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Isolation and Characterization of Flavonoids Isolated from Leaves of Benalu Jeruk (*Scurrula fusca* G. Don) as Antioxidant

HELMINA BR. SEMBIRING[1,*], TONEL BARUS[1], LAMEK MARPAUNG[1] and PARTOMUAN SIMANJUNTAK[2,3]

[1]Department of Chemistry, Faculty of Mathematics and Natural Sciences, University of Sumatera Utara, Medan, Indonesia
[2]Research Center for Biotechnology, Indonesian Institute of Sciences (LIPI), Cibinong, Indonesia
[3]Faculty of Pharmacy, Pancasila University, Jagakarsa, Jakarta Selatan, Indonesia

*Corresponding author: E-mail: helmina02@yahoo.com

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Two flavonoids were isolated from leaves of Benalu Jeruk (*Scurrula fusca* G. Don) by extracting the leaves powder with methanol. The methanol extract was partitioned with ethyl acetate. The ethyl acetate extract was then partitioned further with mixture of methanol and *n*-hexane. The methanolic layer was separated and hydrolyzed with 2 N HCl to give flavonoid aglycon. This flavonoid aglycon was purified by column chromatography (SiO_2, chloroform-methanol = 100:0 ~ 80:20) to produce two flavonoids, flavonoid **A** (3,7-dihydroxy-5-methyl-3',4'-dihydrofurano-flavonol) and flavonoid **B** (3,5,7,4'-tetrahydroxyflavonol or kaempferol). The antioxidant activity test for flavonoids **A** and **B** gave IC_{50} 9.383 and 8.713 mg/mL respectively with ascorbic acid as comparison (IC_{50} 12.08 mg/mL). Chemical structure was determined based on spectroscopy data interpretation [FT-IR, UV, 1-D NMR (^{1}H NMR, ^{13}C NMR and DEPT), 2-D NMR (^{1}H-^{1}H COSY, ^{1}H-^{13}C HMQC, ^{1}H-^{13}C HMBC) and LC-MS].

Keywords: *Scurrula fusca* G. Don, 3,7-Dihydroxy-5-methyl-3',4'-dihydrofurano-flavonol, 3,5,7,4'-Tetrahydroxyflavonol, Antioxidant.

INTRODUCTION

One of the causes of diseases such as cancer, inflammation, artheroschlerosis and aging is reactive oxygen species (ROS) like hydrogen peroxides, superoxides, hydroxyl radicals and other radical compounds. Reactive oxygen and other radical compounds oxidized human body cells, disturbing cells growth and make them growth abnormally that lead to illness and diseases [1]. To prevent this oxidation process we need antioxidant agents to protect cells from those radicals. Antioxidant is a compound that capable in inhibiting and preventing oxidation process of molecules by blocking chain reaction of initiation and propagation [2].

Indonesia is rich in biodiversity which potential to be developed as antioxidant medicine or drug raw material [3,4]. Benalu is a semi-parasite plant [5], that take carbons, water and other nutrition from its host by attaching and penetrating xylem of its host using special organ named haustoria [6]. Benalu is a destructive parasitic plant for commercial plants [7,8]. Nevertheless, traditionally some of Benalu species have been used in treatment of many diseases such as cough, cancer, inflammation, bacterial infection or wounds [9].

Research conducted by Bamane *et al.* [10] for antioxidant activity of methanolic extracts of three parasitic species from Loranthaceae families (*Plicosepalus acacia, Plicosepalus curviflorus* and *Phragmanthera austroarabica*) using free radical scavenging method found antioxidant activity range from 18 to 56 % compared to ascorbic acid as standard.

Scurrula fusca G. Don [Benalu Jeruk (*Citrus sinensis*)] commonly found at orange plantation in Tanah Karo area, Sumatera Utara, Indonesia. This plant caused severe damage to the farmer's commercial plants. The farmers just dispose these parasitic plants without knowing their advantage as medicinal plants. Previous research done to methanol leaves extract of this plant had shown antioxidant activity with IC_{50} value 32.96 µg/mL [11]. The aims of this research are to isolate, characterize and test the antioxidant activity of flavonoids isolated from leaves extract of *Scurrula fusca*.

