3-Prenyl luteolin, a new prenylated flavone with melanin biosynthesis inhibitory activity from wood of Artocarpus heterophyllus

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3-Prenyl luteolin, a new prenylated flavone with melanin biosynthesis inhibitory activity from wood of *Artocarpus heterophyllus*

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Abstract

In our efforts to find new whitening agent from natural resources, we focused on wood of *Artocarpus heterophyllus* which shows anti-melanogenesis activity. By activity-guided fractionation of *A. heterophyllus* wood extract, a new prenylated flavonoid, 3-prenyl luteolin (**1**) was isolated. The IC_{50} of mushroom tyrosinase inhibitotry activity of **1** was 76.3 μM. The results of the comparison with that of luteolin showed the prenyl substituent at C-3 position of **1** play an important role for revealing tyrosinase inhibition. In melanin formation inhibition on B16 melanoma cells, IC_{50} of **1** was 56.7 μM with less cytotoxicity.

Keywords : *Artocarpus heterophyllu*s, tyrosinase, melanin, 3-prenyl-3', 4', 5, 7-tetrahydroxyflavone, 3-prenyl luteolin, B16 melanoma cells

1. Introduction

Jackfruit (*Artocarpus heterophyllus*) is an exotic fruit grown in tropical climates. The various parts of the jackfruit tree have also been reported as an ingredient in the preparations of different *Ayurvedic* and *Yunani* medicines [1]. This plant has properties that remedy disease by supplying cooling, tonic, and pectoral relief (the pulp and seeds), by alleviating diarrhea and fever (the roots), by acting as a sedative in convulsion (the woods), by activating milk in women and animals and acting as an antisyphilitic and vermifuge in humans (the leaves), and by relieving ulcers and wounds (the leaf ash) [2]. The root extract is used in treating skin diseases, asthma, diarrhea, and fever. The leaves and latex treat asthma, ringworm, cracking of the feet; an infusion of mature leaves and bark is used to treat diabetes and gall stones; a tea made with dried and powdered leaves is taken to relieve asthma; heated leaves can treat wounds, abscesses, ear problems and relieve pain. The bark can be used for treatment of dysentery and release of the placenta after calving in cows. The extract from seeds or bark helps digestion while ripe fruits can be used as a laxative [3]. We have already reported that the prenylated flavonoid compounds such as artocarpin, cudraflavone C, 6-prenylapigenin, kuwanon C, norartocarpin, brosimone I and albanin A from wood *A. heterophyllus* suppressed melanin production in B16 melanoma cells with little or no cytotoxicity [4]. Recently, some of prenylated flavonoids such as artocarpesin, artocarpin, cycloartocarpin, cycloartocarpesin, brosimone I, cudraflavone B, carpachromene,

isoartocarpesin were isolated from the wood of *A. heterophyllus* and *Artocarpus incisus* as the anti-browning, anti-tyrosinase, and anti-melanogenesis agents [5, 6, 7, 8, 9].

In our efforts to find new ingredients for whitening agent, we continued to search for the compound with melanin biosynthesis inhibitory activity from the wood of *A. heterophyllus*. By activity-guided fractionation of the extract of the wood of *A. heterophyllus*, a new compound, 3-prenyl-3', 4', 5, 7-tetrahydroxyflavone named as 3-prenyl luteolin (**1**) was isolated. In the present study, we evaluated the isolated compound (**1**) and luteolin (**2**) on mushroom tyrosinase and on melanin biosynthesis in B16 melanoma cells in order to gain the knowledge for structural feature of revealing the inhibitory activity of melanin formation and develop potential depigmenting agents for skin-whitening cosmetics.

