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# Antioxidant Compounds from *Distylium racemosum* Leaves*1

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## ABSTRACT

The leaves of *D. racemosum* showed strong DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging activity and the order of the radical scavenging activity against DPPH radical is ethyl acetate (EtOAc) fraction>crude extracts>residue fraction>hexane fraction>ether fraction, under the experimental conditions. Since EtOAc fraction has highest antioxidative activity among these fractions, the isolation was performed from the EtOAc fraction of the leaves of *D. racemosum* and four phenolic compounds were isolated and identified as follows: methyl gallate, kaempferol, quercetin and quercitrin. The free radical scavenging activities of these compounds were 79.9%, 93.1%, 93.6% and 66.7% at 10 μg/ml, respectively. The $IC_{50}$ of compound 1, compound 2, compound 3 and compound 4 were 6.1, 4.1, 3.6 and 6.5 μg/ml, respectively. These compounds have higher antioxidative activity compared with reference compounds, ascorbic acid ($IC_{50}$ = 9.6 μg/ml).

*Keywords* : *Distylium racemosum*, DPPH free radical scavenging effect, methyl gallate, kaempferol, quercetin, quercitrin

## 1. INTRODUCTION

Active oxygen is the form of superoxide radical ($O_2^-$), hydroxyl radical(HO·), hydrogen peroxide ($H_2O_2$) and singlet oxygen ($^1O_2$), is a product of normal metabolism and attacks biological molecules. As a results of this reaction, this active oxygen can injury to cell or tissue (Quiroga, *et al.*, 2001). Ageing may also be the result of the deleterious free-radical reactions which occur throughout cells and tissues (Maxwell, 1995). For this reason, great concern is focused on natural products including wood extractives as natural antioxidants. Because of the carcinogenic properties of some synthetic antioxidants such as butylated hydroxyltoluene (BHT) and butylated hydroxylanisole (BHA), recently natural antioxidants from plants have received much attention (Cheung, *et al.*, 2003).

In search for antioxidants from several Korean plants, the ethanolic extracts of *Distylium racemosum* was found to exhibit significant

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antioxidative activity, according to DPPH methods. *D. racemosum* (Hamamelidaceae) is small evergreen shrub and is widely distributed in south part of Korea and Japan (Lee, 1979). *D. racemosum* has not been investigated in detail on phytochemical analysis. Recently, only several researches have been reported and showed that proanthocyanidin from the leaves of *D. racemosum* has inhibitory activities at α-amylase and α-glucosidase (Lee, *et al.*, 2002). From the EtOAc soluble fraction, the active compounds were isolated and identified. In present paper, we report the isolation, structure elucidation and antioxidative activities of four compounds which were isolated from the leaves of *D. racemosum*.

# 2. MATERIALS and METHODS

## 2.1. Extractives

### 2.1.1. Materials

The leaves of *D. racemosum* were collected at Hyodon stream, sanghyo-dong, Seogwipo, Jeju-Province, Korea during September, 2002 and dried at room temperature.

### 2.1.2. Extraction, Fractionation and Isolation

Air dried leaves of *D. racemosum* were powdered and extracted twice with 50% acetone and then evaporated to give the crude extracts. The crude extracts was successively partitioned with organic solvents, such as *n*-hexane, ether and ethyl acetate (EtOAc).

The EtOAc soluble fraction was subjected to column chromatography on Sephadex LH-20 eluted with MeOH-EtOH (1:1, v/v) to yield 3 sets of fraction (DRE 1～DRE 3). Fraction DRE 3 was rechromatographed on silica gel column chromatography with $CHCl_3$-MeOH (15:1, v/v) to give 3 subfractions (DRE 3-1～DRE 3-3). Among 3 fractions of DRE 3, second fraction (DRE 3-2) was compound 1 (25 mg). Fraction DRE 2 was further subjected to repeated column chromatography on silica gel eluted with $CH_2Cl_2$-MeOH (50:1～20:1, v/v) to give 4 sets of fractions (DRE 2-1～DRE 2-4). The first fraction (DER 2-1) was compound 2. Among 4 fractions, DRE 2-2 was purified by column chromatography on Sephadex LH-20 and eluted with a solvent system of MeOH to give compound 3. The DRE 1 fraction was also chromatographed with a silica gel column (EtOAc-MeOH, 100:1, v/v) to give 5 subfractions (DER 1-1～DRE 1-5). The second fraction (DRE 1-2) was further subjected to column chromatography over on Sephadex LH-20 and eluted with a solvent system of MeOH to give compound 4.

