DPPH-radical Scavenging Constituents from the Twigs of *Messerschmidia argentea* (III)

Kazuhito Ogihara*, Risa Nakazato*, Yuka Nishi*, Matsutake Higa*, and Seiichi Yogi*

*Department of Chemistry, Biology, and Marine Science, Faculty of Science, University of the Ryukyus, Nishihara, Okinawa 903-0213, Japan

Abstract

Five aromatic compounds possessing DPPH-radical scavenging activity were isolated from a water extract of the twigs of *Messerschmidia argentea*. These compounds were identified as rosmarinic acid and its methyl ester, cafferic acid and its methyl ester, and 3-(3', 4'-dihydroxyphenyl)lactic acid, respectively.

Introduction

Messerschmidia argentea (Japanese name: Monpanoki) is a small tree with large inverted-ovate leaves found on shores of Okinawa Islands. We previously reported the isolation of pyrrolizidine alkaloids and triterpenoids in the twigs of the plant.[1,2] The leaves of *M. argentea* have been used as the first medical treatment against jerryfish venom and known as medicinal plant in Okinawa Islands. We have researched novel biological and phisiological activities for a water extract from the twigs of *M. argentea* and found that the extract possessed the DPPH-radical scavenging effect. Therefore, we examnined DPPH-radical scavenging constituents in the water extract of the twigs and isolated five aromatic compounds possessing DPPH-radical scavenging effect. Herein, we describe the separation and identification of these constituents.

Results and Discussion

DPPH-radical scavenging activity of the water extracts from fresh and dry twigs and leaves.

Scavenging effect of the water extracts from fresh and dry twigs and leaves on DPPH-radicals were exmained. Table 1 shows the DPPH-radical scavenging activity of these water extracts. Most strong scavengining activity was observed with the water extract of dry twigs.

DPPH-radical scavenging constituents in the twigs.

The water extract of the twigs of *M. argentea* was subjected to gel-filteration on

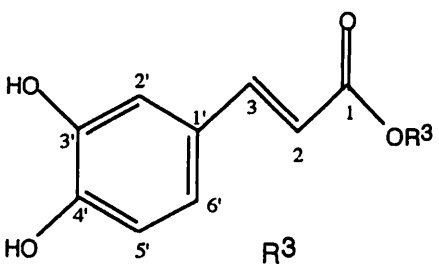
Received: June 28, 2002

Table 1. DPPH radical scavenging activity of water extracts from the leaves and twigs of *Messerschmidia argentea*

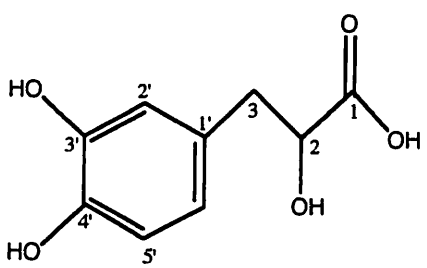
parts	conditions	activity
leaves	fresh	26.0
	dried	45.8
twigs	fresh	33.3
	dried	14.3

$$\text{Activity} = \frac{\text{Sample Abs.} - \text{Sample blank Abs.}}{\text{Reagent blank Abs.}} \times 100$$

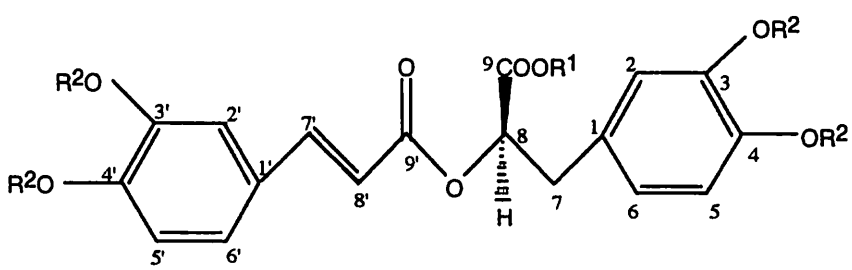
Activity is less than 80: scavenging effect is judged to be active.



