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Isolation, Identification and Tyrosinase Inhibitory Activities of the Extractives from *Allamanda cathartica*

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ABSTRACT

Tyrosinase inhibitory activity of the extractives from A. cathartica was examined and their new bioactivity and potent active compounds were identified. Five compounds, glabridin, new lignan, kaempferol, naringenin, and allamandicin, were isolated by a series of chromatography, and identified by NMR and LC-MS. Among them, glabridin had the highest tyrosinase inhibitory activity (IC_{50}:2.93 *μM*) *which is* 15 *times stronger than that of kojic acid used as positive control* (IC_{50}:43.7 *μM*). *Moreover the lignan was indentified as* 1-[3-(4-*allyl*-2,6-*dimethoxyphenoxy*)-4-*methoxyphenyl*] *propane*-1,2,*diol which was a novel lignan*.

***Keywords*:** *Allamanda c athartica*, *Tyrosinase*, *Glabridin*, *Kaempferol*, *Naringenin*, *Allamandicin*

1. Introduction

Allamanda cathartica is a plant cultivated in tropical area. It is used as decoction in various areas and is used in numerous ways. For instance, the extract is used as cathartic in South America and the stem extract is used as antihypertensive in Bangladesh [1-3]. However, the studies of components which have potent bioactivity in this plant are very few, and the mechanisms of the bioactivity have not been done sufficiently. Therefore, the aim of this study is to search active compounds from extract of this plant, and reveal the bioactive mechanism. We have been interesting to find the bioactive compounds from tropical plant extracts, and have identified some kinds of natural products relating to health and beauty so far. The present study examined tyrosinase inhibitory activity of the extractives from *A. cathartica*.

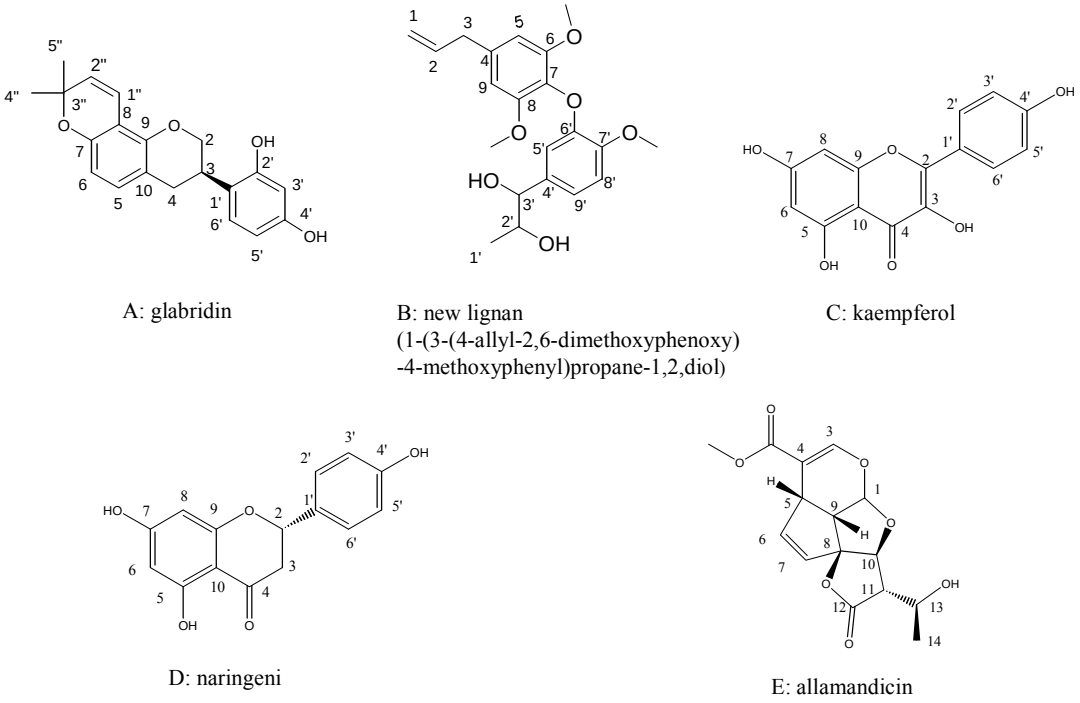
Melanin is a pigment which is biosynthesized from tyrosine by enzymatic oxidation of tyrosinase. Melanin is widely distributed in body surface, retina, nigra of brain, adrenal medullae, and so on. Moreover, it is thought to play an important role in skin cancer prevention by protection of cells from ultraviolent rays. While, it is said that melanin is a reason of sunburn and mottle. Therefore, compounds inhibiting melanin are expected to application of cosmetic as whitening agent. Melanin is biosynthesized in cells called melanocyte, and the starting material of biosynthesis is L-tyrosin. The key enzyme of melanin synthesis is tyrosinase which contains copper, and catalyzes two reactions in the melanin biosynthesis. In the cell, first key step of melanin biosynthesis is the oxidation of L-tyrosine to L-DOPA and second step is L-DOPA to L-DOPA quinine, which are catalyzed by tyrosinase. As the result, pheomelanin and eumelanin are produced. Pheomelanin is red-orange color and eumelanin is blackish brown.

