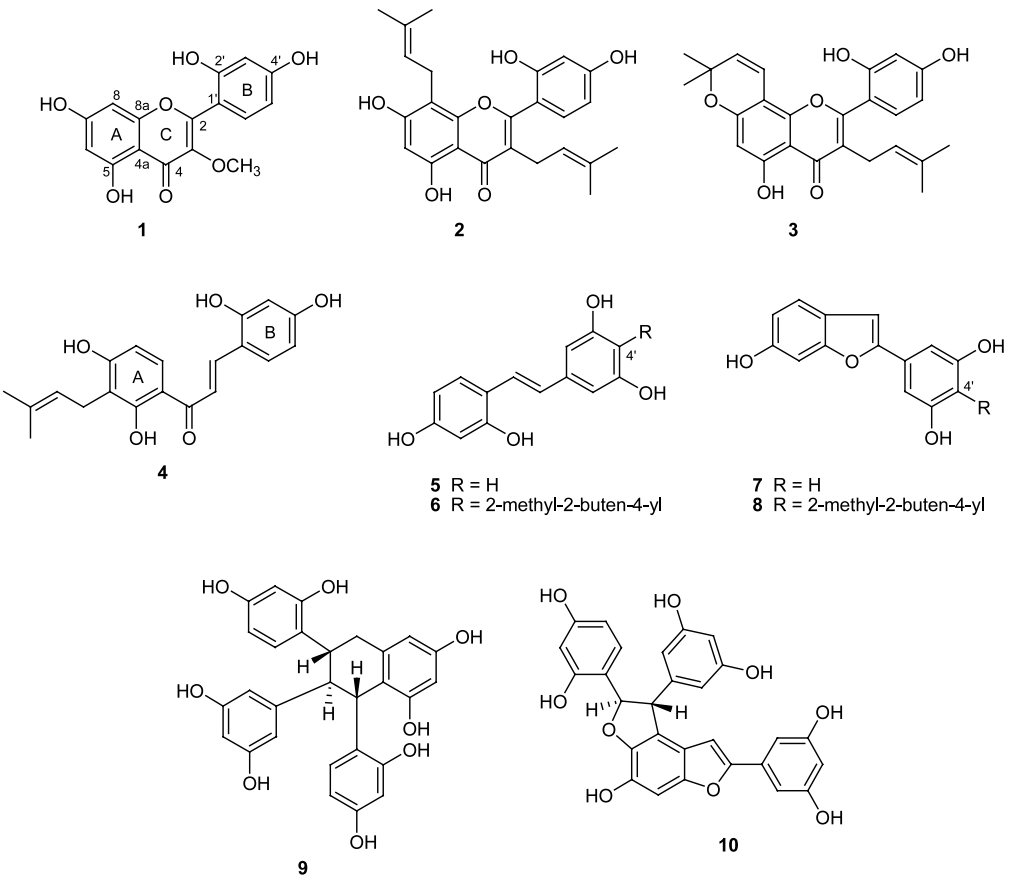

Fig. 1. The phenolic constituents isolated from *M. australis.*

mura, 1988), and moracin M (**7**) (Basnet *et al.*, 1993). Using similar procedures, five other known compounds, *i.e.*, kuwanon C (**2**) (Nomura *et al.*, 1978), morusin (**3**) (Nomura *et al.*, 1976), moracin C (**8**) (Takasugi *et al.*, 1978), alboctalol (**9**) (Bates *et al.*, 1997), and macrourin B (**10**) (Dai *et al.*, 2004), were isolated and identified.

Compound **1**, isolated as a pale yellow solid, showed a quasimolecular ion $[M+H]^+$ in the HRFABMS spectrum at *m/z* 317.0670, corresponding to the molecular formula $C_{16}H_{12}O_7$. The UV and IR spectra of **1** exhibited absorptions (λ_{max} 205, 260, 333 nm; ν_{max} 1653, conjugated C=O) typical for a 3-substituted flavone. The ^{13}C NMR data (Table I) disclosed the presence of 16 well separated carbon signals, one of which was a typical signal for a methoxy carbon atom (δ_C 60.9 ppm), and the rest were signals of sp^2 carbon atoms, including one conjugated carbonyl group (δ_C 179.2 ppm), six oxyaryl (δ_C 164.8, 163.2, 162.0, 158.4, 157.9, 157.7 ppm) carbon atoms, and the C-3 signal (δ_C 139.5 ppm) of the flavon-3-ol (Hussein *et al.*, 2005). These spectroscopic data, therefore, suggested that **1** is a monomethylated tetrahydroxyflavon-3-ol. The proton signals in the ^1H NMR spectrum of **1** (Table I) at δ_H 6.39 and 6.25 ppm as a doublet (J = 2.2 Hz) and at δ_H 12.77 ppm for a chelated phenolic –OH group, indicated that two –OH groups are located at C-5 and C-7. Furthermore, the presence of an ABX spin system of three aromatic signals at δ_H 6.54, 6.55, and 7.38 ppm, in which the later signal contains only an *ortho* coupling (J = 8.2 Hz), allowed the other two –OH groups to be located at C-2′ and C-4′. Though the methoxy group could be located either at C-7, C-2′, or C-4′, the long range correlation found in the HMBC spectrum of **1** between a methoxy proton signal at δ_H 3.80 ppm with the carbon signal at δ_H 139.5 ppm secured the position of the methoxy group at C-3. Thus, compound **1**

Table I. ^{1}H NMR data of compound **1** in $CDCl_3$.