	R^3
1	CH_3
4	H



5



	R^1	R^2
2	CH_3	H
3	H	H
3a	CH_3	Ac

Structures

Sephadex LH-20 and then silica gel to give five compounds (1-5) possessing the DPPH-radical scavenging effect.

Compound 1 was identified as methyl caffeate (1) which was already isolated from a methanol extract from fresh twigs[2].

Compound 3 was obtained as brown amorphous solid, $[\alpha]_D^{25} + 86°$ (c 0.4, MeOH). The IR spectrum of 3 showed bands due to a carboxyl group at 3600 and 1715 cm^{-1}, characteristic bands due to an α, β-unsaturated ester at 1680 and 1350-1100 cm^{-1}, and bands due to aromatic rings at 1600 and 1500 cm^{-1}. The ^1H and ^{13}C NMR spectra of 3 showed a carbonyl carbon signal at δ_C 177.16 due to a ester group, signals at δ_H 7.41 (1H, *d*, J=15.9 Hz) and 6.17 (1H, *d*, J=15.9 Hz) due to each *trans*-configuration olefinic proton conjugated with aromatic ring, AMX pattern signals at δ_H 6.67 (1H, *d*, J=7.8 Hz), 6.81 (1H, *dd*, J =7.8, 2.2 Hz), and 6.93 (1H, *d*, J=2.2Hz) due to aromatic protons, and signals at δ_C 145.85 and 149.30 due to aromatic carbons oxygenated. These spectral data indicate that 3 possesses a 3',4'-dihydroxycinnamic acid moiety as a partial structure. Moreover, the ^1H and ^{13}C NMR spectra of 3 showed another carbonyl carbon signal at δ_C 169.07 due to a carboxyl group, another AMX pattern signals at δ_H 6.53 (1H, *dd*, J=7.8, 2.2 Hz), 6.59 (1H, *d*, J=7.8 Hz), and 6.68 (1H, *d*, J=2.2Hz) due to aromatic protons, signals at δ_C 146.63 and 144.74 due to aromatic carbons oxygenated, a ABX pattern signals at δ_H 5.00 (1H, *dd*, J=9.5, 3.5 Hz), 3.25-2.80 (*m*) due to a oxygenated ethylene group. These spectral data indicate that 3 possesses a 3-(3',4'-dihydroxyphenyl)-lactic acid moiety as a partial structure. These results suggest that 3 is a ester of 3',4'-dihydroxycinnamic acid and 3-(3',4'-dihydroxyphenyl)lactic acid. This suggestion was supported by obsevation of fragment ion peaks at m/z 180 and 198 due to 3',4'-dihydroxycinnamic and 3-(3',4'-dihydroxyphenyl)-lactic acid moieties, respectively, in the mass spectrum (MS) of 3.

In order to determine the condensing position of the two moieties, 3 was acetylated and methylated to give a methylester-tetraacetates derivative (3a) of 3. The ^1H NMR spectrum of 3a showed four singlet at δ_H 2.31, 2.30, 2.28, and 2.27 in a lower field due to four acetyloxy groups and a singlet at δ_H 3.74 due to methyl ester besides the signals of 3, which indicate that a carboxy group of 3',4'-dihydroxycinnamic acid and a hydroxy group at C-2 of 3-(3',4'-dihydroxyphenyl)lactic acid condensed into the ester, rosmarinic acid (3). The physical and spectral data of 3 coincided with those described in references.[3,4] Thus, 3 was identified as *R*-(+)-rosmarinic acid (3).

