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

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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. 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 physiological activities for a water extract from the twigs of *M. argentea* and found that the extract possessed the DPPH-radical scavenging effect. Therefore, we examined 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 examined. Table 1 shows the DPPH-radical scavenging activity of these water extracts. Most strong scavenging 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

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

<table>
<thead>
<tr>
<th>parts</th>
<th>conditions</th>
<th>activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>leaves</td>
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<td>26.0</td>
</tr>
<tr>
<td></td>
<td>dried</td>
<td>45.8</td>
</tr>
<tr>
<td>twigs</td>
<td>fresh</td>
<td>33.3</td>
</tr>
<tr>
<td></td>
<td>dried</td>
<td>14.3</td>
</tr>
</tbody>
</table>

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.

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.*

Compound 3 was obtained as brown amorphous solid, \([ \alpha ]_\text{B} + 86^\circ (c 0.4, \text{MeOH})\). The IR spectrum of 3 showed bands due to a carboxyl group at 3600 and 1715 cm\(^{-1}\), characteristic bands due to an \(\alpha, \beta\)-unsaturated ester at 1680 and 1350-1100 cm\(^{-1}\), and bands due to aromatic rings at 1600 and 1500 cm\(^{-1}\). The \(^1\text{H}\) and \(^{13}\text{C}\) NMR spectra of 3 showed a carbonyl carbon signal at \(\delta_c 177.16\) due to a ester group, signals at \(\delta_h 7.41\) (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 \(\delta_h 6.67\) (1H, d, \(J=7.8\) Hz), 6.81 (1H, dd, \(J=7.8, 2.2\) Hz), and 6.93 (1H, d, \(J=2.2\)Hz) due to aromatic protons, and signals at \(\delta_c 145.85\) 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 \(^1\text{H}\) and \(^{13}\text{C}\) NMR spectra of 3 showed another carbonyl carbon signal at \(\delta_c 169.07\) due to a carboxyl group, another AMX pattern signals at \(\delta_h 6.53\) (1H, dd, \(J=7.8, 2.2\) Hz), 6.59 (1H, d, \(J=7.8\) Hz), and 6.68 (1H, d, \(J=2.2\)Hz) due to aromatic protons, signals at \(\delta_c 146.63\) and 144.74 due to aromatic carbons oxygenated, a ABX pattern signals at \(\delta_h 5.00\) (1H, dd, \(J=9.5, 3.5\) Hz), 3.25-2.80 (m) due to an 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 observation 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 \(^1\text{H}\) NMR spectrum of 3a showed four singlet at \(\delta_h 2.31, 2.30, 2.28,\) and 2.27 in a lower field due to four acetyl groups and a singlet at \(\delta_h 3.74\) due to methyl ester besides the signals of 3, which indicate that a carboxyl 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.*\(^8-10\) Thus, 3 was identified as \(R\)-(+) rosmarinic acid (3).

Compound 2 was obtained as brown amorphous solid, \([ \alpha ]_\text{B} + 36^\circ (c 0.1, \text{MeOH})\). The IR spectrum of 2 showed bands due to hydroxy groups at 3420 cm\(^{-1}\), characteristic bands due to a normal and an \(\alpha, \beta\)-unsaturated esters at 1720, 1650 and 1350-1100 cm\(^{-1}\), and bands due to aromatic rings at 1600 and 1500 cm\(^{-1}\). The \(^1\text{H}\) and \(^{13}\text{C}\) NMR spectra of 2 coincided with those of 3, except for a singlet due to methoxy group at \(\delta_h 3.67\). The EIMS of 2 showed a molecular ion peak at \(m/z\) 374, which is 14 mass units more than 3. These observations 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.\textsuperscript{4,6} Thus, 2 was identified as \textit{R-(+)-}rosmarinic acid methyl ester (2).