No	δ_H (multiplicity, J in Hz)	δ_C	HMBC (H ⇔ C)
1	–	–	–
2	–	157.9	–
3	–	139.5	–
4	–	179.2	–
4a	–	106.1	–
5	–	163.2	–
6	6.25 (*d*, 2.2)	99.3	C-4a, C-5, C-7, C-8
7	–	164.8	–
8	6.39 (*d*, 2.2)	94.5	C-4a, C-6, C-7, C-8a,
8a	–	158.4	–
1′	–	110.6	–
’2′	–	157.7	–
3′	6.54 (*d*, 2.2)	104.4	C-2′, C-4′, C-5′
4′	–	162.0	–
5′	6.55 (*dd*, 2.2, 8.2)	108.5	C-1′
6′	7.38 (*d*, 8.2)	132.4	C-2′, C-4′
3-OCH_3	3.80 (*s*)	60.9	C-3
5-OH	12.77 (*s*)	–	C-4a, C-5, C-6
7-OH	9.67 (*s*)	–	–
2′-OH	8.6 (*s*)	–	C-1′, C-3′
4′-OH	8.9 (*s*)	–	C-3′, C-5′

was assigned as 5,7,2′,4′-tetrahydroxy-3-methoxyflavone. Other HMBC correlations in support for the structure **1** are shown in Table I.

The cytotoxic properties of compounds **1**–**10** (Table II) were evaluated against murine leukemia P-388 cells according to the method described previously (Sahidin *et al.*, 2005). Three prenylated compounds, *i.e.*, morusin (**3**), 4′-(2-methyl-2-buten-4-yl)oxyresveratrol (**6**), and moracin C (**8**), strongly inhibited the cells with IC_{50} values of 10.1, 6.9, and 8.7 μM, respectively. Comparison of the IC_{50} values of compounds **6** and **8** with those of oxyresveratrol (**5**) and moracin M (**7**), respectively, suggested that introducing a prenyl group at C-4′ to a stilbene and 2-arylbenzofuran increases the cytoxicity. In case of compounds **5** and **6**, the same tendency was also demonstrated concerning their activities as inhibitors of the enzyme tyrosinase (Shimizu *et al.*, 2000; Lee *et al.*, 2003). In the flavone series, the strong cytotoxic effect of compound **3** corroborated previous observations (Hakim *et al.*, 2006) showing that prenylation at C-3 of the flavone skeleton significantly contributes to the cytotoxicities against P-388 cells. However, a change from a cyclic form of the prenyl group at C-8 in **3** to a free prenyl group in kuwanon C (**2**) slightly reduced the cytotoxic effect. It is also interesting to note that the presence of a more polar group at C-3 of the flavone in compound **1**, instead of the more hydrophobic prenyl group, greatly reduced the cytotoxicity. A note should also be given to the chalcone **4**; though it contains a prenyl group in the ring A, it failed to give significant cytotoxicity. The stilbene dimer **9** and the adduct of stilbene and 2-arylbenzofuran **10** were shown to be inactive against P-388 cells. Thus, as a general observation, it is attempted to hypothesize that introducing a prenyl group to a phenolic compound could give a significant increase in the cytotoxic effect.

Experimental

General experimental procedures

UV spectra were measured with a Varian Conc. 100 instrument. IR spectra were determined with a Perkin Elmer FTIR Spectrum One spectrometer using KBr pellets. ^{1}H and ^{13}C NMR spectra were recorded with a JEOL ECP400 spectrometer, operating at 400 (^{1}H) and 100 (^{13}C) MHz, using residual and deuterated solvent peaks as reference standards. Mass spectra were obtained with a

Table II. IC_{50} values of compounds **1**–**10** against P-388 cells.