Compound 2 was obtained as brown amorphous solid, $[\alpha]_D^{25} + 36°$ (c 0.1, MeOH). The IR spectrum of 2 showed bands due to hydroxy groups at 3420 cm^{-1}, characteristic bands due to a normal and an α, β-unsaturated esters at 1720, 1650 and 1350-1100 cm^{-1}, and bands due to aromatic rings at 1600 and 1500 cm^{-1}. The ^1H and ^{13}C NMR spectra of 2 coincided with those of 3, except for a singlet due to methoxy group at δ_H 3.67. The EIMS of 2 showed a molecular ion peak at m/z 374, which is 14 mass units more than 3. These obsevations suggest that 2 is a methyl ester derivative of 3, methyl rosmarinate (2).

The physical and spectral data of 2 coincided with those described in references.[5,6] Thus, 2 was identified as *R*-(+)-rosmarinic acid methyl ester (2).

Compound 4 was obtained as brown amorphous solid. The IR spectrum of 3 showed bands due to an α, β-unsaturated carboxyl group at 3420 and 1650 cm^{-1} and bands due to aromatic ring at 1620 cm^{-1}. The ^1H and ^{13}C NMR spectra of 4 showed a carbonyl carbon signal at δ_C 177.16 due to a carboxyl group, signals at δ_H 6.21 (1H, *d*, *J*=15.7 Hz) and 7.52 (1H, *d*, *J*=15.7 Hz) due to each *trans*-configuration olefinic protons conjugated with aromatic ring, AMX pattern signals at δ_H 6.77 (1H, *d*, *J*=8.1 Hz), 6.93 (1H, *dd*, *J*=8.1, 1.9 Hz), and 7.02 (1H, *d*, *J*=1.9Hz) due to aromatic protons, and signals at δ_C 146.82 and 147.03 due to aromatic carbons oxygenated. The EIMS of 4 showed a molecular ion peak at *m/z* 180. These spectral data suggest that 4 is 3',4'-dihydroxycinnamic acid (caffeic acid, 4). The physical and spectral data of 4 coincided with those of an authentic sample. Thus, 4 was identified as 3',4'-dihydroxycinnamic acid (caffeic acid, 4).

Compound 5 was obtained as brown amorphous solid. The IR spectrum of 5 showed bands due to a carboxyl group at 3420 and 1720 cm^{-1} and bands due to aromatic rings at 1600 and 1500 cm^{-1}. The ^1H and ^{13}C NMR spectra of 5 showed a carbonyl carbon signal at δ_C 181.92 due to a carboxyl group, AMX pattern signals at δ_H 6.73 (1H, *dd*, *J*=8.0, 2.0 Hz), 6.83 (1H, *d*, *J*=2.0 Hz), and 6.86 (1H, *d*, *J*=8.0Hz) due to aromatic protons, signals at δ_C 145.29 and 146.55 due to aromatic carbons oxygenated, and ABX pattern signals at δ_H 4.20 (1H, *dd*, *J*=8.0, 4.5 Hz), 3.00-2.73 (*m*) due to an oxygenated ethylene moiety. These spectral data suggest that 5 is 3-(3',4'-dihydroxyphenyl)lactic acid (5). The physical and spectral data of 5 coincided with those described in references.[4,7] Thus, 5 was identified as 3-(3',4'-dihydroxyphenyl)lactic acid (5).

DPPH-radical scavenging activity of 1-5.

Scavenging effects of 1-5 on DPPH-radicals were exmained. Figure 1 shows DPPH-radical scavenging activities of several concentration of 1-5 and ascorbic acid which is well-known as a strong antioxidant. Scavenging effects of 1-5 on DPPH-radicals in several concentration are superior to that of ascorbic acid, which indicate that 1-5 are more available antioxidants than ascorbic acid. Rosmarinic acid (3) was also reported to be an available scavenger against superoxide anion ($O_2\cdot^-$) and 2,2-azinobis (3-ethylbenzothiozoline-6-sulfonate) cation ($ABTS\cdot^+$) radicals.[4,8] Thus, antioxidative effect is newly added to pharmacological ones of *M. argentea* as a medicinal plant.

