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Screening of Indonesian plants for tyrosinase inhibitory activity

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Abstract In our efforts to find new tyrosinase inhibitory materials, we investigated 44 Indonesian plants belonging to 24 families for tyrosinase inhibitory activity. The extracts of 5 *Artocarpus* woods showed potent tyrosinase inhibitory activity (over 80% at 100μg/ml) similar to a positive control, kojic acid. In *Artocarpus* woods, the extracts of the sapwoods showed stronger inhibitory activity than those of the heartwoods. Chlorophorin was isolated as one of the active compounds in the sapwood of *Artocarpus heterophyllus*. The content of chlorophorin in sapwood was higher than that in heartwood.

Key words Indonesian plants · Tyrosinase inhibition · Chlorophorin · *Artocarpus* · Jamu

Introduction

Tyrosinase (EC 1.14.18.1; PPO) is known to be a key enzyme for melanin biosynthesis in plants, microorganisms, and in mammalian cells.[1] This enzyme catalyses two different reactions: the hydroxylation of monophenols to *o*-diphenols (monophenolase activity), and the oxidation of *o*-diphenols to *o*-quinones (diphenolase activity), which, in turn, are polymerized to brown, red, or black pigments.[1] Many tyrosinase inhibitors have been tested in cosmetics and pharmaceuticals as a way of preventing overproduction of melanin in epidermal layers.[2] Alterations in melanogenesis may be responsible for some of the clinical and histopathological features unique to malignant melanoma,[3] a cancer with a rapid increase of incidence.[4] Also, tyrosinase is one of the most important enzymes in the insect molting process,[5] and investigation of its inhibitors may be important in finding alternative insect control agents.[6] Melanin formation is considered to be deleterious to the color quality of plant-derived food and the prevention of this browning reaction has always been a challenge to food scientists.[7] This broadens the possible use of tyrosinase inhibitors as food additives, in addition to insect control agents and whitening agents. Furthermore, it has been reported that tyrosinase could be central to dopamine neurotoxicity as well as contributing to the neurodegeneration associated with Parkinson's disease.[8] Also, tyrosinase is the main enzyme involved in the enzymatic browning of mushrooms, which is responsible for sensory quality loss and loss of nutrient quality.

These observations led us to focus on the exploration of tyrosinase inhibitors from natural products. Several chemicals of plant origin have been reported as tyrosinase inhibitors. Arbutin,[9] ellagic acid,[10] oxyresveratrol,[11] chlorophorin, and norartocarpanone[12] were described for their tyrosinase inhibition properties.

Tropical forest constitutes 40%–50% of all forest area of the world. Indonesia posses an extraordinarily rich flora and great diversity of vegetation types that parallel the diverse physiography of the land. A high proportion of the land area has a forest cover, most of it evergreen rain forest. The great diversity of tropical plants is reflected in the qualitative and quantitative diversity of extractives of plants, or, from a chemical point of view, of the components. The use of tropical herbal medicines has increased in recent years. In Indonesia, some plants are used to obtain traditional medicine called "Jamu." Based on the type of use, Jamu can be distinguished into four categories of medicines; health care, beauty care (cosmetics), tonics or beverages, and body endurance or protection.[13]

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Recently, we have reported antifungal,[14] antityrosinase,[12] antipigmentation,[15] and anti-5α-reductase[16] components from tropical plants. Thus, the tropical woods provide great opportunity to find bioactive components.

This article investigates the tyrosinase inhibitory activity of the extracts of 44 Indonesian plants. The extracts of *Artocarpus* woods showed potent tyrosinase inhibitory activity.

Materials and methods

Reagents

L-Tyrosine and L-dihydroxyphenylalanine (DOPA) were purchased from Wako. Mushroom tyrosinase (2870 units/mg) was purchased from Sigma (St. Louis, MO). Dimethylsulfoxide (DMSO) was purchased from Wako.

Plant materials

Some plant materials (samples no. 4–6, 8, 10, 11, 15–17, 19–22, 25–27, 29–31, 33–37, 39, 40, 50–59, 61–63 in Table 1) were obtained from the Laboratory of Dendrology, Faculty of Forestry, Mulawarman University, Indonesia and samples no. 1–3, 7, 9, 12–14, 18, 23, 24, 28, 32, 38, 49, 60, 64 (in Table 1) were provided from the Research Center for Chemistry, Indonesian Institute of Sciences (LIPI) Indonesia. The voucher specimens of samples are preserved at each institution.