Then, tyrosinase inhibitior makes melanin production diminishing, because the activity of this enzyme is rate-controlling step of melanin synthesis. We found that *A. cathartica* stem extract has tyrosinase inhibitory activity, and tried to search the center active compound. Some kind of tyrosinase inhibitors have been already found from plant extract [4-9]. We isolated 5 compounds (**Figure 1**) from extract of *A.cathartica*, and glabridin had the highest tyrosinase inhibitory activity among the compounds.

2. Materials and Methods

2.1. Materials

The sample was identified by Herbarium Bogoriense, Cibinong, Indonesia and deposited in Biopharmaca Research Center, Bogor Agricultural University no.



A: glabridin

B: new lignan
(1-(3-(4-allyl-2,6-dimethoxyphenoxy)
-4-methoxyphenyl)propane-1,2,diol)

C: kaempferol

D: naringeni

E: allamandicin

Figure 1. Structure of compound A-E.

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2.2. Extraction and Fractionation of *A. cathartica* Stem Powder

A. cathartica stem powder (385.4 g) was extracted with methanol. The methanol extract was fractionated with ethyl acetate. The ethyl acetate soluble fraction was separated with silica gel column chromatography (69 mm φ × 510 mm L). Eluted with *n*-hexane, EtOAc, MeOH to obtain Fr.1-Fr.8. The Fr.3 was separated with preparative HPLC[ODS-3 (20 mm φ × 250 mm L) (MeOH/0.05% TFA aq.soln. = 10/90 (0 min), 100/0 (60 min), 100/0 (80 min)] to obtain Fr.3-1-Fr.3-4. Finally, compound A, B, C, D, and E were isolated from Fr.3-3, Fr.3-1, Fr.3-4, Fr.3-4, and Fr.3-4 respectively by preparative HPLC [ODS-3 (10 mm φ × 250 mm L) (MeOH/0.05% TFA aq.soln. = 10/90 (0 min), 100/0 (60 min), 100/0 (80 min)] (**Figure 2**).

2.3. Tyrosinase Activity Assay

The tyrosinase activity method performed based on Batubara *et al.* (2010) [10]. Briefly, sample 70 µl was put in 96-well plate. Tyrosinase 30 µl (333 unit/ml in phosphate buffer 50 mM pH 6.5) and 110 µl of substrates (L-tyrosine 2mM or L-DOPA 12mM) were added. After incubation at 37˚C for 30 min, the absorbance at 510 nm was determined using a micro plate reader. Moreover IC_{50} value (concentration of inhibitor showing 50% inhibition) was calculated.

2.4. Identification of Compounds

Compound A-E were identified by ^{1}H-NMR, ^{13}C-NMR, ^{1}H-^{1}H-COSY, HMQC, HMBC, and LC-MS. Aceton-d6 was used as the solvent for all compounds. These NMR measurements were performed by using JEOL EC600-NMR.LC-MS measurements (Waters Waters®Xevo™ QTof MS) was performed using column C_{18} (2.1 × 100 mm) with MeOH/water = 60/40 (0 min), 100/0 (10 min), 100/0 (13 min) as eluent.

