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FLAVANONES FROM THE WOOD OF *Morus nigra* WITH CYTOTOXIC ACTIVITY

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ABSTRACT

*Two flavanone derivatives, norartocarpanone (**1**) and euchrenone a7 (**2**) had been isolated for the first time from the methanol extract of the wood of Morus nigra. The structures of these compounds were determined base on spectral evidence, including UV, IR, and NMR. The first compound also confirmed by comparison with the reported data. Cytotoxic properties of these compounds were evaluated against murine leukemia P-388 cells. Euchrenone a7 (**2**) was found more cytotoxic than norartocarpanone (**1**) with their IC_{50} 7.8 and 12.7 μg/mL respectively.*

Keywords: *norartocarpanone; euchrenone a7; Morus nigra; cytotoxic*

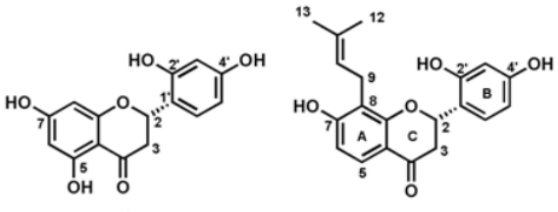
ABSTRAK

*Dua senyawa flavanon yaitu norartokarpanon (**1**) dan eukrenon a7 (**2**) telah berhasil diisolasi untuk pertama kali dari ekstrak metanol kayu batang Morus nigra. Struktur senyawa tersebut ditetapkan dengan cara-cara spektroskopi yang meliputi spektrum UV, IR dan NMR. Senyawa norartokarpanon (**1**) juga dikonfirmasi dengan membandingkannya dengan data yang telah dilaporkan. Sifat sitotoksik kedua senyawa tersebut ditentukan terhadap sel murin leukemia P-388. Eukrenon a7 (**2**) lebih bersifat sitotoksik dibandingkan norartokarpanon (**1**) dengan IC_{50} berturut-turut 7,8 dan 12,7* μg/mL.

Kata Kunci: *norartokarpanon; eukrenon a7; Morus nigra; sitotoksik*

INTRODUCTION

Morus nigra belongs to the economically and medically important genus *Morus*, whose leaves have been an indispensable food source for silk-worms, and its root barks have been used to treat diabetes, arthritis and rheumatism in Chinese herbal medicine [1]. The constituents of its root bark and bark have been studied by many investigators, and a series of isoprenylated phenols have been isolated including flavonoid, stilbene, 2-arilbenzofuran and adduct Diels-Alder [2-3]. Some of these phenolic compounds showed significant antimicrobial [4], antioxidative [5], anti-inflammation [5] and cytotoxic activities [6].



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Some phenolic compounds have been isolated from the bark and root bark of *M. nigra* such as flavanone namely kuwanon E and kuwanon U [7]. However, the wood of *M. nigra* has been rarely studied. Previously, we have been isolated the chalcone and flavone compounds [8-9] from the wood of *M. nigra*. In continuing our investigation on *Morus* plant aiming to find cytotoxic compounds, two known flavanones namely norartocarpanone (**1**) and euchrenone a7 (**2**) have been isolated from the methanol extract of wood of *M. nigra*. All compounds were obtained for the first time from this species, moreover, euchrenone a7 (**2**) were isolated for the first time from *Morus* genus. The cytotoxicity of these compounds was evaluated against murine leukemia P-388 cells and will be briefly discussed.

EXPERIMENTAL SECTION

Materials

The woods of *M. nigra* were collected from Cibeureum Village, Cisurupan, Garut, West Java, Indonesia. The plant species was identified at

Herbarium Bogoriense, Cibinong, Indonesia. Vacuum liquid chromatography (VLC) was carried out using Merck silica gel 60 GF_{254}, while radial chromatography was carried out using Merck silica gel 60 PF_{254}. Sephadex LH-20 (Sigma-Aldrich) was used for column chromatography. For TLC analysis, precoated silica gel plates (Merck Kieselgel 60 GF_{254}, 0.25 mm) were used. Visualization of TLC plates was carried out under UV at 254 nm, as well as by spraying the plates with cerium sulfate 1.5% in sulfuric acid 2 N. The organic solvents were used in this research should be pro analysis (p.a) and distilled, i.e., chloroform, methanol, n-hexane, ethyl acetate and acetone.