Experimental

Analytical TLC was carried out on Merck 60 F_{254} silica gel plate (thickness: 0.25 mm). ^1H (90 and 270 MHz) and ^{13}C NMR (25 and 67.5 MHz) spectra were determined in $CDCl_3$ for 1, in CD_3OD for 2-4 with TMS as int. standard, and in D_2O for 5 with TMSP as int. standard. EIMS were obtained on a Hitachi M-2500 double focusing mass spectrometer at

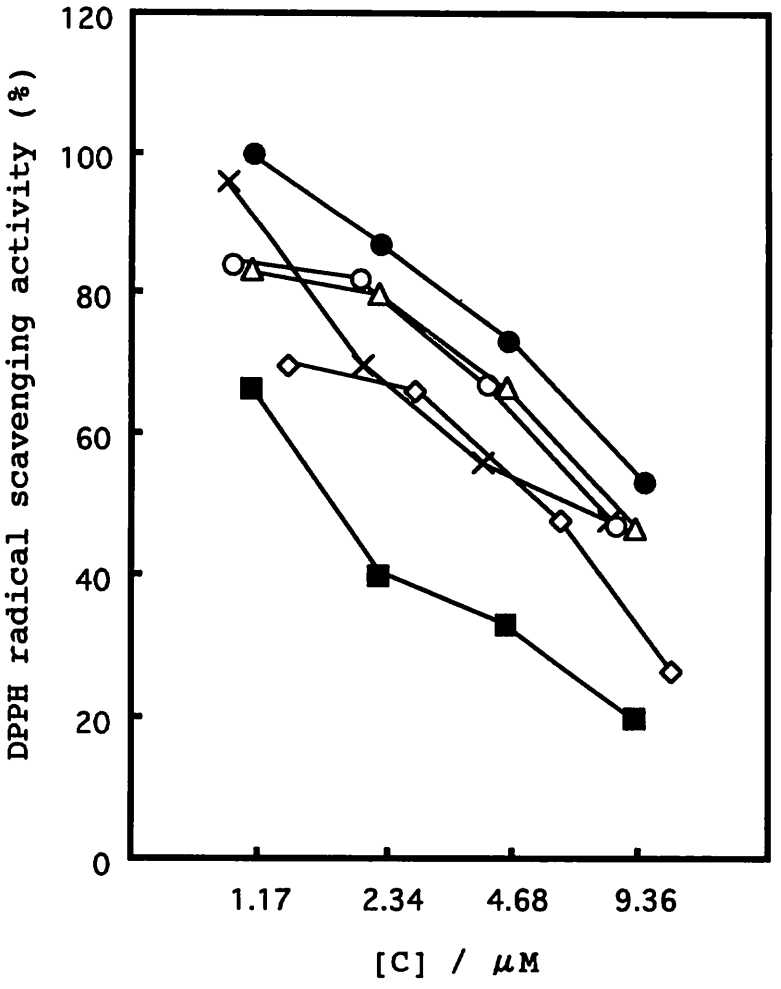


Figure 1. DPPH radical scavenging activities of 1(○), 2(◇), 3(■), 4(△), 5(×), and ascorbic acid(●).

70 eV.

Extraction and isolation. Air-dried twigs (wet weight:3.7 kg, dry weight:1.0 kg) of *M. argentea*, collected at Okinawa Island, Okinawa-prefecture in April, were ground in a mixer after cutting into tip and immersed in distilled water for 2 days. The water soln was concd *in vacuo* and the obtained concentrate (32.4 g) was subjected to gel-filteration column chromatography on Sephadex LH-20 with EtOH. The fractions possessing DPPH-radical scavenging activity were combined and were re-chromatographed on a silica gel column developed with $CHCl_3$-MeOH-H_2O (7:3:0.5) to give compounds 1 (20 mg), 2 (5 mg), 3 (45 mg), 4 (3 mg), and 5 (1 mg) as DPPH-radical scavengers. The compound 1 (20 mg), 4 (18 mg), and 5 (18 mg) were also obtained from a water extract of fresh twigs (2.8 kg).