2. Materials and methods

2.1. Generals

The compound **1** was analyzed by Unity-500 MHz NMR spectrometer (500 MHz, Varian Inc., Palo alto, CA). Compounds were dissolved in methanol-d_4, DMSO-d_6, and methanol-d_4, and then assigned for ^{1}H, ^{13}C, DEPT, HMQC, HMBC, H-H COSY and NOESY. ESI-MS was determined with TOFCS-JMS-T100 CS (JEOL Ltd., Tokyo, Japan). The silica gel column chromatography was conducted using Wakogel C-200. The preparative HPLC column, Inertsil Prep-ODS (20 mm i.d. x 250 mm, GL-Science) has been used. UV spectra were recorded on JASCO-V-530 spectrophotometer. Mushroom tyrosinase (2870 units/mg) was

purchased from Sigma Chemical Co. (St. Louis, MO). A 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and EMEM were obtained from Sigma (St. Louis, MO) and Nissui Chemical Co (Osaka, Japan), respectively. Luteolin was purchased from Extrasynthese (Genay, France). A mouse melanoma cell line, B16, was obtained from RIKEN Cell Bank, Japan.

2.2. Plant material

The wood of *A. heterophyllus* was collected at Samarinda city, Indonesia in August 2003. The plant was identified in Laboratory of Dendrology and the voucher specimen (FHT.LA.13.1H) was deposited at Laboratory of Wood Anatomy of Forestry Faculty, Mulawarman University, Indonesia.

2.3. Extraction and isolation

The sapwood part of wood of *A. heterophyllus* (2.3 kg) was extracted with MeOH at room temperature repeatedly. The MeOH extract was concentrated *in vacuo*, gave a residue (60.6 g). A part of the extract (43.1 g) was suspended in MeOH/H_2O (1:2) and partitioned with *n*-hexane, diethyl ether and EtOAc. The diethyl ether soluble (16.3 g), which showed potent inhibitory effect of melanin production in B16 melanoma cells, was applied to silica gel column (1633 g of Wakogel C-200, 11 x 90 cm) and eluted with *n*-hexane/EtOAc (8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, 1:9, 0:10 and MeOH, each 800 ml) to give sixteen fractions (Fr 1 to Fr 16). Fr 8 (2.04 g) was repeatedly chromatographed over silica gel (400 g of Wakogel

C-200, 6 x 50 cm) and eluted with *n*-hexane/EtOAc (6:4, 5:5, 4:6, 3:7, 2:8, 1:9, 0:10, and 9:1, 7:3, 5:5, 3:7, 1:9, 0:10 of EtOAc/MeOH, each 600 ml and MeOH 1200 ml) to give nine fractions (Fr 8-1 to Fr 8-8). Fr 8-3 (0.9 g) was subjected to silica gel column (400 g of Wakogel C-200, 3 x 40 cm) and eluted with *n*-hexane/EtOAc (10:0 - 0:10, and 9:1 - 1:9 of EtOAc/MeOH, and 5:5, 3:7, 1:9, 0:10 of acetone/MeOH each 50 ml) to give 50 fractions (Fr 8-3-1 to Fr 8-3-50). Fraction Fr 8-3-4 was preparative HPLC (Inertsil Prep-ODS:20 mm i.d. x 250 mm) eluting with MeOH/H_2O (0.1% trifluoroacetic acid, TFA), 20:80, 8 ml/min, yielded compound **1 (**5.1 mg).

2.4. Tyrosinase enzyme assay

Although mushroom tyrosinase differs somewhat from other sources, this fungal source was used for the present experiment due to its ready availability. It should be noted that the commercial tyrosinase was reported to contain numerous proteins besides tyrosinase [10], but was used without purification. The tyrosinase activity was determined with method as previously described [11]. Briefly, all the samples were first dissolved in DMSO and used for the actual experiment at 30 times dilution. First, 333 μL of 200 μM L-DOPA solution was mixed with 600 μL of 0.1M phosphate buffer (pH 6.8), and incubated at 25^{o}C. Then, 33 μL of the sample solution and 33 μL of the aqueous solution of mushroom tyrosinase (1380 units/mL) was added to the mixture and immediately measured the initial rate of linear increase in optical density at 475 nm, on the basis of the formation of DOPAchrome in the

case of using L-DOPA as a substrate. The reaction was started by addition of the enzyme. Although tyrosinase catalyzes a reaction between two substrates, a phenolic compound and oxygen, the assay was carried out in air-saturated solution. Controls, without inhibitor were routinely carried out. Each experiment was carried out in triplicate. Kojic acid was used as a positive control.