Instrumentation

Melting points were determined using Fisher John Apparatus. 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 JEOL ECP400 spectrometer, operating at 400 (^{1}H) and 100 (^{13}C) MHz, using residual and deuterated solvent peaks as reference standards.

Procedure

Extraction and isolation

The dried powder of the heartwoods of *M. nigra* (4.1 kg) was extracted with methanol at room temperature for 24 h (3 x 12.5 L). The methanol extracts were evaporated under reduces pressure to give a dark-brown residue (153 g). A portion of methanol extract (5 x 20 g) was fractionated using vacuum liquid chromatography (VLC) (silica gel, eluted with *n*-hexane:EtOAc = 7:3 → 0:10 and EtOAc:MeOH = 9:1) to give six major fraction A-F (1.2; 2.1; 17.2; 7.2; 20.0; and 7.7 g respectively). Furthermore, fraction C (17.2 g) was refractionated using the same method (silica gel, eluted with *n*-hexane:EtOAc = 7:3 → 4:6, EtOAc, and EtOAc:MeOH = 9:1) yield six fractions C1-C6. Of these, fraction C2 (1.8 g) was further separated by radial chromatography (silica gel, eluted with *n*–hexane:EtOAc = 7:3, 1:1 and 3:7) to give eight fractions C2.1-C2.8. Further separation on fraction C2.8 (144 mg) by column chromatography (sephadex, eluted with MeOH), followed by radial chromatography (silica gel, eluted with $CHCl_3$:MeOH = 97:3), was yield compound **1** (36 mg). Furthermore fraction C2.7 (814 mg) was separated using column chromatography (sephadex, eluted with MeOH) to give four fraction. The 2nd fraction was further separated using radial chromatography (silica gel, eluted with eluen $CHCl_3$:MeOH = 97:3), which followed by using flash column chromatography (silica gel, eluted with *n*-hexane:acetone = 6:5) to yield compound **2** (20 mg).

Cytotoxicity assay

The cytotoxicity assay was conducted according to the method described previously [10]. P-388 cells were seeded into 96-well plates at an initial cell density of approximately 3 x 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 thiazole blue] and the incubation was continued for another 4 h, in which the MTT-stop solution containing SDS (sodium dodecyl sulphate) was added and another 24 h incubation was conducted. Optical density was read by using a micro plate 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.

RESULT AND DISCUSSION

The methanol extract from the dried heartwoods of *M. Nigra* was subjected to a series of chromatographic techniques to obtain compounds **1** and **2**. Their structures were established by UV, IR and NMR spectroscopies.

Compound **1** was obtained as a yellow solid, with m.p. 128-130 °C (decomposed). The UV spectra of **1** exhibited absorption at λ_{max} nm (log ε): 203 (4.02), 226 (sh. 3.76) and 287 (3.69), typical for flavanone skeleton. The bathochromic shift in addition of NaOH as well as $AlCl_3$ indicating the presence of phenolic group and chelating between hydroxyl and carbonyl group respectively. Consistent to UV, the IR spectra showed the functional group of flavanone such as conjugated carbonyl (1642 cm^{-1}), C=C aromatic (1600-1458 cm^{-1}) and hydroxyl group (3402 cm^{-1}). The NMR data (Table 1) disclosed the presence of three protons as ABX spin system at δ_H 2.70 (*dd*, *J* = 2.9 & 17.2 Hz), 3.18 (*dd*, *J* = 13.2 & 17.2 Hz) and 5.70 ppm (*dd*, *J* = 2.9 & 13.2 Hz) that characteristic for proton at C-3eq, C-3ax and C-2 of flavanone skeleton. The proton signal at δ_H 5.94 and 5.96 ppm as pair of doublet (*J* = 2.2 Hz) and at δ_H 12.21 ppm for a chelated phenolic –OH, indicated that two –OH groups are located at C-5 and C-7. Furthermore, the presence of an ABX system of three aromatic signals at δ_H 6.42, 6.46