Compound 4 was obtained as brown amorphous solid. The IR spectrum of 3 showed bands due to an \(\alpha, \beta\)-unsaturated carboxyl group at 3420 and 1650 cm\(^{-1}\) and bands due to aromatic ring at 1620 cm\(^{-1}\). The \(^1\text{H}\) and \(^13\text{C}\) NMR spectra of 4 showed a carbonyl carbon signal at \(\delta\text{c} 177.16\) due to a carboxyl group, signals at \(\delta\text{H} 6.21\) (1H, \(d, J=15.7\) Hz) and 7.52 (1H, \(d, J=15.7\) Hz) due to each \textit{trans}-configuration olefinic protons conjugated with aromatic ring, AMX pattern signals at \(\delta\text{H} 6.77\) (1H, \(d, J=8.1\) Hz), 6.93 (1H, \(dd, J=8.1, 1.9\) Hz), and 7.02 (1H, \(d, J=1.9\) Hz) due to aromatic protons, and signals at \(\delta\text{c} 146.82\) 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 \(^1\text{H}\) and \(^13\text{C}\) NMR spectra of 5 showed a carbonyl carbon signal at \(\delta\text{c} 181.92\) due to a carboxyl group, AMX pattern signals at \(\delta\text{H} 6.73\) (1H, \(dd, J=8.0, 2.0\) Hz), 6.83 (1H, \(d, J=2.0\) Hz), and 6.86 (1H, \(d, J=8.0\) Hz) due to aromatic protons, signals at \(\delta\text{c} 145.29\) and 146.55 due to aromatic carbons oxygenated, and ABX pattern signals at \(\delta\text{H} 4.20\) (1H, \(dd, J=8.0, 4.5\) Hz), 3.00-2.73 (\textit{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.\textsuperscript{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 examined. 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\textsubscript{2}\textsuperscript{−}) and 2,2-azinobis (3-ethylbenzothiozoline-6-sulfonate) cation (ABTS\textsuperscript{·+}) radicals.\textsuperscript{4,6} Thus, antioxidantive effect is newly added to pharmacological ones of \textit{M. argentea} as a medicinal plant.

**Experimental**

Analytical TLC was carried out on Merck 60 \(F_25\) silica gel plate (thickness: 0.25 mm). \(^1\text{H}\) (90 and 270 MHz) and \(^13\text{C}\) NMR (25 and 67.5 MHz) spectra were determined in CDCl\(_3\) for 1, in CD\(_3\)OD for 2-4 with TMS as int. standard, and in D\(_2\)O for 5 with TMSP as int. standard. EIMS were obtained on a Hitachi M-2500 double focusing mass spectrometer at
DPPH-radical scavenging constituents from the twigs of *M. argentea*

![Graph](image_url)  
**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-filtration 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 CHCl₃-MeOH-H₂O (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'-Dihydroxycinamic acid methyl ester (methyl caffeate: 1). White needles, mp 287.5-288° (EtOH); IR ν max cm⁻¹: 3400 and 1670 (COOH), 3020 (=CH), 1610 and 1510 (aromatic ring); ¹H (270 MHz): δ 3.78 (3H, s, COOCH₃), 4.74 (2H, brs, 2×OH), 6.23 (1H, d, J=15.8 Hz, CH=CH-COOCH₃), 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; [α]D +36° (c 0.1, MeOH); IR ν max 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 (CD₃OD, 270 MHz): δ 7.04 (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, COOCH₃), 3.02 (2H, m, H-7'); ¹³C-NMR (CD₃OD, 270 MHz): 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-11), 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'), 52.7 (OCH₃). These physical and spectral data coincided with those described in references.8,9

R-(+)-rosmarinic acid (3). Brown amorphous solid, [α]D +86° (c 0.4, MeOH); IR ν max 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)₂C₆H₅CH-C(=O)-O), 180 ((OH)₂C₆H₄=CH(=O)-O), 15; ¹H-NMR (CDCl₃): 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); ¹³C-NMR (CDCl₃): 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.8,9

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 CHCl₃ and 0.2M sulfuric acid. The CHCl₃ layer was subjected to column chromatography on silica gel with solvent system of EtOAc-MeOH-H₂O (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 (CDCl₃): 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
DPPH-radical scavenging constituents from the twigs of *M. argentea*

$\delta = 8.6, 1.9$ Hz; 7.64 (1H, d, $J=15.9$ Hz); $^{13}$C-NMR (CDCl$_3$): $\delta$ 20.66 (4×O=CCH$_3$), 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 $\nu$$_{max}$ cm$^{-1}$: 3420 (OH), 1650 (C=O), 1620 (aromatic ring); ElMS $m$/z (rel. int.): 180 (M+, 13), 136 ([M-COO$^+$]+, 77); $^1$H-NMR (CD$_3$OD): $\delta$ 6.21 (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$_3$OD): $\delta$ 115.05 (C-2'), 115.48 (C-2), 116.47 (C-6'), 122.85 (C-5'), 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 $\nu$$_{max}$ cm$^{-1}$: 3420 and 1720 (COOH), 1600 and 1500 (aromatic ring); $^1$H-NMR (D$_2$O): $\delta$ 2.73-3.00 (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'), 6.96 (1H, d, $J=8.0$ Hz, H-5'); $^{13}$C-NMR (D$_2$O): $\delta$ 40.36 (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.$^{17}$

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 X 10$^{-4}$ 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.

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References