2.5. Determination of melanin content and cell viability on B16 melanoma cells

A mouse melanoma cell line, B16, was obtained from RIKEN Cell Bank. The cells were maintained in EMEM supplemented with 10% (v/v) fetal bovine serum (FBS) and 0.09 mg/ml theophylline. The cells were incubated at 37^{o}C in a humidified atmosphere of 5% CO_2. These assays were determined as described [12].

Compound **1** (Figure 1), 3-prenyl-3', 4', 5, 7-tetrahydroxyflavone (3-prenyl luteolin) Yellow powder; UV (MeOH) λ_{max} (log ε) 260 (4.62), 320(4.31), 401 (3.83). ^{1}H-NMR and ^{13}C NMR data, see Table 1; ESI-MS *m/z* 355.12 $[M+H]^{+}$.

3. Results and Discussion

In our previous screening it has been found that the methanol extract of the wood part of *A. heterophyllus* showed the inhibitory activity of melanin formation in B16 melanoma cells[12]. Some compounds such as artocarpin, cudraflavone C, 6-prenylapigenin, kuwanon C, norartocarpin, and albanin A have been isolated as melanin biosynthesis inhibitor. In our continuous searching for inhibitors of melanin biosynthesis from *A. heterophyllus*, we became

aware that some inhibitors in *A. htererophyllus* remained to be not isolated. Therefore, reinvestigation of the methanol extract of *A. heterophyllus* has been done. The repeated silica gel column chromatography and preparative HPLC against the diethyl ether soluble of the methanol extract of *A. heterophyllus* led to isolate one melanin biosynthesis inhibitor, such as a new compound, 3-prenyl luteolin (**1**).

The structure of 3-prenyl luteolin was determined as follows.

Compound **1**: obtained as a yellow powder, and based on the ^{13}C NMR spectrum (DEPT: 11 x C, 6 x CH, 1 x CH_2 and 2 x CH_3) of **1** revealed 20 carbon atoms. The ESI-MS spectral data ($[M+H]^+$ = *m*/*z* 355.12) together with the ^{13}C NMR data suggested the molecular formula $C_{20}H_{18}O_6$. In the ^{1}H-NMR spectrum (Table 1), Two aromatic protons on the A ring, a characteristic set of signals of meta correlation at δ 6.17 (1H, d, *J* = 2.1 Hz, H-6) and δ 6.25 (1H, d, *J* = 2.1 Hz, H-8). Three aromatic protons on the B ring revealed a clear ABX system [δ 6.37 (1H, d, *J* = 2.3 Hz, H-2') and δ 6.39 (1H, dd, *J* = 2.3 and 8.2 Hz, H-6'] showed meta correlation its proton, and ortho correlation with proton in signal δ 7.05 (1H, d, *J* = 8.2 Hz, H-5'). In ^{13}C-NMR spectrum (Table 1), the presence of signals at δ 17.6, 25.8, 132.6, 122.8, 24.8 indicated as a characteristic of isoprenyl moiety. ^{1}H-^{1}H COSY relationships permitted the assignment of isoprenoid proton chemical shifts and also supported the substitution pattern. In HMBC spectrum (Table 1), correlations of H-1" (δ 3.07) to C-4 (δ 183.6), C-3 (δ 163.2) and C-2 (δ 121.9) indicated that the isoprenyl moiety is attached in C-3. This is also

proved by NOESY spectrum, which NOE interaction showed between the 6' (δ 6.39) and 5' (δ 7.05) protons and the proton at δ 3.07. Full proof of the skeleton of **1** was obtained by a combination of HMBC, DEPT, 'H-'H COSY, and NOESY experiments. Therefore, **1** was elucidated as 3-prenyl-3', 4', 5, 7-tetrahydroxyflavone (Figure 1). To our knowledge, the 3-prenyl-3', 4', 5, 7-tetrahydroxyflavone or 3-prenyl luteolin is a new naturally occurring compound and there is no report on the occurrence of this compound in this plant or other plants. Also, prenylated luteolins seem to be rare compound so far. Diaz et al., [13] isolated 6-prenylated luteolin from callus of *Hypericum perforatum.* Botta et al., reported that a reaction catalyzed by a dimethylallyl transferase or prenyl transferase is important for biosynthesis of prenyl flavonoids. The introduction of the γ ,γ -dimethylallyl (isoprenyl) chain at C-6 and C-8 positions in flavonoid by prenyl transferase have been investigated in biogenetic studies, but not at C-3 position [14]. Therefore, complete biosynthesis mechanism of prenylation at C-3 position in luteolin remains unclear.