Extraction of samples

All samples were chipped and milled. Then, they were extracted with methanol at room temperature for 24 h or soxhlet-extracted with 70% aqueous ethanol for 24 h (see Table 1).

Enzyme assays

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,[17] but was used without purification. The temperature was controlled at 25°C using an Ecoline E100 circulating bath (Lauda, Germany) with a heater and a digital thermometer. The reaction was started by addition of the enzyme. Although tyrosinase catalyzes a reaction between two substrates, i.e., a phenolic compound and oxygen, the assay was carried out in air-saturated solution. Controls, without inhibitor, containing DMSO at inhibitor concentration were routinely carried out. Kojic acid was used as a positive control.[2]

All the samples were first dissolved in DMSO and used for the actual experiment at 30 times dilution. The assay was performed as previously described.[18] First, 333 μl of 2.5 mM L-DOPA or L-tyrosine solution was mixed with 600 μl of 0.1 M phosphate buffer ($Na_2HPO_4 \cdot 12H_2O/NaH_2PO_4 \cdot 2H_2O$) (pH 6.5), and incubated at 25°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 the initial rate of linear increase in optical density at 470 nm, based on the formation of dopachrome, was measured immediately. The extent of inhibition by the addition of samples is expressed as the concentration necessary for 50% inhibition (IC_{50}).

Isolation of active compounds from *Artocarpus heterophyllus*

The wood meal of sapwood of *Artocarpus heterophyllus* (2.3 kg) was repeatedly extracted with methanol at room temperature. The methanol extract was concentrated in vacuo to give a residue (60.6 g). A part of the extract (5 g) was suspended in methanol–water (1:2) and successively partitioned with *n*-hexane, diethyl ether, and ethyl acetate to give *n*-hexane-soluble (0.19 g, 3.8% of the methanol extract), diethyl ether-soluble (2.4 g, 48%), ethyl acetate-soluble (0.43 g, 8.6%), and aqueous portions (1.8 g, 36%). The diethyl ether-soluble portion (2.4 g) was fractionated by column chromatography on silica gel (480 g) by elution with *n*-hexane-ethyl acetate gradient [*n*-hexane : ethyl acetate = 8:2, 7:3, 6:4, 400 ml (fraction 1: 72 mg) → 5:5, 400 ml (fraction 2: 55 mg) → 4:6, 400 ml (fraction 3: 336 mg, fraction 4: 236 mg) → 3:7, 400 ml (fraction 5: 174 mg) → 2:8, 400 ml (fraction 6: 244 mg, fraction 7: 288 mg) → 1:9, 400 ml (fraction 8: 150 mg) → 0:10, 400 ml (fraction 9: 55 mg, fraction 10: 32 mg) → methanol, 400 ml (fraction 11: 40 mg, fraction 12: 425 mg, fraction 13: 6.7 mg, fraction 14: 4.2 mg, fraction 15: 4.3 mg, fraction 16: 6.8 mg]. Sixteen fractions were collected. Fractions 4 and 5 were combined (350 mg) on the basis of thin-layer chromatography (TLC) analysis and tyrosinase inhibitory activity, and the resultant material was then fractionated by column chromatography on silica gel (175.5 g). Thirty-two fractions were collected and fraction 17 (30 mg) were subjected to preparative high performance liquid chromatography (HPLC) (Inertsil Prep-ODS : 20 mm i.d. × 250 mm), eluting with aqueous 0.1% trifluoroacetic acid (TFA)/methanol (20/80) at 12 ml/min to give 17 fractions (factions A–Q). Compound A (1.4 mg) was isolated as the main active compound from fraction F (5.2 mg) by preparative HPLC eluting with aqueous 0.1% TFA /methanol (30/70) at 12 ml/min, and was identified as chlorophorin by HPLC co-chromatography with authentic sample.[12]

Quantitative determination of chlorophorin in methanol extract of *Artocarpus heterophyllus*

The methanol extracts of sapwood and heartwood of *A. heterophyllus* were each dissolved in methanol. An aliquot (200 μl) of each sample solution was injected into the HPLC system under the following conditions: pump, Waters 626; column, packed column (150 × 4.6 mm i.d.) of Inertsil ODS-3 (GL Science, Japan); detector, Waters photodiode array