### 2.1.3. Instrumental Analysis

For the determination of molecular weights of the isolated compounds, EI-MS was performed at 70 eV ionization energy by direct inlet probe method, using JEOL JMS-600W mass spectrometer. NMR spectra were obtained using a Varian UI 500 spectrometer at the operating frequency of 500 MHz ($^1$H) and 125 MHz ($^{13}$C) at Korea Basic Science Institute in Seoul.

### 2.1.4. Spectral Data of Compounds

#### 2.1.4.1. Compound 1

EI-MS *m/z* : 184 ($M^+$, molecular ion) 153, 125, 79, 51. $^1$H-NMR (500 MHz, $CD_3OD$) : δ 3.82 (3H, *s*, 7-$OCH_3$), 7.06 (2H, *s*, H-2, 6). $^{13}$C-NMR (125 MHz, $CD_3OD$) : δ 51.2 (7-$OCH_3$), 108.9 (C-2, 6), 120.3 (C-1), 138.6 (C-4), 145.3 (C-3, 5), 167.8 (C-7).

#### 2.1.4.2. Compound 2

EI-MS *m/z* : 286 ($M^+$ , molecular ion), 258, 121. $^1$H-NMR (500 MHz, $CD_3OD$) : δ 6.20 (1H, *d*, $J$ = 2.2 Hz, H-6), 6.44 (1H, *d*, $J$ = 2.2 Hz, H-8), 6.94 (2H, *dd*, $J$ = 2.2, 6.8 Hz, H-3',

5'), 8.13 (2H, *dd*, $J$ = 2.2, 6.8 Hz, H-2', 6'). $^{13}$C-NMR (125 MHz, $CD_3OD$) : δ 93.6 (C-8), 98.2 (C-6) 103.4 (C-10), 115.4 (C-3', 5'), 122.6 (C-1'), 129.7 (C-2', 6'), 136.1 (C-3), 146.6 (C-2), 157.1 (C-9), 159.6 (C-4'), 161.4 (C-5), 164.5 (C-7), 176.1 (C-4).

2.1.4.3. Compound 3

EI-MS *m/z* : 302 ($M^+$ , molecular ion), 275, 165, 153, 137, 123. $^1$H-NMR (500 MHz, $CD_3OD$) : δ 6.19 (1H, *d*, $J$ = 1.8 Hz, H-6), 6.41 (1H, *d*, $J$ = 1.8 Hz, H-8), 6.91 (1H, *d*, $J$ = 8.5 Hz, H-5'), 7.65 (1H, *dd*, $J$ = 2.0, 8.5 Hz, H-6'), 7.76 (1H, *d*, $J$ = 2.0 Hz, H-2'). $^{13}$C-NMR (125 MHz, $CD_3OD$) : δ 94.3 (C-8), 98.2 (C-6), 103.4 (C-10), 114.9 (C-2'), 115.2 (C-5'), 120.6 (C-6'), 123.0 (C-1'), 136.2 (C-3), 145.2 (C-3'), 146.7 (C-2), 147.7 (C-4'), 157.1 (C-9), 161.4 (C-5), 164.5 (C-7), 176.1 (C-4).

