JURNAL ILMU KEFARMASIAN INDONESIA, April 2011, hlm. 72-77 Vol. 9, No. 2
ISSN 1693-1831

Antioxidant Activities of Compounds from Wood of *Artocarpus heterophyllus*

(Aktivitas Antioksidan Senyawa yang berasal dari Kayu *Artocarpus heterophyllus*)

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Accepted 28 December 2010, Approved 4 April 2011

Abstract: Isoprenoid-substituted flavonoids were isolated from the wood of *Artocarpus heterophyllus* by biologically activity-guided fractionation, namely antioxidant. The isolated compounds are cudraflavone C, kuwanon C, norartocarpin, albanin A and artocarpanone have more hydroxyl groups seem to be specifically to scavenge H_2O_2. These experimental results demonstrated that each scavenging compound different in its scavenge capacity toward different form of radicals.

Keywords: flavonoid, *Artocarpus heterophyllus*, antioxidant.

Abstrak: Beberapa senyawa flavonoid yang mengandung isoprenoid telah diisolasi dari kayu tanaman *Artocarpus heterophyllus* melalui fraksinasi yang dipandu oleh aktifitas antioksidan. Senyawa-senyawa seperti *cudraflavone* C, *kuwanon* C, *norartocarpin*, *albanin* A dan *artocarpanone* memiliki gugus hidroksil yang lebih banyak dan terlihat sangat spesifik dalam menangkap H_2O_2. Hasil-hasil penelitian ini menunjukkan bahwa terdapat perbedaan kapasitas aktifitas antiosidannya terhadap sumber radikal bebasnya.

Kata kunci: flavonoid, *Artocarpus heterophyllus*, antioksidan.

INTRODUCTION

THE skin is the largest and the first line of defense that protects the internal organs from various chemical and physical environmental insults. Ultra violet radiation (UVR) is a major environmental factor that influences the type, survival, and proliferation of many cell types. Exposure of the skin to UVR affects the survival and the proliferation of epidermal and dermal cells and alters various cutaneous functions. Epidermal melanocytes play a key role in determining the response of skin to UVR exposure. Melanocytes represent 8-10% of epidermal cells, yet they are serving a critical function in protecting the skin from UV-induced photodamage. The skin has developed two main defense mechanisms to guard against the damaging effects of UVR: epidermal thickening and hyperkeratosis, and stimulating of melanin biosynthesis by epidermal melanocyte[(1)].

The region representing UVR, the most significant region of sunlight with respect to skin cancer, lies in the range of 200-400 nm, just above visible light of 400-700 nm. UVR can be further subdivided into UVA (320-400 nm), UVB (290-320 nm with a peak at 305 nm) and UVC (200-290 nm with a peak at 254 nm). The UVC contribution to the development of skin cancers is considered negligible, since it is prevented from reaching the surface of the Earth by the atmospheric ozone layer that blocks UVR below about 300 nm. Unlike UVC, UVA and UVB both reach the Earth's surface in sufficient amounts to cause harmful biological effects on the skin. It is estimated that 5% of solar UVR is UVB [(2)].

It could be understand, the melanocyte is under continuous low-grade oxidative insult from the sun. Indeed, melanin synthesis generates hydrogen peroxides that, if inappropriately inhibited or treated, can lead to the generation of hydroxyl radicals and other reactive oxygen species (ROS). Low concentrations of ROS may

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be beneficial or even indispensable in processes such as cell signaling and regulation. In human epidermis, several non-enzymatic and enzymatic antioxidation mechanisms are available as a safeguard against ROS accumulation and these are finely regulated in consequence of physical or chemical oxidative stresses [3].

Free radicals are species with incomplete shells electron that make them more chemically reactive than those with complete shells electrons. They are byproducts of metabolic processes. In cells, in fact, oxidation processes use oxygen to produce energy for biochemical reactions. During these reactions, free radicals are produced and react with biomolecules such as proteins, lipids, and DNA, causing irreversible damages to the cells. Exposure to various environmental factors, including nitrogen dioxide and ozone in polluted air, heavy metals, halogenated hydrocarbons, ionizing radiation, and cigarette smoke, can increase free radical formation[4]. Overtime, such damage can become irreversible, and recent studies have demonstrated the involvement of free radicals in the pathology of human disease such as atherosclerosis, cardiovascular diseases, and diabetes[5-7]. Protective therapeutic intervention might include natural or synthetic pharmacological agents with antioxidant activity. Antioxidants are often described as free radical scavengers, meaning that they will neutralize the electrical charge and prevent free radicals from taking electrons from other molecules. Antioxidants are abundant in fruits and vegetables, as well as in other foods including nuts, grain, and some meats, poultry, and fish[8].