Table 1. The effects of Indonesian plant extracts on mushroom tyrosinase activity

Number	Scientific name	Family	Plant organ	Extraction solvent	Relative activity (%)[a]
1	*Strobilanthes crispus*	Acanthaceae	Leaves	70% EtOH	130
2	*Averhoa bilimbi*	Apiaceae	Leaves	70% EtOH	75
3	*Averhoa carambola*	Apiaceae	Leaves	70% EtOH	51
4	*Alstonia scholaris*	Apocynaceae	Root	MeOH	102
5	*Alstonia scholaris*	Apocynaceae	Bark	MeOH	111
6	*Dyera costulata*	Apoynaceae	Wood	MeOH	100
7	*Bixa orellana*	Bixaceae	Leaves	70% EtOH	70
8	*Terminalia catappa*	Combretaceae	Wood	MeOH	33
9	*Kalanchoe hirta*	Crassulaceae	Leaves	70% EtOH	98
10	*Shorea balangeran*	Dipterocarpaceae	Heartwood	MeOH	27
11	*Aleurites moluccana*	Euphorbiaceae	Wood	MeOH	142
12	*Euphorbia hirta*	Euphorbiaceae	Leaves	70% EtOH	103
13	*Excoecaria cochinchinensis*	Euphorbiaceae	Leaves	70% EtOH	112
14	*Phyllanthus fraternus*	Euphorbiaceae	Leaves	70% EtOH	90
15	*Castanopsis javanica*	Fagaceae	Heartwood	MeOH	82
16	*Castanopsis javanica*	Fagaceae	Sapwood	MeOH	80
17	*Eusideroxilon zwageri*	Lauraceae	Wood	MeOH	74
18	*Persea americana*	Lauraceae	Leaves	70% EtOH	95
19	*Gliciridia sepium*	Leguminosae	Heartwood	MeOH	84
20	*Gliciridia sepium*	Legminosae	Sapwood	MeOH	98
21	*Leucaena glauca*	Legminosae	Heartwood	MeOH	26
22	*Leucaena glauca*	Legminosae	Sapwood	MeOH	53
23	*Pachyrhizus erosus*	Leguminosae	Bulb	70% EtOH	115
24	*Pachyrhizus angulatus*	Leguminosae	Leaves	70% EtOH	102
25	*Pterocarpus indicus*	Legminosae	Bark	MeOH	98
26	*Pterocarpus indicus*	Legminosae	Root	MeOH	113
27	*Pterocarpus indicus*	Leguminosae	Sapwood	MeOH	114
28	*Hibiscus rosa-sinensis*	Malvaceae	Leaves	70% EtOH	105
29	*Hibiscus thylliace*	Malvaceae	Heartwood	MeOH	121
30	*Hibiscus thylliace*	Malvaceae	Sapwood	MeOH	101
31	*Xylocapus granatum*	Meliaceae	Wood	MeOH	58
32	*Cyclea barbata*	Menispermaceae	Leaves	70% EtOH	105
33	*Acacia mangium*	Mimosaceae	Bark	MeOH	26
34	*Acacia mangium*	Mimosaceae	Wood	MeOH	133
35	*Artocarpus altilis*	Moraceae	Heartwood	MeOH	13
36	*Artocarpus altilis*	Moraceae	Sapwood	MeOH	3
37	*Artocarpus communis*	Moraceae	Branch (Wood)	MeOH	4
38	*Artocarpus communis*	Moraceae	Leaves	70% EtOH	158
39	*Artocarpus elasticus*	Moraceae	Heartwood	MeOH	8
40	*Artocarpus elasticus*	Moraceae	Sapwood	MeOH	3
41	*Artocarpus heterophyllus*	Moraceae	Heartwood	MeOH	50
42	*Artocarpus heterophyllus*	Moraceae	Sapwood	MeOH	4
43	*Artocarpus integra*	Moraceae	Heartwood	MeOH	6
44	*Artocarpus integra*	Moraceae	Sapwood	MeOH	3
45	*Piper aduncum*	Piperaceae	Leaves	MeOH	140
46	*Anthocephalus cadamba*	Rubiaceae	Sapwood	MeOH	88
47	*Anthocephalus cadamba*	Rubiaceae	Heartwood	MeOH	93
48	*Anthocephalus chinensis*	Rubiaceae	Wood	MeOH	109
49	*Morinda citrifolia*	Rubiaceae	Leaves	70% EtOH	100
50	*Santalum album*	Santalaceae	Wood	MeOH	100
51	*Duabanga moluccana*	Sonneratiaceae	Wood	MeOH	142
52	*Sonneratia acida*	Sonneratiaceae	Leaves	MeOH	99
53	*Kleinhovia hospita*	Sterculiaceae	Bark	MeOH	103
54	*Kleinhovia hospita*	Sterculiaceae	Flower	MeOH	105
55	*Kleinhovia hospita*	Sterculiaceae	Fruit	MeOH	96
56	*Kleinhovia hospita*	Sterculiaceae	Leaves	MeOH	103
57	*Kleinhovia hospita*	Sterculiaceae	Root Bark	MeOH	100
58	*Kleinhovia hospita*	Sterculiaceae	Root	MeOH	114
59	*Kleinhovia hospita*	Sterculiaceae	Wood	MeOH	104
60	*Thea sinensis*	Theaceae	Leaves	70% EtOH	58
61	*Aquilaria malaccensis*	Thymelaeaceae	Root	MeOH	100
62	*Gmelina arborea*	Verbenaceae	Heartwood	MeOH	93
63	*Peronema canescen*	Verbenaceae	Wood	MeOH	106
64	*Curcuma domestica*	Zingiberaceae	Leaves	70% EtOH	78
Kojic acid					20
Control (DMSO)					100

