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Isolation and Identification of Active Antioxidant Compound from Star Fruit (*Averrhoa carambola*) Mistletoe (*Dendrophthoe pentandra* (L.) Miq.) Ethanol Extract

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Abstract: Indonesia is a biodiversity rich country. Mistletoe is one of the unique Indonesian biodiversity. As a semi-parasitic plant, mistletoe is considers as an unwanted plant to economically important horticultural plant, however in the other side, mistletoe is known as one of medicinal plant used in traditional therapy. Mistletoe potency as drug material should be studied so the utilization of mistletoe could be developed. Compare to mistletoe that grown on tea as the host plant, relatively less studies are conducted in mistletoe that grown on other host plants, although it was reported the species of host plant could affected the chemical constituents in the mistletoe. Previous study shows that the antioxidant activity (based on measurement using DPPH free radical scavenger method) of leaf extracts of mistletoe (*Dendrophthoe pentandra*) that grown on star fruit (*Averrhoa carambola*) were depend on the solvent used for extraction. In this study we reported antioxidant activity guided isolation of chemical constituent from ethanol extract of this mistletoe leaves. Separation was conducting using vacuum column chromatography and characterization was conducted using TLC, LC-MS, UV-VIS and IR spectrophotometer and melting point apparatus. From the results obtained it was suggest that the isolated compound is a flavonol glycoside, quercitrin (quercetin-3-O-rhamnoside) which an active antioxidant with IC_{50} value of 5.19 μg mL^{-1}.

Key words: Mistletoe, dendrophthoe, DPPH, antioxidant, flavonol glycoside

INTRODUCTION

Indonesia is a biodiversity rich country. Mistletoe is a semiparasitic plant that belong to the order of Santalales. There are many species of mistletoe. It was reported there were 44 species of mistletoe in Java which belong to the family of Loranthaceae, Santalaceae and Viscaceae (Windari and Rahajoe, 1998). However people in Indonesia usually called the mistletoe depend on the host plant where it grow, such as *benalu the* (mistletoe that grown on tea tree), *benalu belimbing* (mistletoe that grown on star fruit tree) and *benalu mangga* (mistletoe that grown on mango), etc (Pitoyo, 1996). This way of naming mistletoe can be misleading, many species of mistletoes can grow on the same host tree. For example, mistletoe species found grown on tea are *Scurulla oortiana*, *S. junghunii*, *Dendropththoe pentandra, S. phillippensis*, *Lepeostegues gemmiflorus* dan *S. parasitica* (Dr. Partomuan Simanjuntak, personal communication). Besides mistletoe mention before, other mistletoe species that found to be grown on many horticultural species are *D. falcata*, *Elytranthe albida*, *Macrosolen cochinchinensis* and *Viscum articulatum* (Pitoyo, 1996). Hence, mistletoe is one of the unique Indonesian biodiversity. In one side mistletoe is consider as an unwanted plant due to is parasitic property to economically important horticultural plant, however in the other side, mistletoe is known as one of medicinal plant used in traditional therapy such as cough medicine, cancer treatment, diuretic and after birth treatment (PT. EISAI Indonesia, 1995; Pitoyo, 1996; Murwani and Subroto, 2001; Ishizu *et al*., 2002).

In Europe mistletoe extract are commercially available as alternative anticancer drug, they were sales with the brand such as Iscador, Eurixor and Isorel. The mistletoe extract is from the species *Viscum album* L. (Viscaceae) which is grown on various tree such as aple, oak, maple, elm and pine. It was suggest that the chemical constituents of the commercial mistletoe extracts are depend on the host tree (NCI, 2003). At present, at least

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2 US investigators have Investigational New Drug approval to study mistletoe as a treatment for cancer (NCI, 2003). Although the mistletoe species commonly found in Indonesia is different from the one used in Europe, however, here, as mention earlier, mistletoe also used in traditional/alternative medicine. Therefore, mistletoe potency as drug material should be studied so the utilization of mistletoe could be developed.

Compare to mistletoe that grown on tea, there are not many studies are conducted on mistletoe that grown on star fruit. Here we reported our antioxidant activity guided isolation studies on *Dendrophthoe pentandra* (L.) Miq. (Loranthaceae) grown on star fruit (*Averrhoa carambola*) as host trees. Antioxidant is a compound that has ability to inhibit oxidation rate or to neutralize a free radicals. Oxidative damage caused by free radicals may be related to aging and diseases, such as atherosclerosis, diabetes, cancer and cirrhosis (Halliwell and Gutteridge, 1984). Antioxidant supplements, or foods containing antioxidants, may be used to help the human body reduce oxidative damage (Yang *et al*., 2002). Our earlier study shows that the antioxidant activity (based on measurement using DPPH free radical scavenger method) of leaf extracts of star fruit mistletoe was depend on the solvent used for extraction (water, hexane, ethyl acetate, methanol and ethanol (Artanti *et al*., 2003). In this study we reported isolation and identification of active antioxidant compound from the aqueous ethanol leaves extracts of this mistletoe based on analysis of the compound using liquid chromatography mass spectroscopy (LC-MS), Infra Red (IR) and UV-Vis spectroscopy and melting point apparatus.