Based on above facts and the results of melanin biosynthesis inhibitors from compounds **1-10** (see Table 1), isolated from sapwood of *Artocarpus heterophyllus* led author to examine their scavenging activities in order to evaluate the ability of those compounds as antioxidant. In this chapter, author reports the scavenging activity that evaluated by DPPH (1,1-diphenyl-2-picrylhydrazyl) and H_2O_2 assays.

MATERIALS AND METHODS

MATERIALS. Reagents. The DMSO and silica gel (Wakogel C-200) were purchased from Wako (Japan). The 1,1-diphenyl-2-picrylhydrazyl (DPPH) was from TCI (Japan), The Horse radish enzyme was Sigma (Germany). Other chemicals were the highest grade and commercially available.

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.

METHODS. Isolation of Compounds from Artocarpus heterophyllus. The milled sapwood of *A. heterophyllus* (sapwood, 2.3 kg) was extracted with methanol and filtrated with appropriate filter paper after 5 days at room temperature. The methanol extracts were concentrated by rotary evaporator under vacuo to dryness, gave a 60.6 g of extract. A portion 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) and eluted with step gradient using n-hexane-EtOAc (8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, 1:9, 0:10 and MeOH,

Table 1. The scavenging activities of isolated compounds from sapwood of *Artocarpus heterophyllus.*

Compounds	Scavenging activities	
	DPPH radical [IC_{50}(μM)]	H_2O_2 [IC_{50}(μM)]
Artocarpin (1)	718	>724
Cudraflavone C (2)	724.2	47.4
6-prenylapigenin (3)	>724	>724
Kuwanon C (4)	>724	55.8
Norartocarpin (5)	>724	40.2
Albanin A (6)	>724	44.8
Artocarpanone (7)	137.3	19.4
Chlorophorin (8)	24.8	>724
Cudraflavone B (9)	>526.4	>724
Brosimone I (10)	176.5	>724
Vitamin E (positive control)	12	>724
Vitamin C (positive control)	9.1	0.1

each 800 mL) to give sixteen fractions [Fr 1 (0.30g), Fr 2 (0.13g), Fr 3 (0.35g), Fr 4 (0.37g), Fr 5 (1.01g), Fr 6 (2.02g), Fr 7 (2.10g), Fr 8 (2.04g), Fr 9 (2.14g), Fr 10 (2.03g), Fr 11 (0.37g), Fr 12 (2.47g), Fr 13 (0.20g), Fr 14 (0.05g), Fr 15 (0.03g), Fr 16 (0.05g)]. Fr 7 (2.10 g) was repeatedly chromatographed over silica gel (410 g of Wakogel C-200, 6 x 50 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 5:5 of EtOAc-MeOH, each 600 mL and MeOH 1200 mL) to give nine fractions (Fr 7-1 to Fr 7-9). Fr 7-2 (500 mg) was subjected to preparative HPLC (Inertsil Prep-ODS:20 mm i.d. x 250 mm) eluting with H2O/MeOH, 15:85, 10 mL/min, to afford compounds **1** (artocarpin, 19.5 mg), **2** (cudraflavone C, 41.0 mg), and **3** (6-prenylapigenin, 3.6 mg). Fr 7-3 (400 mg) was applied to preparative HPLC eluting with H_2O-MeOH(0.1% trifluoroacetic acid, TFA), 30:70, 10 mL/min, to give compounds **4** (kuwanon C, 15.3 mg), **5** (norartocarpin, 14.9 mg), **6** (albanin A, 53.4 mg), **7** (artocarpanone, 13.5 mg), **8** (chlorophorin, 39.1 mg). Thus, Fr 7-1 (500 mg) was separated by preparative HPLC (Inertsil Prep-ODS:20 mm i.d. x 250 mm) eluting with H_2O/MeOH (0.1% trifluoroacetic acid, TFA (15:85), 10 mL/min, to obtain compounds **9** (cudraflavone B, 35.5 mg) and **10** (brosimone I, 10.4 mg).

Structure Elucidation of Isolated Compounds from *Artocarpus heterophyllus*. All compounds were analyzed by 400 MHz on JNM-AL400 FT NMR spectrometer (Jeo1). All compounds were dissolved in DMSO-*d*6, methanol-*d*4, and acetone-*d*6. The compounds were assigned for 1H, 13C, HMQC, HMBC, DEPT, and 13C-1H COSY. All compounds (1-10) were also determined by FAB-MS (LX1000-JEOL). The obtained data were compared with the previous reports, compounds **1**[9], **2-6**[10], **7**[11], **8**[12], **9**, and **10**[13], see Figure 3.