Sample concentrations were 100 μg/ml
EtOH, ethanol; MeOH, methanol; DMSO, dimethylsulfoxide
[a] Relative activity (%) = (Activity of sample/activity of control) $\times$ 100

Table 2. Tyrosinase inhibitory activity of the extracts from *Artocarpus* plants

Number[a]	Scientific name	Plant organ	IC_{50} (μg/ml)
35	*Artocarpus altilis*	Heartwood	29.9
36	*Artocarpus altilis*	Sapwood	8.6
37	*Artocarpus communis*	Branch (wood)	7.1
38	*Artocarpus communis*	Leaves	No inhibition at 100μg/ml
39	*Artocarpus elasticus*	Heartwood	12.5
40	*Artocarpus elasticus*	Sapwood	8.0
41	*Artocarpus heterophyllus*	Heartwood	125
42	*Artocarpus heterophyllus*	Sapwood	7.0
43	*Artocarpus integra*	Heartwood	16.8
44	*Artocarpus integra*	Sapwood	6.6
Kojic acid			2.8

IC_{50}, 50% inhibitory concentration
[a] Corresponding to Table 1

detector 996 set at 329 nm; mobile phase, aqueous 0.1% TFA/0.1% TFA methanol (35/65); flow rate, 1 ml/min. The amount of chlorophorin was calculated by the peak area ratio with respect to the standard.

Results and discussion

Table 1 summarizes the results of mushroom tyrosinase inhibition of several plant extracts tested. Values are presented as percentage of tyrosinase activity. This study revealed that 52 of 64 extracts showed poor tyrosinase inhibitory activity (less than 50% inhibition). Twelve of them, which belong to a variety of species (*Terminalia catappa*, *Shorea balengaran*, *Leucaena glauca*, *Acacia mangium*, *Artocarpus altilis*, *Artocarpus communis*, *Artocarpus elasticus*, *Artocarpus heterophyllus*, *Artocarpus integra*), showed tyrosinase inhibitory activity at more than 50%. Out of 64 samples, 8 samples, which were prepared from *Artocarpus altilis* (sapwood and heartwood), *Artocarpus communis* [branch (wood)], *Artocarpus elasticus* (sapwood and heartwood), *Artocarpus heterophyllus* (sapwood), and *Artocarpus integra* (sapwood and heartwood), showed more potent inhibitory activity than kojic acid at the concentration of 100 μg/ml. It should be noted that kojic acid,[2] which is known to be a potent tyrosinase inhibitor, had an IC_{50} of 0.02 mM (2.8 μg/ml) in our assay.