MATERIALS AMD METHODS

General: LC-MS instrument used was Mariner Biospectrometry. Spectrophotometer used is Hitachi 2000. IR and UV-Vis Analysis were analysed at Department of Physic, University of Indonesia, Jakarta.

Plant material: The plant material is mistletoe (*Dendrophthoe pentandra* (L.) Miq.) grown on star fruit (*Averrhoa carambola*) tree in Jakarta, collected on April 2003 by Ratih Seksiati and determination was conducted at Herbarium Bogoriense Bogor.

Extraction and isolation: Dried powdered leaves of star fruit mistletoe (200 g) were extracted with aqueous 80% ethanol (v/v) (10×350 mL) at room termperature. The extract was concentrated using rotary evaporator *in vacuo*. Fractionation was conducted using silica gel vacuum column chromatography using increasing solvent polarity (hexane, ethyl acetate, methanol). Activity guided isolation was conducted by analysing the antioxidant activity using DPPH method (free radical scavenging activity). Fractions with high antioxidant activity were further purified by rinsing with hexane and ethyl acetate. Thin Layer Chromatography (TLC) was conducted through out the fractionation and purification process. Isolates with highest antioxidant activity was analysed using LC-MS, IR and UV-vis spectroscopy and melting point apparatus for identification.

Antioxidant analysis: Antioxidant analysis was conducted using modification of DPPH free scavenging activity" method (Yen and Chen, 1995). Various concentrations of the mistletoe extract/fractions in 0.8 mL methanol were mixed with 0.2 mL of methanolic solution containing 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals, resulting in a final concentration of the DPPH of 0.2 mM and sample concentrations up to 100 μg mL^{-1}. The mixture was shaken vigorously and left to stand for 30 min in room temperature, the absorbance was then measured using spectrophotometer at 515 nm. Percentage of inhibition (free radical scavenging activity) was calculated by the equation: [1 - (B/A)]×100%; whereas A is absorbance in the absence of sample and B is absorbance in the presence of sample. IC_{50} value denote the concentration of sample required to scavenge 50% DPPH free radicals.

TLC analysis: TLC was performed on Silica Gel 60 GF_{254} plate and dichloromethane/methanol (4:1) as eluent. Spots were visualized by spraying 10% H_2SO_4 solution in ethanol, followed by heating.

LC-MS analysis: Sample was dissolved in methanol at concentration 1 mg mL^{-1} and introduced by injection through a 2×150 mm silica C-18 at flow rate 0.5 mL min^{-1} with the injection volume was 10 μL and eluted with methanol/water (90:10) containing 0.3% acetic acid, then analysis by positive electrospray ionization mass spectrometry.

RESULTS AND DISCUSSION

Crude aqueous ethanol extract of star fruit mistletoe leaves has IC_{50} value 29.89 μg mL^{-1} and fractionation using silica gel vacuum column chromatography of this extracts gave 10 fractions. Antioxidant activity guided isolation showed that Fr. 1-5 were not active (IC_{50}>100 μg mL^{-1}) whereas Fr. 6-10 were active (IC_{50}<100 μg mL^{-1}) as antioxidant based on DPPH free radical scavenging method (Table 1). Further purification