Scavenging of 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical. The assay was performed as previously described[14] with minor modification. The assay was done by using method and the temperature was controlled at 25 ^{0}C by circulating bath (Ecoline E100 Lauda. Co, Germany). The reaction mixture contained 467 µL of 60 µM DPPH (1,1-diphenyl-2-picrylhydrazyl) in 500 µL ethanol and 33 µL of sample solution (the extract and compounds) in DMSO. After the reaction was carried out at room temperature for 20 minutes, the free radical scavenging activity of sample was quantified by UV Spectrophotometer (V530 Spectrophotometer, JASCO, Japan) at 514 nm.

Scavenging of hydrogen peroxide (H_2O_2). The assay was performed by the method of Bahorun *et al.* with minor modification[15]. The assay was done by using V530 Spectrophotometer (JASCO, Japan). The reaction mixture containing 400 µL of PBS (pH 7.4), 50 µL of 0.002% H_2O_2 and 50 µL of sample solution in MeOH. After 15 min at 37 ^{0}C, reaction tubes were then overlaid with 500 µL of phenol red (0.2 mg/mL) containing horseradish peroxidase (HRP) type II (Sigma, 8U/mL), to permit the specific oxidation of phenol red by H_2O_2. After 15 min at 37 ^{0}C, was followed by an addition of 50 µL of 1N NaOH, then the amount of H_2O_2 was determined by the changing color in reaction tubes which read by UV Spectrophotometer (V530 Spectrophotometer, JASCO, Japan) at 610 nm.

RESULTS AND DISCUSSION

The research on regulation of melanogenesis has focused on factors that affect tyrosinase, the rate-limiting enzyme in the melanogenic pathway, including research on chemicals that inhibit the tyrosinase function. Considering the importance of counteracting oxidative stress caused by ultra violet radiation (UVR) as a means of preventing skin damage, it is also important to find out a multifunctional skin-whitening agent with antioxidant properties.

It was reported that UV radiation has a strong oxidative component, and photo-oxidative stress has been directly linked to the onset of skin photodamage 16. More specific, Sander *et al.*[17] reported that UVB radiation to keratinocytes lead to dose-dependent intracellular production of H_2O_2. Moreover, H_2O_2 treatment is similar to UVB-irradiation which induced apoptosis of human keratinocytes dose-dependent manner. These facts, lead us to determine the effect of H_2O_2 in B16 melanoma cells and the result can be seen in Figure 1. The result indicated that 50 µM of H_2O_2 concentration lead to 50% cytotoxicity of cells. Above 50 µM of H_2O_2 concentration will lead more cytotoxicity of cells, around 95%.

The result on scavenging activity of DPPH radical of Fr 1 - Fr 16 extracts (Figure 2) showed that fraction 7 (85 %) was one of the most potent fractions. Based on this results, lead the author to evaluate antioxidant activity (radical and ROS scavenging activity) of isolated compound from fraction 7. The evaluation of isolated compounds on radical scavenging activity (DPPH assay) and ROS scavenging activity (H_2O_2 assay) were done by using DPPH and H_2O_2 assays, and the results were presented in Table 1.

The IC_{50} DPPH radical of isolated compounds (**1-6**, **9** and **10**) was weak (> 700 µM) except compound **8** (24.8 µM) which show stronger to scavenge the radical as well as vitamin E and C, the positive controls. The compound **8** may have a labile of H atom that can be transferred to the radical of DPPH and neutralized the radical which shown by decolorization (violet to yellow) of the assay solution. As reported by Goupy *et*