These extracts prepared from *Artocarpus* wood described above could be potential sources of potent tyrosinase inhibitory materials. Therefore, we focused on the extracts of *Artocarpus* wood. It should be noted that some tyrosinase inhibitory compounds isolated from *Artocarpus* wood have been reported by Shimizu et al.[12] and Likhitwitayawuid and Sritularak.[19] In addition, *Artocarpus* plants are known to exhibit other biological properties including cytotoxic,[20–22] antimalarial,[23] anti-inflammatory, antiplatelet, antioxidant, anticomplementary,[24] antibacterial,[23,25] and antinepheritis[26] activities. They are also known inhibitors of cathepsin K,[27] TNF-α formation,[28] 5α-reductase,[16] UV-B induced pigmentation,[15] cyclooxygenase,[28] and lymphocyte proliferation.[29]

Fig. 1. *Artocarpus heterophyllus* (wood)

To our knowledge, comparison of tyrosinase inhibitory activity of the extracts prepared from sapwood and heartwood of *Artocarpus* plants has not been reported so far (Fig. 1). In our investigation, *Artocarpus* leaves had no inhibitory activity on tyrosinase. Interestingly, the extracts of sapwood showed higher inhibitory activity than those of heartwood or leaves, as shown in Table 2 (L-DOPA was used as a substrate). These results indicated that tyrosinase inhibitory compounds might accumulate in sapwood rather than heartwood.

Also, we have focused on the extracts prepared from sapwood and heartwood of *A. heterophyllus*, because they showed distinctly different inhibitory activity, more specifically, the extract of sapwood showed much more potent inhibitory activity than that of heartwood (as shown in Fig. 2). The extract of sapwood of *A. heterophyllus* exhibited a dose-dependent inhibitory effect on the oxidation of L-tyrosine and L-DOPA, as shown in Fig. 3. The experiment using L-tyrosine as a substrate showed a monophenolase activity, which was indicated by a lag phase before the maximum of the hydroxylation step was reached.[1] In contrast, the experiment using L-DOPA as a substrate showed a diphenolase activity, which was indicated by the lack of a lag phase.[30] In addition, the lag phase depends on both enzyme and substrate concentrations.[31,32] Thus, the extract of sapwood of *A. heterophyllus* showed potent inhibitory activity against monophenolase and diphenolase activities.

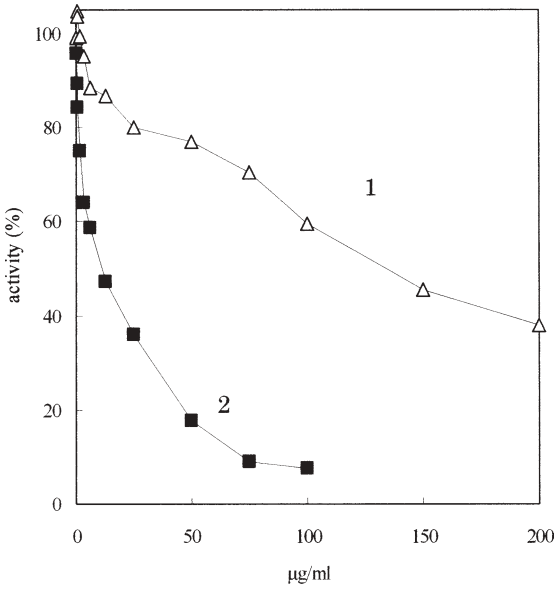

Fig. 2. Effects of the methanol extracts of sapwood and heartwood of *A. heterophyllus* on tyrosinase for the catalysis of L-dihydroxyphenylalanine (DOPA). *1*, Heartwood; *2*, sapwood