Table 1. NMR data of compound **1** in acetone-d_6

No	δ_H (multiplicity, *J* in Hz)	
	1	1*
2	5.70 (*dd*, 2.9 & 13.2)	5.70 (*dd*, 2.9 &13.2)
3	3.18 (*dd*, 13.2 & 17.2) H-ax 2.70 (*dd*, 2.9 & 17.2) H-eq	3.18 (*dd*, 13.2 & 17.2) H-ax 2.70 (*dd*, 2.9 & 17.2) H-eq
4	-	-
5	-	-
6	5.96 (*d*, 2,2)	5.96 (*d*, 2.2)
7	-	-
8	5.94 (*d*, 2.2)	5.93 (*d*, 2.2)
1'	-	-
2'	-	-
3'	6.46 (*d*, 2.6)	6.46 (*d*, 2.4)
4'	-	-
5'	6.42 (*dd*, 2.6 & 8.4)	6.42 (*dd*, 2.4 & 8.4)
6'	7.31 (*d*, 8.4)	7.31 (*d*, 8.4)
5-OH	12.21 (*s*)	12.22 (*s*)
7/2'/4'-OH	8.6 (*s*), 8.4 (*s*), 9.6 (*s*)	-

1*: [11]

and 7.31 ppm, in which the later signal contain only an *ortho* coupling (*J* = 8.0 Hz), allowed the two –OH groups to be located at C-2' and C-4'. These spectroscopic data, therefore, suggested that **1** is norartocarpanone. This suggestion was supported by NMR data of norartocarpanone [11]. The comparison of these data showed high similarity, in conclusion, the compound **1** is nortocarpanone

Compound **2** was isolated as yellow solid with m.p. 118-119 °C. The UV spectra of **2** exhibited absorption at λ_{max} nm (log ε): 203 (4.64), 221 (sh, 4.48) and 285 (4.19) typical for flavanone skeleton. The bathochromic shift in addition of NaOH indicating the presence of phenolic group, but there is no bathochromic effect in addition of $AlCl_3$ indicating there is no chelating between hydroxyl and carbonyl group. Consistent to UV, the IR spectra showed the functional group of flavanone such as conjugated carbonyl (1645 cm^{-1}), C=C aromatic (1602-1441 cm^{-1}), hydroxyl group (3394 cm^{-1}) and C-H aliphatic group (2978-2927 cm^{-1}). The ^{13}C-NMR data (Table 2) disclosed the presence of 20 well separated carbon signals, one of which was a typical signal for conjugated carbonyl (δ_C 191.6 ppm), 14 signal for C-sp^2 atom including four oxyaryl (δ_C 162.4, 162.1, 159.3 and 156.1 ppm), and the rest were signal for C-sp^3 atom including one oxymetin signal (δ_C 75.8 ppm). The ^{1}H-NMR spectra (Table 2) displayed three proton signal as ABX spin system at δ_H 2.71 (*dd*, *J* = 2.9 & 16.8 Hz), 2.96 (*dd*, *J* = 13.2 & 16.8 Hz) and 5.69 ppm (*dd*, *J* = 2.9 & 13.2 Hz) that characteristic for proton at C-3eq, C-3ax and C-2 of flavanone skeleton. Another ABX spin system was appeared for three aromatic protons signal at δ_H 6.43, 6.47 and 7.37 ppm, in which the later signal contain only an ortho coupling (*J* = 8.0 Hz) allowed two –OH group to be located at C-2' and C-4' that identical to compound 1. Furthermore, the proton signals at δ_H 7.59 and 6.61 ppm as a pair of doublet (*J* = 8.8 Hz) without signal for chelated phenolic -OH, indicated only one –OH group is located at C-7 with one more substituent at C-8. The substituent should be an isoprenyl which presence of two *broad-singlet* signal of vinylic methyl (δ_H 1.60 and 1.64 ppm), a doublet signal of methylene (δ_H 3.34 ppm, *J* = 7.3 Hz) and a triplet signal of olefin (δ_H 5.25 ppm, *J* = 7.3 Hz). The long range correlation in the HMBC spectrum of **2** between a proton signals at δ_H 7.59 ppm with the carbonyl signal at δ_C 191.6 ppm as well as correlation between methylene proton at δ_H 3.34 ppm with oxyaryl carbon at δ_C 162.4 and 162.1 ppm, secured the position of an isoprenyl at C-8. The NOESY spectra was supported this an isoprenyl position at C-8 with correlation between a methylene proton at δ_H 3.34 ppm with one of *ortho* proton at δ_H 6.61 ppm .Thus, compound **2** was assigned as 7,2',4'-trihydroxy-8-isoprenylflavanone or euchrenone a7 [12]. Other HMBC and NOESY correlations in support for the structure **2** are shown in Table 2.