The NMR data of compounds isolated from *A. cathartica* stem is shown in **Table 1**. LC-MS: ES^{-}data of Compoun A, B, C, D, and E were m/z: 323 (M-1), 373, 285, 271, 307 respectively.

3. Results and Discussion

3.1. Compounds Identification

Allamanda cathartica contains hydrocarbons(long chain esters), e.g. 1-triacontanol, 1-dotriacontanol, docosanoic-, tetracosanoic- and hexacosanoic acid in the root; β-sitosterol and triterpenes e.g. ursolic acid and β-amyrin in the leaves or stem, and lupeol in the roots [1-3]. Other components isolated from the roots include series of iridoid lactones: allamadin, allamandicin, plumericin, isoplumericin, plumeieride and fluvoplumierin [11,12].

Compound A concluded as glabridin, while compound C, D and E was kaempferol, naringenin, and allamandicin respectively. Interestingly, Kaempferol have

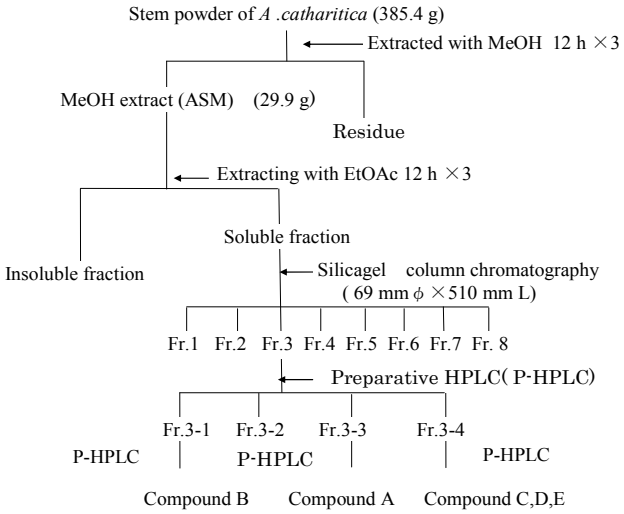

Figure 2. Isolation scheme of the compounds from *A. catharitica* stem powder.

been found in petals of this plant, and allamandicin have been found in roots[4]. However, it was revealed that the two compounds are also contained in stem. Moreover, glabridin and naringenin are found the first time in this plant.

NMR spectrum of glabridin was also searched, and tried to compare to data of compound A and glabridin. The NMR spectrum data from glabridin was similar to that of compound A. Equally, compound C, D, E were identified as keampferol, naringenin, allmandicin respectively [13-15].

Compound B was found to be a novel compound. According to NMR data for compound B, 5.11 and 5.07ppm protons were geminal and alkene protons because of chemical shift and HMQC data. The two protons of 6.55 ppm peeks were equivalen in aromatic ring protons, because it appeared as singlet proton. The three protons of 6.72, 6.94, 6.68 ppm were also the aromatic protons indicating ortho-metha, ortho, and metha coupling. According to the HMBC spectrum of compound B (**Figure 3**), long-range correlations were observed between H-1 and C-2, H-2 and C-1, C-3, C-4, H-3 and C-1, C-2, C-4, H-5 and C-3, C-4, C-6, 6-OMe and C-6, 8-OMe and C-8, H-9 and C-8, C-4, C-3, H_3-1' and C-2', C-3', H-2' and C-3', H-3' and C-4', C-9', H-5' and C-3', C-4', C-6', C-7, 7'-OMe and C-7', H-8' and C-7', C-9' and between H-9' and C-8', C-3', C-4'. These NMR and MS data showed us the compound B is lignan 1-[3-(4-allyl-2, 6-dimetoxyphenoxy)-4-methoxyphenyl]propane-1,2,diol shown **Figure 1**.

3.2. Tyrosinase Inhibitory Activity

Allamanda crude extract and the fractions were analyzed for their activity against tyrosinase. The IC_{50} values of MeOH extract and fractions are shown in **Table 2**. Kojic acid is used as a positive control, because kojic acid is included in whitening agent of cosmetic products. Table 1 shows that IC_{50} value of MeOH extract is 98.4 μg/ml, and that of Fr.3 is 8.35 μg/ml. Thus, tyrosinase inhibition is becoming strong with following fractionation. Among to Fr.3-1 till Fr.3-4, Fr.3-3 had the strongest activity (IC_{50} 0.589 μg/ml).