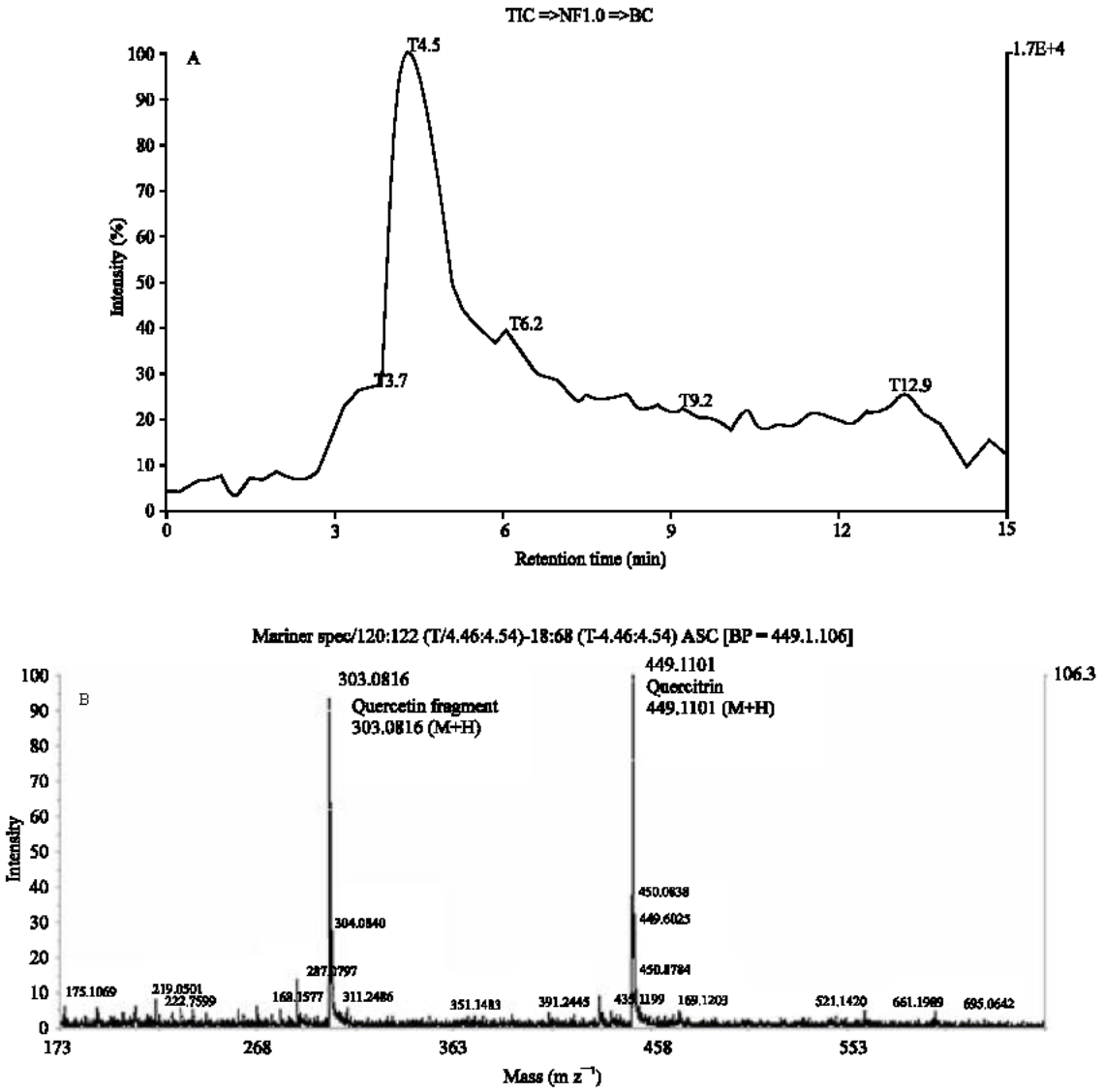

Fig. 1: LC chromatogram (A) and MS spectrum (B) of powder Fr. 6

Table 1: Antioxidant activity (DPPH free radical scavenging activity) of star fruit mistletoe fractions

	DPPH free radical scavenging activity IC_{50} (μg mL)*		
Sample	Total**	Powder***	Soluble fraction****
Fr. 1	>100	-	-
Fr. 2	>100	-	-
Fr. 3	>100	-	-
Fr. 4	>100	-	-
Fr. 5	>100	-	-
Fr. 6	32.5	5.19	9.50
Fr. 7	13.0	8.62	24.72
Fr. 8	20.3	7.48	10.89
Fr. 9	11.6	25.33	9.05
Fr. 10	19.9	29.64	11.94

*Extract is consider active if IC_{50}<100 μg mL^{-1}, ** Total is the whole fraction obtained, ***Powder is purified powder obtained after rinsing the fraction with hexane and ethyl acetate, **** Soluble fraction is part of the fraction that soluble in hexane and ethyl acetate

Table 2: Comparison of IR wave number of powder Fr. 6 with reported quercitrin data

	Wave number (v cm^{-1})	
Powder Fr. 6	Quercitrin*	Functional group
3363.8	3378	O-H
2933.3	2932	C-H
1651.1	1651	C=O
1604.6	1607	C=C
1502.4	1505	C=C

*From (Jeong and Shim, 2004)

conducted on Fr. 6-10 by rinsing with hexane and ethyl acetate. The results show that both powder and soluble fraction are active as antioxidant, although the IC_{50} value are varied (Table 1). Powder obtained from Fr. 6 was the most active with IC_{50} value 5.19. μg mL^{-1} (Table 1). TLC of