2.1.4.4. Compound 4

EI-MS *m/z* : 302 ($M^+$-rhamnose, molecular ion), 275, 165, 153, 137, 123. $^1$H-NMR (500 MHz, $CD_3OD$) : δ 0.95 (3H, *d*, $J$ = 6.0 Hz, H-6"), 3.33 (1H, *m*, H-4"), 3.45 (1H, *m*, H-5"), 3.74 (1H, *dd*, $J$ = 3.2, 9.6 Hz, H-3"), 4.23 (1H, *dd*, $J$ = 1.8, 3.2 Hz, H-2"), 5.96 (1H, *d*, $J$ = 1.8 Hz, H-1"), 6.20 (1H, *d*, $J$ = 2.3 Hz, H-6), 6.37 (1H, *d*, $J$ = 2.3 Hz, H-8), 6.93 (1H, *d*, $J$ = 8.3 Hz, H-5'), 7.30 (1H, *dd*, $J$ = 1.8, 8.3 Hz, H-6') 7.35 (1H, *d*, $J$ = 1.8 Hz, H-2'). $^{13}$C-NMR (125 MHz, $CD_3OD$) : δ 16.6 (C-6"), 70.7 (C-5"), 70.9 (C-2"), 71.0 (C-3"), 72.1 (C-4"), 93.5 (C-8), 98.6 (C-6), 102.9 (C-1"), 104.7 (C-10) 115.1 (C-2'), 115.7 (C-5'), 121.6 (C-1'), 121.8 (C-6'), 135.0 (C-3), 145.1 (C-3'), 148.5 (C-4'), 157.2 (C-2), 158.0 (C-5), 161.9 (C-9), 164.5 (C-7), 178.3 (C-4).

## 2.2. Antioxidative Activity

MeOH solutions (4 ml) of samples at various concentrations were added to a solution of DPPH in MeOH ($4.5\times10^{-4}$ M, 1 ml) and the reaction mixture were shaken vigorously. After storing mixtures for 30 min at room temperature, the remaining amounts of DPPH were determined by colorimetry (8452A Diode Array Spectrophotometer, Hewlett Packard Co.) at 520 nm (Blois, M. S, 1958). The mixture of 4 ml MeOH with a solution of 1 ml DPPH was used as control. The mean values were obtained from triplicate experiments. Free radical scavenging activity was calculated as follows :

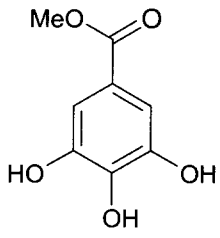
Free radical scavenging activity (%) =

$$\left(1-\frac{\text{Absorbance of sample at 520 nm}}{\text{Absorbance of control at 520 nm}}\right)\times 100$$

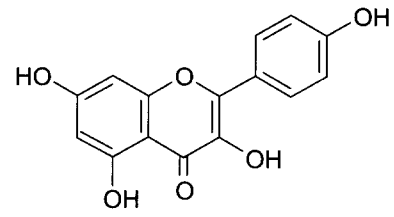
# 3. RESULTS and DISCUSSION

## 3.1. Identification of the Compounds

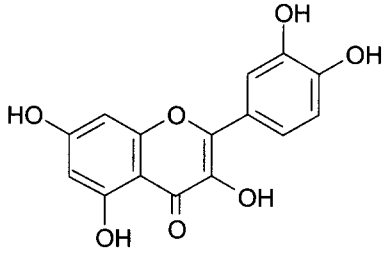
The compound 1 was obtained as a white amorphous powder. The EI-MS presented a signal at *m/z* 184, corresponding to molecular formula $C_8H_8O_5$. The $^1$H-NMR spectrum indicated the presence of a methoxyl group at δ 3.82 (3H, *s*, OMe). In $^1$H-NMR spectrum, one set of singlet at δ 7.06 (2H, *s*) that were assigned to H-2, 6. A $^{13}$C-NMR signal at δ 167.8 was assigned to carboxyl carbon (C=O, C-7). In $^{13}$C-NMR spectrum C-1 at δ 167.8, C-4 at δ 138.6 and C-2, 6 at δ 108.9, respectively, were assigned. The assigned carbon chemical shifts signals were compared with the literature values (Ham *et al.*, 2001; Larjis and Khan, 1994). Consequently the structure of **compound 1** was concluded to be methyl gallate (Fig. 1). The $^1$H-NMR spectrum of compound 2 exhibited two set of doublets at δ 6.94 (2H, *dd*, $J$ = 2.2, 6.8 Hz) and 8.13 (2H, *dd*, $J$ = 2.2, 6.8 Hz) that were assigned to H-3', H-5' and H-2', H-6',