In Table 2, it showed tyrosinase inhibitory activity of 3-prenyl luteolin (**1**) and kojic acid. In this study, we used kojic acid as positive controls for tyrosinase inhibition [15, 16, 17]. Arbutin is not suitable as positive control because arbutin is reported to be oxidized by tyrosinase [18]. The 3-prenyl luteolin (**1**) showed tyrosinase inhibitory activity with IC_{50} of 76.3 μM (Table 2). To obtain the structural feature for revealing tyrosinase inhibition of **1**, the effect of luteolin (**2**) which is a structurally related compound of **1** on tyrosinase was

examined. Interestingly, luteolin (**2**) acted as a substrate of tyrosinase rather than did as an inhibitor because luteolin (**2**) was easily oxidized by tyrosinase due to the presence of catechol moiety at B ring (data not shown). This behavior is similar with that of quercetin [19]. On the other hand, no change of UV-Vis spectrum of 3-prenyl luteolin (**1**) in the presence of tyrosinase was observed regardless of having catehol moiety at B ring as shown in Fig. 2. It means that 3-prenyl luteolin was not oxidized by tyrosinase. We speculated that the steric hindrance of prenyl substituent at C-3 position prevent the binding of catechol moiety at B ring of **1** to the active site of tyrosinase appropriately, but unclarified part of **1** still bind to tyrosinase for revealing inhibitory activity. However, the reason of no oxidation of 3-prenyl luteolin with tyrosinase and its tyrosinase inhibitory activity remains unclear. These behaviors led us to conclude that the prenyl substituent at C-3 position of lutelin moiety is important for revealing the tyrosinase inhibition. Furthermore, in our previous paper, albanin A (**3**) showed much less tyrosinase inhibitory activity with IC_{50} of 463 μM [12] than that of 3-prenyl luteolin (**1**). In the case of 3-prenyl flavones such as 3-prenyl luteolin (**1**) and albanin A (**3**), the catechol moiety at B ring is preferred to the resorcinol (2', 4'-dihydroxyphenyl) moiety for revealing tyrosinase inhibition.

Next, we investigated the melanin biosynthesis inhibitory of these compounds (**1** and **2**) by using B16 melanoma cells. Unexpectedly, 3-prenyl luteolin (**1**) showed lower inhibition of melanin formation on B16 melanoma cells than luteolin (**2**) regardless of showing more

potent tyrosinase inhibitory activity of **1** than that of **2**. It seems that 3-prenyl luteolin (**1**) which has an isoprenoid moiety, tend to reduce the ability of luteolin (**3**) to inhibit melanin formation on B16 melanoma cells. This result may not support with our previous finding that isoprenyl-derived substituents (prenyl) may be important structural moieties as far as the melanin biosynthesis inhibitory activity of polyphenols are concerned [4]. Several reasons of explaining these behaviors were raised as follows. Luteolin (**3**) has been reported as melanin biosynthesis inhibitor on B61 melanoma cells by reducing the signal of cAMP (cyclic adenosine monophosphate) which decreased the melanin content [20, 21]. It should be noted that luteolin (**4**) has been reported to have several biological activities such as antioxidant, anti-inflammatory, and as hypoglycemic agent to reduce blood glucose levels [22, 23, 24]. These pharmaceutical properties of luteolin moiety might be related to melanin biosynthesis inhibitory activity. Therefore, the inhibitory activity of luteolin (**2**) on melanin formation in B16 melanoma cells became higher than that of 3-prenyl luteolin (**1**).

The inhibition mechanism of melanin formation on B16 melanoma cells by 3-prenyl luteolin (**1**) is remaining unclear. Therefore, further experiments are needed in order to determine the exact mechanism of melanin inhibition of 3-prenyl luteolin (**1**).