Both of the isolated flavanones are not new compounds, but they are new for this species, in fact euchrenone a7 (**2**) is the first reported from this genus. Norartocarpanone (**1**) was isolated from *Morus multicaulis* [13], meanwhile euchrenone a7 (**2**) was isolated from *Euchresta horsfieldii* [12]

As a part of our research on phytochemistry compounds of *Morus* and their cytotoxic activity against murine leukemia P-388 cells, therefore, the cytotoxic properties of these compounds were evaluated according to the method described previously [10]. Euchrenone a7 (**2**) was found more cytotoxic rather than

Table 2. NMR data of compound **2** in acetone-d_6

No	δ_H (multiplicity, *J* Hz)	δ_C	HMBC ($^1H \Leftrightarrow ^{13}C$)	NOESY($^1H \Leftrightarrow ^1H$)
2	5.69 (*dd*, 2.9 & 13.2)	75.8	C-1', C-6', C-2'	H-3eq, H-3ax
3	2.96 (*dd*, 13.2 & 16.8) H-ax 2.71 (*dd*, 2.9 & 16.8) H-eq	43.7	C-2, C1', C-4 C-4a, C-4	H-3eq, H-2 H-3ax, H-2
4	-	191.6	-	-
4a	-	115.3	-	-
5	7.59 (*d*, 8.8)	126.3	C-7/C-8a, C-4	H-6
6	6.61 (*d*, 8.8)	110.3	C-4a, C-8	H-9, H-5
7	-	162.4[a]	-	-
8	-	116.4	-	-
8a	-	162.7	-	-
9	3.34 (*d*, 7.3)	22.7	C-8, C-10, C-11, C-7/C-8a	H-6, H-12, H-13
10	5.25 (*t*, 7.3)	123.1	C-13, C-12	H-9
11	-	131.6	-	-
12	1.64 (*br s*)	18.0	C-12, C-10, C-11	H-9
13	1.60 (*br s*)	26.0	C-13, C-10, C-11	H-9
1'	-	118.2	-	-
2'	-	156.7	-	-
3'	6.47 (*d*, 2.2)	103.4	C-5', C-1', C-2', C-4'	-
4'	-	159.3	-	-
5'	6.43 (*dd*, 2.2 & 8.0)	107.7	C-3', C-1'	H-6'
6'	7.37 (*d*, 8.0)	128.6	C-2, C-2', C-4'	H-5'
7/2'/4' -OH	8.41/8.66/9.32 (*brs*)	-	-	-

a: can be changed

norartocarpanone (**1**) with their IC_{50} 7.8 and 12.7 μg/mL respectively, suggested the presence of isoprenyl group on flavanone increases the cytotoxicity. The same tendency was also demonstrated on screening promotor antitumor of some flavanone [14].

CONCLUSION

Two flavanones derivatives, norartocarpanone (**1**) and euchrenone a7 (**2**) have been isolated from the methanol extract of wood of *M. Nigra* for the first time. These compounds showed cytotoxic activity with their IC_{50} were 12.7 and 7.8 μg/mL respectively.

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