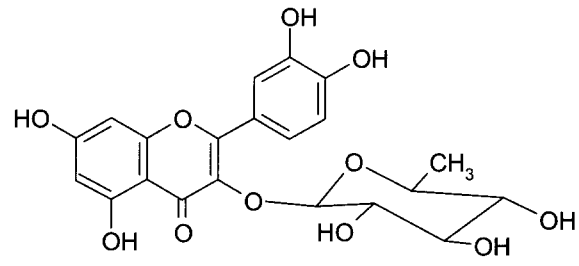

**methyl gallate ( 1 )**




**kaempferol (2 )**




**quercetin (3)**




**quercitrin (4)**

Fig. 1. Structures of compounds isolated from *D. racemosum* leaves.

respectively. In $^{13}$C-NMR spectrum, C-8 at δ 93.6, C-6 at δ 98.2, C-10 at δ 103.4, C-1' at δ 122.6, C-3 at δ 136.1, C-9 at δ 157.1, C-4' at δ 159.6, C-5 at δ 161.4, C-7 at δ 164.5, C-4 at δ 176.1 and two sets of symmetrical carbons, C-2', C-6' and C-3', C-5' at δ 129.7 and δ 115.4, respectively, were assigned. The identity of **compound 2** was thus confirmed to be 3,4',5,7-tetrahydroxyflavone, kaempferol (Park, 2002) (Fig. 1). The compound 3 was obtained as a yellow powder. The EI-MS presented a signal at *m/z* 302, corresponding to molecular formula $C_{15}H_{10}O_7$. The $^1$H-NMR spectrum of compound 3 and 4 showed two meta-coupled doublets assigned to H-8 and H-6 of ring A in the flavonoid skeleton. The $^1$H-NMR spectrum of these compounds also exhibited, one *meta*-coupled aromatic proton, one *ortho, meta*-coupled aromatic proton and one *ortho*-coupled aromatic proton attributable to H-2', H-6' and H-5' of B ring, respectively, and the $^1$H-NMR spectra of compound 4 showed one anomeric proton signal at δ 5.96 ($J$ = 1.8 Hz). These data indicated that compound 3 was quercetin and compound 4 was quercetin glycoside. The sugar moiety of compound 4 was determined to be α-L-rhamnopyranose by the $J$ values of the anomeric proton signal and the $^{13}$C-NMR data. Consequently the structure of **compound 3** was concluded to be 3, 3', 4', 5, 7-pentahydroxyflavone, quercetin (Shen *et al.*, 1993; Agrawal, 1989) (Fig. 1) and the structure of **compound 4** was concluded to be quercitrin