EXPERIMENTAL

The infrared spectra were recorded with Perkin Elmer 1600 Series FT-IR instrument, UV spectra were measured on Shimadzu UV-160A Spectrophotometer, ^{1}H, ^{13}C and 2-D NMR spectra were obtained on JEOL JNM-FX400 spectrophotometer using TMS as internal standard (500 MHz) and chemical shifts are given in δ (ppm) values relative to those of the solvent [methanol-d_3 (δ_H 3.31; δ_C 48.7)]. Mass spectra were obtained

on JMS 700 spectrophotometer. TLC analysis used pre-coated Kiesel gel 60 F_{254} (0.2 mm, Merck). Chromatographic column process was carried out using silica gel 60G F_{254} (Merck).

Leaves of *S. fusca* G. Don were collected from orange plantation in Sukarame Village, District of Munte, County of Karo, North Sumatra Province, Indonesia and identified and determined at Herbarium Bogorienses, Research Center for Biology, Indonesian Institute of Sciences (LIPI), Cibinong, Indonesia.

Extraction: Dried leaves powder of *S. fusca* (3 kg) were extracted by method performed by Ginting *et al.* [12] to get total flavonoid aglicon (3 g).

Flavonoid compounds purification: Flavonoid aglycons were separated by column chromatography method (SiO_2, chloroform-methanol = 100:0 ~ 80:20) and monitored by TLC analysis. Based on TLC chromatograms we obtained 7 fractions. Compound **A** was isolated from fraction 3 and compound **B** from fraction 4. Both compounds were purified by recrystallization using *n*-hexane.

Antioxidant activity test: Antioxidant activity test of flavonoids **A** and **B** was run by free radical scavenging method using 1,1-diphenyl-2-pycril-hydrazil (DPPH) reagent according to Molyneux [2] using ascorbic acid as standard comparison.

RESULTS AND DISCUSSION

Flavonoid **A** as amorphous brownish green solid (10 mg) and flavonoid **B** as amorphous yellow solid (14 mg) showed high antioxidant activity with IC_{50} 9.38 and 8.71 µg/mL, respectively compared to ascorbic acid (12.08 µg/mL) as standard. The antioxidant activity of methanolic leaves extract of *S. fusca*, flavonoid aglycon, flavonoid **A**, flavonoid **B** and ascorbic acid are given in Table-1.

TABLE-1
ANTIOXIDANT ACTIVITY OF METHANOLIC LEAVES EXTRACT OF *S. fusca*, FLAVONOID AGLIKON, COMPOUND **A**, COMPOUND **B** AND ASCORBIC ACID

Sample	Regression line formula	IC_{50} value (µg/mL)
Methanolic extract	Y = 1.474x + 1.4230	32.96
Flavonoid aglycon	Y = 2.957x + 8.0830	14.18
Flavonoid **A**	Y = 3.032x + 21.550	9.38
Flavonoid **B**	Y = 3.069x + 23.260	8.71
Ascorbic acid	Y = 3.188x + 11.480	12.08

Table-1 showed that IC_{50} value of flavonoids **A** and **B** were lower than IC_{50} value of ascorbic acid that indicated very high antioxidant activity. This results might caused by flavonoids contained with their hydroxyl groups which easily donor hydrogen atoms to radicals supported by conjugated aromatic system that could delocalized single electron without pair. Flavonoid compounds have Δ 2,3 double bonds that conjugated with very active 4-keto groups at C ring which dislocated electrons from B ring and increased its capacity as radical scavenger [13].