Compound	Type	IC_{50} [μM]
5,7,2′,4′-Tetrahydroxy-3-methoxyflavone (**1**)	Flavon-3-ol	37.3 ± 5.4
Kuwanon C (**2**)	3-Prenylated flavone	14.0 ± 1.0
Morusin (**3**)	3-Prenylated flavone	10.1 ± 0.8
Morachalcone A (**4**)	Prenylated chalcone	17.8 ± 1.4
Oxyresveratrol (**5**)	Stilbene	35.3 ± 3.7
4′-(2-Methyl-2-buten-4-yl)oxyresveratrol (**6**)	Prenylated stilbene	6.9 ± 0.7
Moracin M (**7**)	2-Arylbenzofuran	24.8 ± 0.0
Moracin C (**8**)	Prenylated 2-arylbenzo-furan	8.7 ± 0.3
Alboctalol (**9**)	Stilbene dimer	74.7 ± 4.3
Macrourin B (**10**)	2-Arylbenzofuran stilbene adduct	62.4 ± 7.8

JEOL JMS-AM20 mass spectrometer, using the FAB mode. Vacuum liquid (VLC) and column chromatography were carried out using Merck silica gel 60 GF_{254} and silica gel G60 35–70 mesh. For TLC analyses, precoated silica gel plates (Merck Kieselgel 60 GF_{254}, 0.25 mm) were used.

Plant material

The wood of *M. australis* was collected at Cibeureum Village, Cisurupan, Garut, West Java, Indonesia, in July 2005. The plant was identified at the Herbarium Bogoriense, Bogor, Indonesia.

Extraction and isolation

The powdered dried wood (4.3 kg) of *M. australis* was macerated with methanol at room temperature for 24 h (3 × 12.5 L). The mixture was filtered and the solvent was evaporated under reduced pressure to give a dark-brown methanol extract (240 g). A portion (60 g) of the extract was fractionated using vacuum liquid chromatography (silica gel; eluted with *n*-hexane/ethyl acetate 8:2 → 0:10, EtOAc/MeOH 8:2) to give nine major fractions A–I (1.4, 0.8, 1.4, 0.6, 4.4, 0.6, 18.5, 6.1, and 2.5 g, respectively). Fraction E was refractionated using the same method (silica gel; eluted with *n*-hexane/ethyl acetate 7:3 → 0:10 → EtOAc/MeOH 9:1) to give seven fractions E1–E7 (0.05, 0.06, 0.06, 0.34, 1.14, 2.0, and 1.0 g, respectively). Purification of fraction E4 using centrifugal planar chromatography (silica gel; eluted with *n*-hexane/ethyl acetate 7:3 → 1:1 and $CHCl_3$/MeOH 97:3) yielded compounds **4** (10 mg), **6** (15 mg), **1** (3 mg), and **7** (12 mg). From a portion of fraction E6 (250 mg), after purification by centrifugal planar chromatography (silica gel; eluted with $CHCl_3$/MeOH 9:1), compound **5** (90 mg) was isolated. Using similar procedures, fractions C and D gave compounds **3** (17 mg), **8** (40 mg), and **2** (27 mg) and fraction H afforded compounds **9** (30 mg) and **10** (5 mg).

5,7,2′,4′-Tetrahydroxy-3-methoxyflavone (**1**): Pale yellow solid. – UV: λ_{max}(MeOH) (log ε) = 205 (4.22), 260 (3.88), 333 (3.73) nm; λ_{max}(MeOH + NaOH) = 203 (4.50), 269 (3.96), 324 (3.71), 385 (3.76) nm; λ_{max}(MeOH + $AlCl_3$) = 203 (4.30), 267 (3.99), 388 (3.59) nm; λ_{max}(MeOH + $AlCl_3$ + HCl) unchanged from (MeOH + $AlCl_3$). – IR (KBr): ν_{max} = 3376, 3137, 2934, 1653, 1625, 1606, 1577, 1500, 1468, 1366, 1305, 1240, 1212, 1169, 1107, 1085, 1006, 806 cm^{-1}. – ^1H NMR (400 MHz, $CDCl_3$): see Table I. – ^{13}C NMR (100 MHz, $CDCl_3$): see Table I. – HRFABMS: *m/z* = 317.0670 $[M+H]^+$ (calcd. for $C_{16}H_{12}O_7$ 317.0661).

Cytotoxicity assay

The cytotoxicity assay was conducted according to the method described previously (Sahidin *et al.*, 2005). P-388 cells were seeded into 96-well plates at an initial cell density of approximately $3 \cdot 10^4$ cells cm^{-3}. After 24 h of incubation for cell attachment and growth, varying concentrations of samples were added. The compounds added were first dissolved in DMSO at the required concentration. Subsequent six desirable concentrations were prepared using PBS (phosphoric buffer solution, pH 7.30–7.65). Control wells received only DMSO. The assay was terminated after a 48 h incubation period by adding MTT reagent [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; also named as thiazol blue], and the incubation was continued for another 4 h, in which the MTT-stop solution containing SDS (sodium dodecyl sulfate) was added and another 24 h incubation was conducted. Optical density was read by using a microplate reader at 550 nm. IC_{50} values were taken from the plotted graph of percentage live cells compared to control (%), receiving only PBS and DMSO, versus the tested concentration of compounds (μM). The IC_{50} value is the concentration required for 50% growth inhibition. Each assay and analysis was run in triplicate and averaged, and the results are shown in Table II.

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