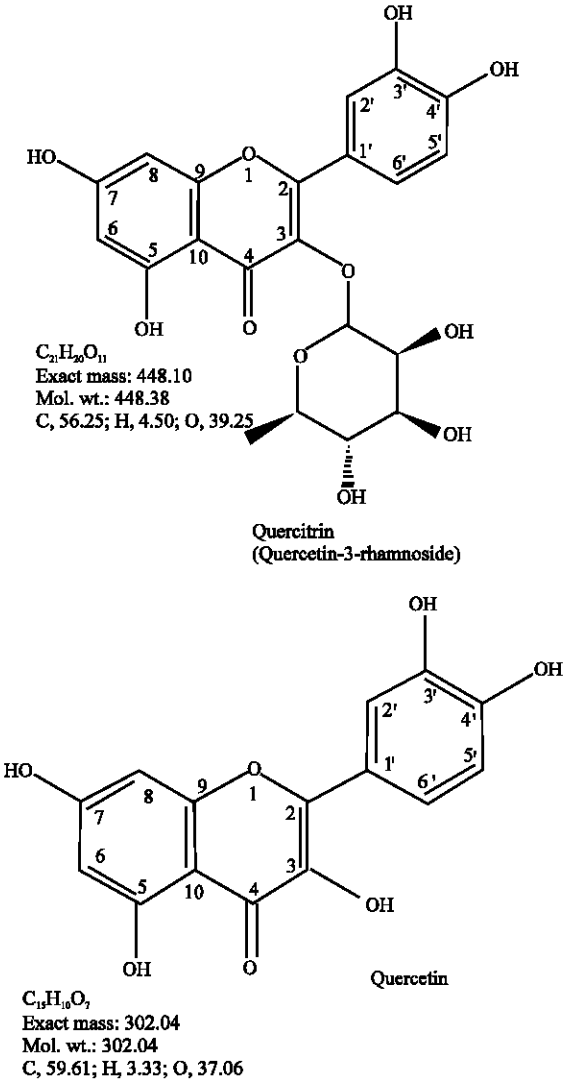

Fig. 2: Chemical structure of quercitrin and quercetin

this isolates show one brown spot with Rf value of 0.675. Powder from Fr. 7-10 also have spot with the same Rf, however, probably powder from other fractions have more impurity because the IC_{50} value is less then powder from Fr. 6. TLC of hexane and ethyl acetate soluble fractions of Fr. 6-8 show two spot with Rf value 0.6 (light brown) and 0.7 (dark green) whereas this portion of Fr. 9 and 10 show only one spot with Rf value 0.8 (light green). This suggests that impurities present in Fr. 6- 8 different from Fr. 9 and 10. Powder from Fr. 6 was subjected to LC-MS, IR, UV-vis and melting point analysis for identification of the active antioxidant compound.

Analysis using LC-MS show that powder Fr. 6 gave a single big peak on LC chromatogram and 2 peak on MS spectrum with m/z 302 and 448 (Fig. 1). Based on references, m/z 448 is correspond with molecular weight (MW) of quercitrin (Winhollz *et al*., 1983), which reported as a taxonomic marker of the Loranthaceae family (Devehat *et al*., 2002). The star fruit mistletoe (*D. pentandra*) is also belongs to the Loranthaceae family. More present studies showed this compound was found abundant in mistletoes species *Ligaria cunelfolia* (R. et P.) Tiegh (Fernandez *et al*., 1998), *S. ferruginea* Danser (Devehat *et al*., 2002) and *S. atropurpurea* (Ohashi *et al*., 2003). Figure 2. shows the chemical structure of quercitrin, this compound is a quercetin glycosilated with rhamnose (quercetin-3-rhamnoside). Molecular weight of quercetin (302) was also detected in the MS spectrum (Fig. 1), this suggest as fragmentation of quercitrin in the analysis, because TLC analysis did not indicate the present of other compound in powder Fr. 6. This suggestion also support by reported quercitrin analysis using LC-MS-MS which also shown the present of quercetin fragment (Sanchez-Rababeda *et al*., 2003).

Suggestion that powder Fr. 6 is quercitrin was also supported by IR, UV-Vis and melting point results. Table 2 show the IR analysis result of powder Fr. 6 and comparison with IR data of quercitrin in published paper (Jeong and Shim, 2004). The result suggest that the result obtained in this study is similar with quercitrin published data. UV-Vis analysis showed that powder Fr. 6 has peak at 256 and 371 nm, flavonoid compound is reported have two optimum wave length at around 250-280 nm (I) and at around 330-380 nm (II) (Markham, 1982). Melting point analysis showed that powder Fr. 6 has melting point 178-179°C, whereas melting point for quercitrin is 175-179°C (Winhollz *et al*., 1983).

CONCLUSIONS

Based on analysis using LC-MS, IR, UV-Vis and melting point and comparison with published references, it was concluded that the isolated compound from star fruit mistletoe (*D. pentandra*) is a flavonol glycoside, quercitrin (quercetin-3-O-rhamnoside) which is an active antioxidant with IC_{50} value of 5.19 μg mL^{-1}. Star fruit mistletoe might contain other active antioxidant compound(s) since the soluble hexane and ethyl acetate fraction also show relatively high antioxidant activity.

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