Flavonoid A: UV spectroscopy data showed two maximum absorbance at λ 370.0 nm (band I) and 256.0 (band II) that indicated flavonoid **A** belonged to flavonol-type of flavonoid structure with hydroxyl group at C-3 [14].

The FT-IR spectra for flavonoid **A** showed (KBr, ν_{max}, cm^{-1}): 3470.7, 1362.9, 1248.6, 3078.8, 1228.6, 1660.6, 1614.3, 2761.8, 2933.5 and 1451.3 which indicated that flavonoid **A** had flavonoid functional group with substituent $-CH_3$ and $-O-CH_2-CH_2-O-$ [15].

Based on ^1H NMR data, flavonoid **A** had five aromatic protons, four oxy-ethylene protons and three methyl protons. ^{13}C NMR data revealed that flavonoid **A** had 18 carbon atoms including one methyl carbon substituent, four oxy-ethylene carbons, five methine carbons and 10 quaternary carbons. DEPT data revealed that compound **A** had two carbon atoms that bonds protons in even number [16]. The chemical shift 1-D ^1H NMR of flavonoid **A** are presented in Table-2.

2-D ^1H-^1H COSY data analysis showed that methyne proton H-6 was coupled with methyne protons H-8; H-6' was coupled with H-5' and H-2' and methylene proton H-2" was coupled with H-3" [17].

2-D ^1H-^{13}C HMBC data analysis showed that proton H-6′ (δ_H 7.3501 ppm) was correlated with C-1′ ()δ_C 140.9439 ppm), C-4′ and C-3′ (δ_C 159.8584 ppm). Proton H-5′ and H-2′ (δ_H 6.7469 ppm) were correlated with C-6′ (δ_C 130.1465 ppm).

LC-MS data analysis showed that flavonoid **A** had molecular ion peak $[M^+]$ at *m/z* 310 and indicated that compound **A** had molecular weigh 310 with molecular formula $C_{18}H_{14}O_5$. Fragmentation at *m/z* 295 $[M-15]^+$ showed lost of methyl group and fragmentation at *m/z* 267 $[M-43]^+$ showed lost of oxy ethylene group. Based on FT-IR, UV, 1-D NMR (^1H NMR, ^{13}C NMR, DEPT), 2-D NMR (^1H-^1H COSY, ^1H-^{13}C HMQC, ^1H-^{13}C HMBC) and LC-MS data analysis we convinced that flavonoid **A** is 3,7-dihydroxy-5-methyl-4,3-dihydrofurano-flavonol as shown in Fig. 1.

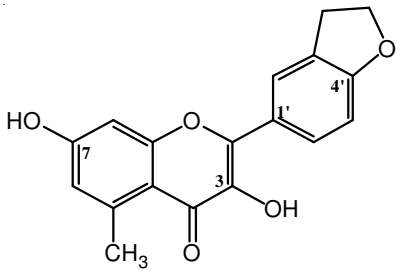

Fig. 1. Chemical structure of flavonoid **A** (3,7-dihydroxy-5-methyl-4,3-dihydrofurano-flavonol)

Flavonoid B: UV spectra showed two maximum absorbance at λ 364.5 nm (band I) and 266.0 (band II) that indicated flavonoid **B** belongs to flavonol type of flavonoid structure with hydroxyl group at C-3 [14].

IR (KBr, ν_{max}, cm^{-1}): 3314.4, 3078.2, 1660.6, 1614.3, 1382.9, 1226.0 and 1177.4. Based on those data, flavonoid **B** had functional group with characteristic for flavonoid compounds [15]. Based on 1-D ^1H NMR and ^{13}C NMR data, compound **B** had six methyne protons and 15 carbon atoms including six methyne carbons and nine quaternary carbons. DEPT data analysis showed that compound **B** had not had carbon atom that bond proton in even number [18].

2-D ^1H-^1H COSY data analysis revealed that methyne proton H-6 was coupled with methyne proton H-8; H-5'and H-3' were coupled with H-6' and H-2' respectively at (δ_H(ppm) : δ_H (ppm)): (6.27: 6.54); (8.16 : 7.03) and (7.02 : 8.16) [17].