In conclusion, 3-prenyl luteolin (**1**) is a promising compound that could be useful for treating hyperpigmentation, as a skin-whitening agent. However, it should be noted that safety is a primary consideration for its practical use in human.

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Tables Legend

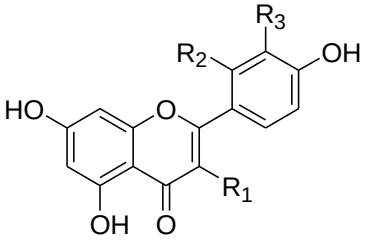
Table 1. NMR spectroscopic data for 3-prenyl luteolin (**1**)

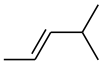
Table 2. Effect of isolated compounds on mushroom tyrosinase activity, melanin formation and cell viability in B16 melanoma cells.

Figures Legend

Figure 1. Chemical structures of 3-prenyl luteolin (**1**), luteolin (**2**) and albanin A (**3**)

Figure 2. UV-Vis spectra obtained in the reaction medium with of 3-prenylated luteolin (70.4 μM) in the presence of mushroom tyrosinase. Scans were at 0 (a) and 10 (b) min, respectively.



1: R_1 =  , R_2 = H, R_3 = OH

2: R_1 = H, R_2 = H, R_3 = OH

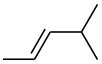
3: R_1 =  , R_2 = OH, R_3 = H

Figure 1.

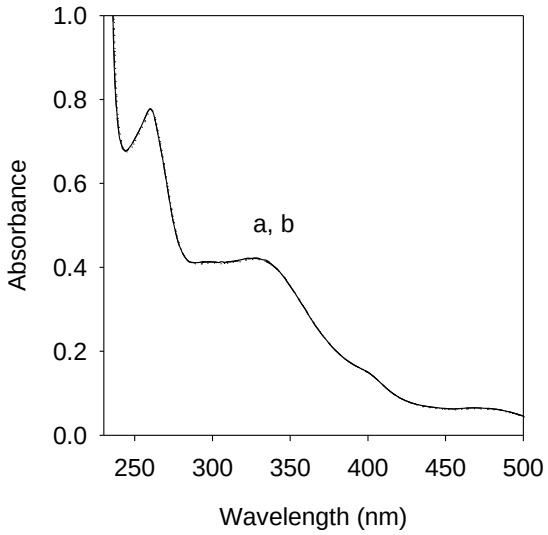

1.0
0.8
0.6
0.4
0.2
0.0
Absorbance
a, b
250
300
350
400
450
500
Wavelength (nm)

Figure 2.

Table 1.

Position	δ_C	$\delta_{H\ (J,\ Hz)}$	HMBC
2	163.2		
3	121.9		
4	183.6		
5	163.5		
6	99.5	6.25, d (2.1)	C10
7	165.5		
8	94.5	6.17, d (2.1)	C10
9	159.8		
10	105.4		
1’	113.4		
2’	107.9	6.37, d (2.3)	C6’ , 1’
3’	157.8		
4’	161.8		
5’	132.3	7.05, d (8.2)	C3’ ,4’, 6’
6’	103.8	6.39, dd (8.2, 2.3)	C2’ ,1’
1”	24.8	3.07, d (7.1)	C2 ,3,4,2”,3”
2”	122.8	5.07, m (7.1)	C3” ,5”
3”	132.6		
4”	25.8	1.57, s	C2” ,3”,5”
5”	17.6	1.38, s	C2” ,3”,4”

Note : The sample dissolved in methanol-d_4

Table 2.

Compound	tyrosinase [a]	melanin inhibition	cell viability
	IC_{50}(μM)	IC_{50} (μM)	(% vs control) [b]
3-prenyl luteolin (**1)**	76.3	56.7	95
luteolin (**2**)	- [c]	10.1	96
arbutin (positive control)	- [c]	198.3	95
kojic acid (positive control)	14.1	>3,521 [d]	90 at 3,521 μM

[a]: substrate: L-DOPA

[b]: cell viability (%) at the concentration of IC_{50} for melanin formation on B16 melanoma cells

[c]: not determined

[d]: 40% inhibition of melanin production at 3,521 μM