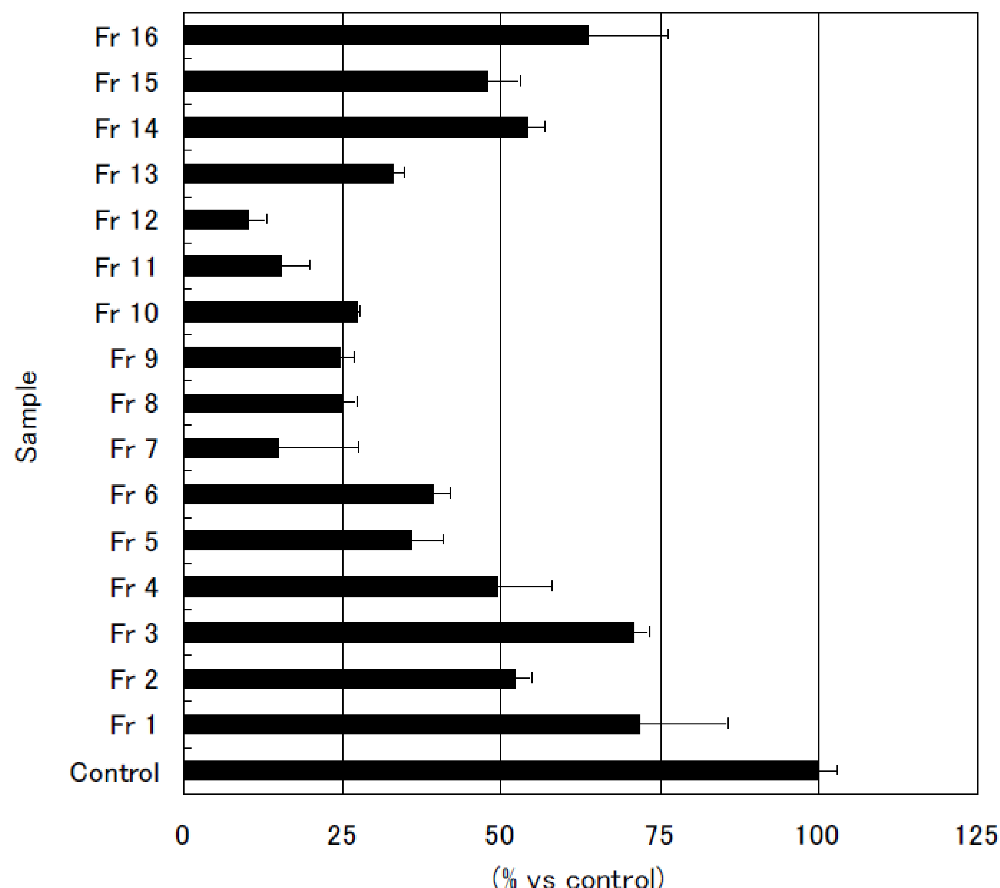

Figure 1. The scavenging activities on DPPH of fractions 1-16 of diethyl ether extract of *Artocarpus heterophyllus* sapwood (Sample concentration : 100 μg/mL).

al.[18] in radical scavenging activity (DPPH assay), the antioxidant compound will transfer its labile H atom to radical of DPPH in order to neutralized the radical. In addition, Cefarelli *et al.*[4] reported that DPPH radical solution was intensely violet colored. The color intensity of DPPH radical will decrease when the radical is reduced by an antioxidant compound.

Interestingly, compounds **2**, **4-6** and **7** exhibited weak on scavenging the radical in DPPH assay but strong to neutralize or scavenge the reactive oxygen species in H_2O_2 assay with IC_{50} of 47.4, 55.8, 40.2, 44.8, and 19.4 μM, respectively. The assay is based on the horseradish peroxidase (HRP) mediated oxidation of phenol red by H_2O_2 and the final product of this reaction will produce purple-mauve color after adding the NaOH as a color indicator into the solution. If the compound has ability to scavenge H_2O_2, it will react with H_2O_2 and block the H_2O_2 to oxidize phenol red. This reaction will mark by turning the color intensity from purple-mauve to reddish.

The results in Table 1 are similar to the results which reported by Cefarelli *et al.*[4] on some flavonoids in DPPH radical and H_2O_2 assays, and suggested that hydroxyl groups play important as radical scavenge activity. Furthermore, Yu *et al.*[19] informed that number and location of hydroxyl groups in flavonoids are also essential to scavenge the radicals. Arung *et al.*[20] also reported that the presence of catechol moiety in ring B of flavonoids (quercetin and quercetin-4’-O-glucoside) is the most important in revealed the antioxidant activity. Moreover, the hydroxyl groups in 4-substituted resorcinol moiety both in flavonoid and stilbene might play in part as scavenger of free radicals. In contrast, vitamin E exhibited weak scavenging activity in H_2O_2 than vitamin C, this result might be caused by its solubility in test medium as suggested by Yu *et al.,*[19] that solubility of test sample in test medium affects their ability to scavenge the radical.

It is generally agreed that a major and important contributors to skin aging, skin disorders and skin diseases are reactive oxygen species (ROS)[21]. Furthermore, considering the importance of counteracting oxidative stress caused by UVR as a mean to prevent skin damage, it is important that a compound with skin-whitening agent properties also have antioxidant activity.

CONCLUSION

Since UVR can cause photocarcinogenesis through ROS generated, it is important to protect the skin from UVR. The experiment in B16 melanoma cells treated with H_2O_2 at certain concentration proved the hazardeous

Figure 2. The chemical structure of compounds 1 – 10 isolated from sapwood of *Artocarpus heterophyllus*.

of this radical which lead cells to cytotoxicity. The isolated compounds such as **2**, **4-6** and **7** have more hydroxyl groups seem to be specifically to scavenge H_2O_2. These experimental results demonstrated that

each scavenging activity is different in its scavenged capacity toward different source of radicals. Test of compounds using multiple in vitro assay system are considered advantageous to assess the antioxidant or scavenging properties of potential active compounds as different reaction mechanism.

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