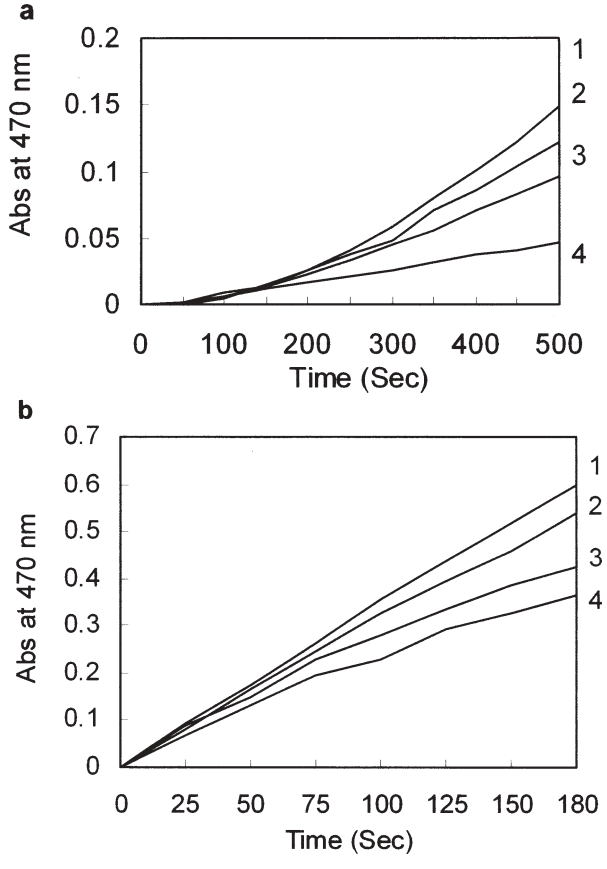

Fig. 3a,b. The dose-dependent inhibitory effects of the extract of sapwood of *A. heterophyllus*. **a** The oxidation of L-tyrosine: *1*, control; *2*, 0.7 μg/ml; *3*, 3 μg/ml; *4*, 6 μg/ml. **b** The oxidation of L-DOPA: *1*, control; *2*, 0.7 μg/ml; *3*, 3 μg/ml; *4*, 6 μg/ml

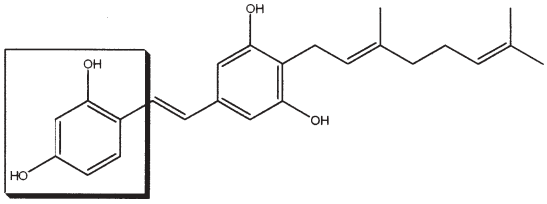

Fig. 4. Structure of chlorophorin (*boxed part*, 4-substituted resorcinol moiety)

It should be noted that the extract of sapwood of *A. heterophyllus* showed stronger inhibition on monophenolase than diphenolase activity as shown in Fig. 3 (the IC_{50} is 3.7 μg/ml for L-tyrosine and 7.0 μg/ml for L-DOPA). These results suggest that the tyrosinase inhibitory components may accumulate in sapwood rather than in heartwood. Therefore, the sapwood of *A. heterophyllus* was selected for further investigation.

The methanol extract of the sapwood of *A. heterophyllus* was partitioned with *n*-hexane, diethyl ether, and ethyl acetate. The tyrosinase inhibitory activities of the *n*-hexane-soluble, diethyl ether-soluble, ethyl acetate-soluble, and aqueous portions were 52, 94, 72, and 49 at the concentration of 100 μg/ml, respectively. The diethyl ether-soluble portion, which showed the strongest tyrosinase inhibitory activity, was subjected to further separation by repeated column chromatography on silica gel. Further separation to give the active fraction (fraction F) was achieved by preparative HPLC gave an active compound, chlorophorin (Fig. 4), which showed 97% inhibition at 50 μg/ml (130 μM) on the oxidation of L-DOPA by tyrosinase. It should be noted that there were several active compounds in the methanol extract of the sapwood of *A. heterophyllus*. The contents of chlorophorin in the methanol extracts of sapwood and heartwood are 13.0 and 7.60 mg/g, respectively, according to HPLC analysis. This difference in the content of chlorophorin in the methanol extracts of the sapwood and heartwood may be, at least in part, related to their different tyrosinase inhibitory activities. However, to clarify this difference completely, further experiments are needed because there are several compounds in *Artocarpus* woods other than chlorophorin that affect tyrosinase activity.

The plants of *Artocarpus* (Moraceae) comprise approximately 50 species and are widely distributed in tropical and subtropical regions, and have been used as traditional folk medicine called "Jamu" in Indonesia against inflammation, malarial fever, and so on.[33] These plants are known to produce a variety of isoprenoid-substituted polyphenols containing the unique feature of the 4-substituted resorcinol skeleton[24,34] (Fig. 4). Also, our previous report on structure–activity relationships showed that the 4-subtituted resorcinol moiety is important for revealing tyrosinase inhibitory activity.[35] Therefore, the extract of *Artocarpus* plants should contain several tyrosinase inhibitory compounds with the 4-substituted resorcinol moiety. Also, these active compounds with the 4-substituted resorcinol moiety might be accumulated in sapwood rather than in heartwood, such as

in the case of chlorophorin described above. These assumptions about the tyrosinase inhibitory activity of *Artocarpus* plants should be justified by further investigation into the isolation and structure elucidation of tyrosinase inhibitor from *Artocarpus* plants. These kinds of experiments are in progress.

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