From Fr.3-3, glabridin was isolated. Moreover, from Fr.3-1, lignan was isolated and from Fr.3-4, naringenin, kaempferol, and allamandicin were isolated. Their tyrosinase inhibitory activity are shown in **Table 3**. According to **Table 3**, only glabridin has potent activity. The tyrosinase inhibitory activity of glabridin is shown in **Figure 4** for L-tyrosine as substrate and **Figure 5** for L-DOPA as substrate. According to **Figure 4**, glabridin had about 93% of tyrosinase inhibition at concentration 19.3 μM for reaction with L-tyrosine as substrate. While, Kojic acid had only about 28% inhibition at 54.9 μM concentration. IC_{50} value of glabridin was 2.93 μM, and this value was lower than that of Kojic acid value (43.7 μM).

Moreover, in **Figure 5** (tyrosinase inhibitory activity using DOPA as the substrate), IC_{50} value of glabridin was 25.5 μM, and this value was lower than that of Kojic

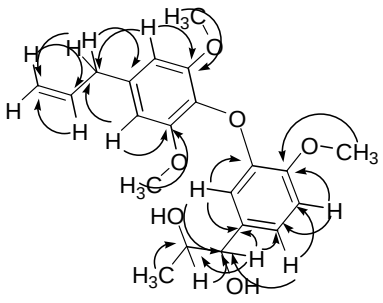

Figure 3. Key HMBC correlations of compound B.

Table 1. ^{1}H-NMR and ^{13}C-NMR data of compound A-E.

Position	Compound A δ_H	J(Hz)	δ_C	Position	compound B δ_H	J(Hz)	δ_C
2	4.31 m		70.1	1	5.11 dd	17.2, 2.10	115.1
	3.97 t	10.3			5.01 dd	10.3, 2.04	
3	3.47 m		31.7	2	5.97 m		137.7
4	2.95 dd	15.8,10.9	30.4	3	3.34 d	6.90	40.2
	2.77 ddd	15.7,5.7,1.8		4			137.7
5	6.81 d	8.05	129.3	5	6.55 s		105.8
6	6.25 d	8.55	108.4	6			153.7
7			152.0	7			136.0
8			109.6	8			153.7
9			149.8	9	6.55 s		105.8
10			114.8	OMe	3.83 s (6H)		55.7
1’			118.4	1’	1.00 d	6.18	12.6
2’			156.1	2’	4.29 m		82.1
3’	6.45 d	2.3	102.8	3’	4.70 s		73.0
4’			157.4	4’			145.4
5’	6.31 dd	8.3,1.75	106.7	5’	6.72 d	8.22	114.5
6’	6.93 d	15.5	128.7	6’			133.4
1”	6.68 d	10.2	116.9	7’			147.3
2”	5.57 d	10.3	127.8	8’	6.94 d	1.38	109.7
3”			75.1	9’	6.68 dd	8.22, 1.38	118.6
4”	1.33 s		27.0	OMe	3.78 s (3H)		55.4
5”	1.35 s		27.1				

Position	Compound C δ_H	J(Hz)	δ_C	Position	Compound D δ_H	J(Hz)	δ_C
2			146.2	2	5.42 dd	2.76,13.1	79.1
3			135.8	3	2.70 dd	2.5,17.1	42.7
					3.20 m		
4			175.8	4			196.4
5			161.5	5			164.2
6	6.23 s		98.4	6	5.93 d	2.04	96.0
7			164.2	7			166.6
8	6.50 s		93.7	8	5.94 s		95.0
9			157.0	9			163.6
10			103.3	10			102.3
1’			122.5	1’			129.9
2’	8.11 d	8.28	123.0	2’	7.36 d	8.28	128.2
3’	6.98 d	8.22	115.5	3’	6.87 d	8.28	115.3
4’			159.4	4’			157.9
5’	6.98 d	8.22	115.5	5’	6.87 d	8.28	115.3
6’	8.11 d	8.28	123.0	6’	7.36 d	8.28	128.2

Position	Compound E δ_H	J(Hz)	δ_C
1	5.67 d	6.18	102.0
3	7.40 s		152.6
4			109.2
5	3.92 m		38.2
6	5.92 dd	2.04,5,46	140.0
7	5.79 dd	2.10,5.52	127.9
8			106.5
9	3.46 dd	6.18,9.60	53.6
10	4.70 s		83.6
11	2.70 d	1.18	55.2
12			175.9
13	4.33 m		66.0
14	1.32 d	6.18	21.5
CO			166.4
OMe	3.69 s(3H)		50.9

Table 2. Tyrosinase inhibitory activity of extract and fractions from *A. cathartica* stem.