3', 4'-Dihydroxycinnamic acid methyl ester (*methyl caffeate*: 1). White needles, mp 287.5-288° (EtOH) ; IR ν_{max}^{KBr} cm^{-1}: 3400 and 1670 (COOH), 3020 (=CH), 1610 and 1510 (aromatic ring); ^1H (270 MHz): δ 3.78 (3H, *s*, $COOCH_3$),4.74 (2H, *brs*, 2×OH), 6.23 (1H, *d*, *J*=15.8 Hz, CH=CH-$COOCH_3$), 6.83 (1H, *d*, *J*=8.4 Hz, 5'-H), 6.93 (1H, *dd*, *J*=8.4 and 1.3 Hz, 6'-H), 7.06 (1H, *d*, *J*=1.3 Hz, 2'-H), and 7.56 (1H, *d*, *J*=15.8 Hz, CH=CH-COOH); EIMS *m/z* (rel. int.): 194 $[M]^+$ (100), 452 (7), 423 (22), 248 (100), 203 (45), 175 (8), and 43 (12). These physical and spectral data coincided with those of 1 already isolated from a methanol extract of the plant.

R-(+)-*rosmarinic acid methyl ester* (2). Brown amorphous solid; $[\alpha]_D^{25}$ + 36° (*c* 0.1, MeOH); IR ν_{max}^{KBr} cm^{-1}: 3420 (OH), 1720, 1650, and 1350-1100 (COO, =CHCOO), 1600 and 1500 (aromatic ring); EIMS *m/z* (rel. int.): 374 (M^+, 5), 212 (18), 194 (18), 163 (12), 123 (100); ^1H-NMR (CD_3OD, 270 MHz): δ7.04 (1H, *d*, *J*=2 Hz, H-2), 6.69 (1H, *d*, *J*=8 Hz, H-5), 6.95 (1H, *dd*, *J*=8, 2 Hz, H-6), 7.55 (1H, *d*, *J*=16 Hz, H-7), 6.26 (1H, *d*, *J*=16 Hz, H-8), 6.70 (1H, *d*, *J*=2 Hz, H-2'), 6.77 (1H, *d*, *J*=8 Hz, H-5'), 6.57 (1H, *dd*, *J*=8 Hz, H-6'), 5.19 (1H, *dd*, *J*=5.0, 8.0 Hz, H-8'), 3.67 (3H, *s*, $COOCH_3$), 3.02 (2H, *m*, H-7'); ^{13}C-NMR (CD_3OD, 270 MHz): δ127.6 (C-1), 114.1 (C-2), 146.8 (C-3), 149.8 (C-4), 116.5 (C-5), 123.2 (C-6), 148.0 (C-7), 115.2 (C-8), 168.3 (C-9), 128.7 (C-1'), 117.5 (C-2'), 146.2 (C-3'), 145.4 (C-4'), 116.3 (C-5'), 121.8 (C-6'), 37.9 (C-7'), 74.7 (C-8'), 172.2 (C-9'), 52.7 (OCH_3). These physical and spectral data coincided with those described in references.[5-6]