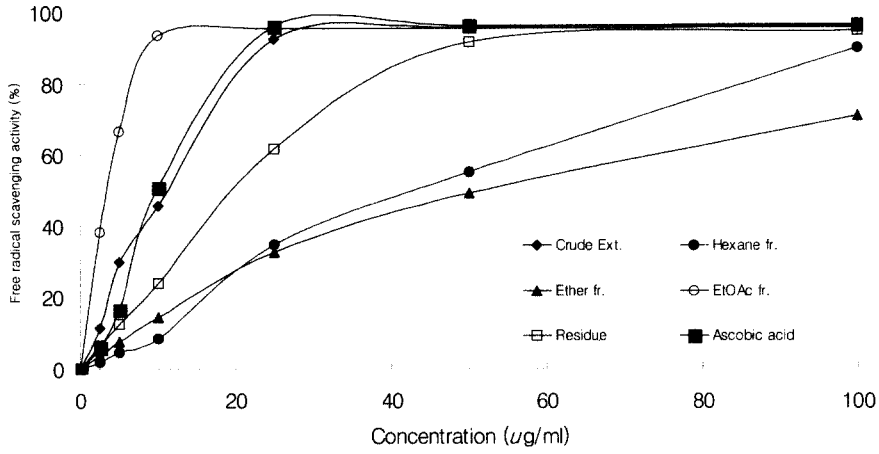



Fig. 2 Free radical scavenging activities of extracts and its fractions from *D. racemosum* leaves.

Table 1. Free radical scavenging activities and $IC_{50}$ values of compounds from *D. racemosum* leaves

| Compounds | Free radical scavenging activity (%) | | IC50 (μg/ml)[1)] |
|---|---|---|---|
| | 5 μg/ml | 10 μg/ml | |
| methyl gallate (1) | 40.5 | 79.8 | 6.1 |
| kaempferol (2) | 57.8 | 93.2 | 4.1 |
| quercetin (3) | 68.2 | 93.6 | 3.6 |
| quercitrin (4) | 43.5 | 66.7 | 6.5 |
| gallic acid | 90.8 | 94.2 | 2.0 |
| ascorbic acid | 16.7 | 50.8 | 9.6 |

[1)] The values indicate 50% decrease of DPPH radical and are the means of triplicate data.

(Park, *et al.*, 1996 : Jang, *et al.*, 2002) (Fig. 1). These four compounds are the first report from *D. racemosum*.

## 3.2. Antioxidative Activity

The free radical scavenging activity of crude extracts and its solvent partitioned fractions from *D. racemosum* leaves was shown in Fig. 2. The order of the radical scavenging activity against DPPH radical is EtOAc fraction>crude extracts>residue>hexane fraction>ether fraction, under the experimental conditions. The $IC_{50}$ values of crude extracts, hexane fraction, ether fraction, EtOAc fraction and residue were 11.2, 43.5, 51.7, 4.3 and 19.5 μg/ml, respectively.

The free radical scavenging activity of four compounds obtained from *D. racemosum* leaves was also shown in Table 1. Among four isolated compounds, compound 3 (quercetin) exhibited highest scavenging activity value on DPPH with $IC_{50}$ value 3.6 μg/ml. These results suggest that the radical scavenging effect of EtOAc fraction was partially attributable to compound 1, 2, 3 and 4. From the results of Table 1, the radical scavenging activity of gallic acid which has hydroxyl groups on C-7 was higher ($IC_{50}$=2.0 μg/ml) than that of methyl gallate which has methoxyl group

on C-7. According to the reports of Cooper- Drive *et al.* (1998), the antioxidant activity of flavonoids is based on the polyphenolic structure and the presence of both 4-carbonyl and 5- or 3-hydroxyl group.

Nowadays, natural antioxidants are receiving much attention and flavonoids are regarded as efficient antioxidants by scavenging oxygen radicals. Therefore, this study indicates that these compounds isolated from *D. racemosum* may be useful for the treatment of oxidative damage and have potential possibility to be natural antioxidants.

## 4. CONCLUSIONS

From EtOAc fraction of the leaves of *D. racemosum*, four compounds were isolated by column chromatography using Sephadex LH-20 and/or silica gel and identified using EI-MS and $^{1}H$- and $^{13}C$-NMR spectroscopy as follows: methyl gallate, kaempferol, quercetin and quercitrin. This is the first report of the isolation and identification of these compounds from *D. racemosum*. The free radical scavenging activities of four compounds obtained from *D. racemosum* leaves were 79.9%, 93.1% 93.6% and 66.7% at 10 $\mu$g/ml, respectively. These compounds have higher antioxidative activity compared with reference compounds, ascorbic acid (50.8%).

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