	Kojic acid	MeOH extract	Fr.1	Fr.2	Fr.3	Fr.4	Fr.5	Fr.6	Fr.7	Fr.8
IC_{50}(μg/ml)	6.18	98.4	100<	95.7	8.35	89.1	100<	100<	100<	100<
	Fr.3-1	Fr.3-2	Fr.3-3	Fr.3-4						
IC_{50}(μg/ml)	100<	56.6	0.589	100<						

Table 3. Tyrosinase inhibitory activity of compounds isolated from *A. cathartica* stem.

compounds	Kojic acid	Glabridin	Lignan	Kaempferol	Naringenin	Allmandicin
IC_{50}(μM)	43.7	2.93	100<	100<	100<	100<

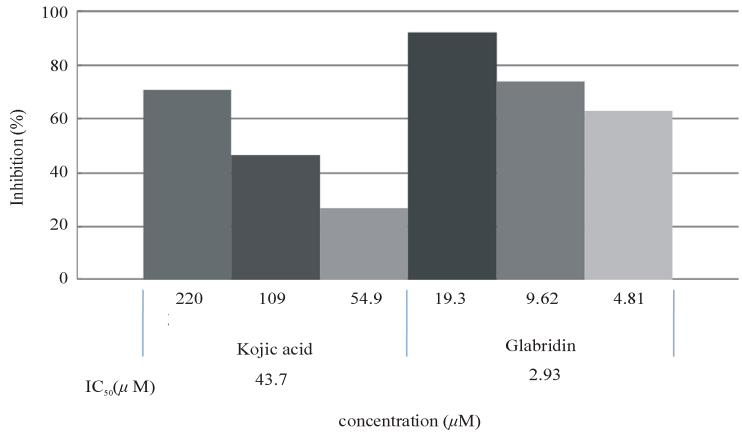

Figure 4. Tyrosinase inhibitory activity of glabridin using tyrosine as the substrate.

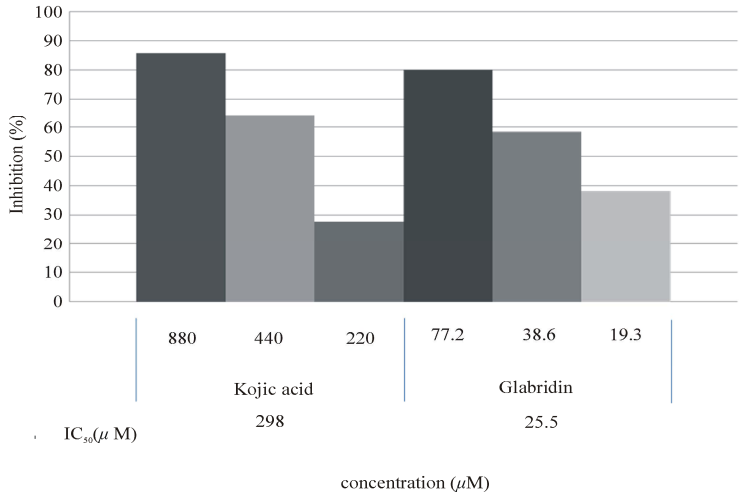

Figure 5. Tyrosinase inhibitory activity of glabridin using DOPA as the substrate.

acid value 298 μM. Tyrosinase inhibitory activity of glabridin was more than 10 times stronger than that of kojic acid. Thus, glabridin is the center active compound, and possibly has a potent activity for cosmetic as whitening agent.

4. Conclusions

In this study, 5 compounds were newly found in *A. cathartica* stem, namely glabridin, new lignin, naringenin, kaempferol and allamandicin. Among all the isolated compounds, glabridin has the most potent tyrosinase inhibitory activity.

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