R-(+)-rosmarinic acid (3). Brown amorphous solid, $[\alpha]_D^{25}$ + 86° (*c* 0.4, MeOH); IR ν_{max}^{KBr} cm^{-1}: 3600 and 1715 (COOH), 1680 and 1350-1100 (C=C-C(=O)-O), 1600 and 1500 (aromatic ring); EIMS *m/z* (rel. int.): 198 ($(OH)_2C_6H_3CH_2$-CH(COOH)-O, 18), 180 ($(OH)_2C_6H_3C$=C-C(=O)-O, 15); ^1H-NMR (CD_3OD): δ 2.80-3.25 (2H, *m*), 5.00 (1H, *dd*, *J*=9.5, 3.5 Hz, H-8'), 6.17 (1H, *d*, *J*=15.9 Hz, H-8'), 6.53 (1H, *dd*, *J*=7.8, 2.2 Hz, H-6), 6.59 (1H, *d*, *J*=7.8 Hz, H-5), 6.67 (1H, *d*, *J*=7.8 Hz, H-5'), 6.68 (1H, *d*, *J*=2.2 Hz, H-2), 6.81 (1H, *dd*, *J*=7.8, 2.2 Hz, H-6'), 6.93 (1H, *d*, *J*=2.2 Hz, H-2'), 7.41 (1H, *d*, *J*=15.9 Hz, H-7); ^{13}C-NMR (CD_3OD): δ 38.63 (C-7), 77.35 (C-8), 115.16 (C-2'), 115.43 (C-8'), 116.22 (C-5), 116.47 (C-2), 117.52 (C-5'), 121.76 (C-6), 122.91 (C-6'), 127.89 (C-1'), 130.94 (C-1), 144.74 (C-3), 145.85 (C-3'), 146.63 (C-4), 149.30 (C-4'), 149.77 (C-7'), 169.07 (C-9'), 177.16 (C-9). These physical and spectral data coincided with those described in references.[3-4]

Compound 3 (10 mg) was dissolved in MeOH (2 ml). To this solution was added pyridine (0.5 ml) and acetic anhydride (0.5 ml), and then stirred for 1h at 80°C. The reaction mixture was partitioned with $CHCl_3$ and 0.2M sulfic acid. The $CHCl_3$ layer was subjected to column chromatography on silica gel with solvent system of EtOAc-MeOH-H_2O (8:2:0.1) to give methyl rosmarinate tetracetate (3a, 15 mg). White amorphous solid; EIMS *m/z* (rel. int.): 452 (M^+, 20); ^1H-NMR ($CDCl_3$): δ 2.27 (3H, *s*, OAc), 2.28 (3H, *s*, OAc), 2.30 (3H, *s*, OAc), 2.31 (3H, *s*, OAc), 3.19 (1H, *dd*, *J*=14.5, 8.0 Hz), 3.23 (1H, *dd*, *J*=14.5, 5.0 Hz), 3.74 (3H, *s*, COOMe), 5.34 (1H, *dd*, *J*=8.0, 5.0 Hz), 6.41 (1H, *d*, *J*= 15.9 Hz), 7.11-7.15 (3H, *m*), 7.23 (1H, *d*, *J*=8.6 Hz), 7.38 (1H, *d*, *J*=1.9 Hz), 7.42 (1H, *dd*, *J*

=8.6, 1.9 Hz), 7.64 (1H, *d*, *J*=15.9 Hz); ^{13}C-NMR ($CDCl_3$): δ 20.66 (4×O=C$\underline{C}H_3$), 36.73, 52.51, 72.69, 118.09, 122.98, 123.41, 123.92, 124.47, 126.63, 127.37, 133.06, 134.61, 141.11, 141.92, 142.42, 143.70, 144.26, 165.66, 167.94, 168.03, 168.14, 168.25, 169.84.

3',4'-dihydroxycinnamic acid (*caffeic acid*, 4). Brown amorphous solid; IR ν_{max}^{KBr} cm^{-1}: 3420 (OH), 1650 (C=O), 1620 (aromatic ring); EIMS *m/z* (rel. int.): 180 (M^+, 13), 136 ([M-COO]$^+$, 77); ^{1}H-NMR (CD_3OD): δ6.21 (1H, *d*, *J*=15.7 Hz, H-2), 6.77 (1H, *d*, *J*=8.1 Hz, H-5'), 6.93 (1H, *dd*, *J*=8.1, 1.9 Hz, H-6'), 7.02 (1H, *dd*, *J*=1.9 Hz, H-2'), 7.52 (1H, *d*, *J*=15.7 Hz, H-3); ^{13}C-NMR (CD_3OD): δ115.05 (C-2'), 115.48 (C-2), 116.47 (C-5'), 122.85 (C-6'), 127.77 (C-1'), 146.82 (C-3'), 147.03 (C-4'), 149.45 (C-3), 177.16 (C-1). These physical and spectral data coincided with those of authentic sample.