TABLE-2
CHEMICAL SHIFT 1-D ^{1}H NMR OF FLAVONOIDS **A** AND **B**

Carbon No.	Flavonoid **A**		Flavonoid **B**	
	1-D ^{1}H NMR (Methanol-D3; δ_H (ppm); *J* (Hz))	1-D ^{1}C NMR (Methanol-D3; δ_H (ppm); *J* (Hz))	1-D ^{1}H NMR (Aceton-D3; δ_H (ppm); *J* (Hz))	1-D ^{1}C NMR (Aceton-D3; δ_H (ppm); *J* (Hz))
1	–	–	–	–
2	–	170.43	–	157.86
3	–	132.38	–	136.67
4	–	180.42	–	176.62
5	–	115.31		160.19
6	6.34 (d, *J* = 15.5)	123.45	6.54 (d, *J* = 1.9)	99.17
7	–	175.76	–	165.08
8	6.34 (d, *J* = 15.5)	123.45	6.27 (d, *J* = 1.9)	94.56
9	–	141.06	–	147.00
10	–	128.88	–	104.14
1'	–	140.94	–	123.37
2'	6.75 (dd, J_1 = 9.1; J_2 = 1.95)	116.63	8.17(d, *J* = 9.1)	130.52
3'	–	159.86	7.03(d, *J* = 8.4)	116.35
4'	–	170.43	-	160.19
5'	6.75 (dd, J_1 = 9.1; J_2 = 1.95)	116.63	7.03(d, *J* = 8.4)	116.35
6'	7.35 (dd, J_1 = 15.6; J_2 = 8.4)	130.15	8.17(d, *J* = 9.1)	130.52
1"	–	–	–	–
2"	3.68 (m)	64.51	–	–
3"	3.49 (m)	30.83	–	–
CH_3	1.89 (s)	24.31	–	–

^{1}H-^{13}C HMBC data analysis results showed that proton H-2' and H-6' (δ_H 8.19 ppm) were correlated with C-3' and C-4' (δ_C 147.00 ppm). Proton H-3' and H-5' (δ_H 7.03 ppm) were correlated with C-1' (δ_C 123.37 ppm). Proton H-6 (δ_H 6.54 ppm) was correlated with C-10, C-9 and C-7 at δ_C 104.14 ppm, δ_C 157.85 ppm and δ_C 165.08 ppm respectively. 1-D ^{1}H NMR and 1-D ^{1}H NMR spectroscopy data analysis results of flavonoid **B** was shown in Table-2.

LC-MS data analysis revealed that flavonoid **B** (Fig. 2) had molecular ion peak $[M+H]^+$ at *m/z* 287.09 that indicated that flavonoid **B** had molecular weight 286 with molecular formula $C_{15}H_{10}O_6$. Fragmentation at *m/z* 153 $[M-133]^+$ showed lost of ring B and ring C.

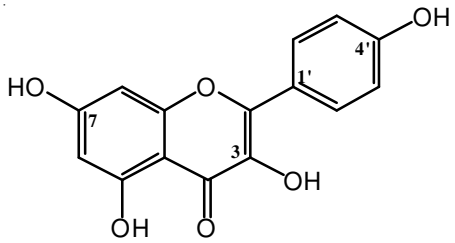

Fig. 2. Chemical structure of **B** (3,5,7,4'-tetrahydroxyflavonol or kaempferol)

Conclusion

Two flavonoids were isolated from leaves of *S. fusca* G. Don, *i.e.* compound **A** (3,7-dihydroxy-5-methyl-3',4'-dihydro-furano-flavonol) with IC_{50} value 9.383 µg/mL and compound **B** (3,5,7,4'-tetrahydroxyflavonol or kaempferol) with IC_{50} value 8.713 µg/mL.

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