3-(3',4'-dihydroxyphenyl)lactic acid (5). Brown amorphous solid; IR ν_{max}^{KBr} cm^{-1}: 3420 and 1720 (COOH), 1600 and 1500 (aromatic ring); ^{1}H-NMR (D_2O): δ2.73-3.00 (2H, *m*), 4.20 (1H, *dd*, *J*=8.0, 4.5 Hz, H-2), 6.73 (1H, *dd*, *J*=8.0, 2.0 Hz, H-6'), 6.83 (1H, *d*, *J*=2.0 Hz, H-2'), 6.86 (1H, *d*, *J*=8.0 Hz, H-5'); ^{13}C-NMR (D_2O): δ40.36 (C-3), 75.96 (C-2), 119.02 (C-5'), 120.06 (C-2'), 124.69 (C-6'), 133.54 (C-1'), 145.29 (C-4'), 146.55 (C-3'), 181.92 (C-1). These physical and spectral data coincided with those described in references.[4,7]

Scavenging effect on DPPH-radicals. An EtOH solution (0.05 ml) of each test compound at various concentrations was added to a 2.95 ml EtOH solution containing DPPH (1,1-diphenyl-2-picrylhydrazyl, 3.33 × 10^{-5} M) and prepared to pH 7.5 with a buffer solution. The reaction mixture was shaken vigorously and then kept at room temperature for 30 min in air. The absorbance of the remaining DPPH was measured in 1 cm cuvettes with UV spectrophotometer at 517 nm, and the radical-scavenging activity of each compound is expressed by the ratio of decrease in the absorbance of DPPH (%) relative to the absorbance (100%) of a DPPH solution in the absence of the sample, in the following equation.

$$\text{Activity} = \frac{\text{Sample Abs.} - \text{Sample blank Abs.}}{\text{Reagent blank Abs.}} \times 100$$

Activity is less than 80: scavenging effect is judged to be active.
Activity is more than 80: scavenging effect is judged to be inactive.

Acknowledgements---The authors thank Professor Tatsuo Higa, Faculty of Science, University of the Ryukyus, for the use of ^{1}H (270 MHz) and ^{13}C NMR (67.5 MHz) spectrometer and Professor Yosei Uehara, Faculty of Science, University of the Ryukyus, for his helpful advice and useful comments on the manuscript.

References

1) K. Ogihara, Y. Miyagi, M. Higa and S. Yogi, *Phytochemistry*, 44, 545-547 (1997).
2) K. Ogihara, R. Iraha, M. Higa and S. Yogi, *Bull. Coll. Sci. Univ. Ryukyus*, No.64, 53-59 (1997).

3) S. M. Lee, J. S. Lai and Y. H. Kuo, *J. Chi. Chem. Soc.*, **40** (1), 87-91 (1993).
4) Y. Nakamura, Y. Ohto. A. Murakami, and H. Ohigashi, *J. Agric. Food Chem.*, **1998** (46), 4545-4550.
5) T. Isobe, Y. Noda, T. Kubota, *Nippon Kagaku Kaishi*, **1985** (7), 1513-1515.
6) H. Kohoda, O. Takeda, S. Tanaka, K. Yamasaki, A. Yamashita, T. Kurokawa, S. Ishibashi, *Chem. Pharm. Bull.*, **37** (5), 1287-1290 (1989).
7) C. J. Kelly, R. C. Harruff and M. Carmack, *J. Org. Chem.*, **41** (3), 449-455 (1976).
8) M. Wang, J. Li, M. Rangarajan, Y. Shao, E. J. LaVoie, T. Huang and C. Ho, *J. Agric. Food Chem.*, **1998** (46), 4869-4873.