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Antioxidative Constituents of the Aerial Parts of *Galium spurium*

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Abstract

As part of an ongoing search for natural plants with antioxidant compounds by measuring the radical scavenging effect on 1,1-diphenyl- 2-picrylhydrazyl (DPPH), a total extract of the twigs of *Galium spurium* L. (Rubiaceae) was found to show potent antioxidant activity. Subsequent activity-guided fractionation of the methanolic extract led to the isolation of nine compounds, asperulosidic acid methyl ester (1), asperuloside (2), caffeic acid (3), kaempferol-3-*O*-L-rhamnopyranoside (4), quercetin-3-*O*-[α-L-rhamnopyranosyl(1→6)-β-D-glucopyranoside] (5), isorhamnetin-3-*O*-glucopyranoside (6), quercetin-3-*O*-α-L-rhamnopyranoside (7), kaempferol-3-*O*-[α-L-rhamnopyranosyl(1→6)-β-D-glucopyranoside] (8), and quercetin (9). Their structures were elucidated by spectroscopic studies. Compounds 1, 3-8 were isolated for the first time from this plant. Among them, compounds 3 and 9 showed the significant radical scavenging effects on DPPH, and compounds 3 and 7 showed the potent riboflavin originated superoxide quenching activities.

Key Words: *Galium spurium* L., Rubiaceae, Antioxidant activity, DPPH radical, Superoxide quenching activity

INTRODUCTION

Reactive oxygen species (ROS), in the form of superoxide anion(O_2^-), hydroxyl radical (·OH) and hydrogen peroxide (H_2O_2), are generated in living organisms through many pathways (Chung *et al.*, 2005). An imbalance between production of ROS and their elimination in the organisms cause oxidative stress (Monika *et al.*, 2010). Oxidative stress has been implicated as a possible factor in the etiology of several human diseases, including cancer, cardiovascular disease, Alzheimer's disease and aging (Suganya *et al.*, 2007). Antioxidants are the important defense factors against oxidative stress caused by ROS. However, the use of synthetic antioxidant components, such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), has been limited because of their possible toxic and carcinogenic effects (Branen, 1975). Thus, there is a growing interest in finding natural herbal plants with low in toxicity and high in radical scavenging activity (Branen, 1975). In the course of screening for antioxidant from Korean natural plants by measuring the radical scavenging effect on 1,1-diphenyl-2-picrylhydrazyl (DPPH), a total extract of the *Galium spurium* L. (Rubiaceae) was found to show a potent antioxidant activity. *G. spurium* is annual or biannual plant. Phytochemical studies on the genus have shown it to be chemically diverse, yielding among others, triterpenoid saponins, iridoid glucosides (Salvatore *et al.*, 2000). Some iridoids (Deliorman *et al.*, 2001), anthraquinones (Koyama *et al.*, 1993) and flavonoids (Cai *et al.*, 2009) were analyzed from *G. spurium*. Its aerial parts have been used for the treatment of the bones and sinews pain and hematuria. Crude extract of *G. spurium* was also used as a anticancer agent in folk medicine. It has been reported to have immunostimulant and anti-tumor activity (Yoon *et al.*, 2005). However, information concerning the antioxidant activity of *G. spurium* is unavailable. This paper deals with the isolation and structural characterization of these compounds and their scavenging activity of the stable DPPH free radical and superoxide quenching activities.

MATERIALS AND METHODS

General experimental procedures

NMR spectra were determined on a JEOL JMN-EX 400 spectrometer. Sephadex LH-20 was used for column chromatography (25-100 μm; Fluka, Buchs, Switzeland). Prep-HPLC was carried out on a Jaigel GS310 column (Tokyo, Japan). TLC was carried out on Merck (Darmstadt, Germany) precoated silica gel F_{254} plates, and silica gel for column chromatography was Kiesel gel 60 (230-400 mesh, Merck). Spots were detected under UV and by spraying with 10% H_2SO_4 in

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ethanol followed by heating at 100-120°C for 3 min. All other chemicals and solvents were of analytical grade and used without further purification. Ascorbic acid, butylated hydroxyanisole (BHA) and 1,1-diphenyl-2-picrylhydrazyl (DPPH) were obtained from Sigma Chemical Co. (St. Louis, USA).

Plant materials

The aerial parts of *G. spurium* were collected and air-dried in August 2010 at Wanju, Jeonbuk, Korea. A voucher specimen was deposited in the herbarium of College of Pharmacy, Woosuk University (WSU-10-012).

Extraction and isolation

The shade dried plant material (1.8 kg) was extracted three times with MeOH at 50°C and filtered. The extracts were combined and evaporated *in vacuo* at 50°C. The resultant methanolic extract (306 g) was subjected to successive solvent partitioning to give *n*-hexane (50.4 g), methylene chloride (1.9 g), ethyl acetate (3.0 g), *n*-BuOH (30.0 g) and H_2O soluble fractions. Each fraction was tested for the radical scavenging effect on 1,1-diphenyl-2-picrylhydrazyl (DPPH). Among these fractions, the ethyl acetate fraction showed the most significant free radical scavenging effect on DPPH (Fig. 1). The ethyl acetate soluble extract was subjected to chromatography on a Sephadex LH-20 column and give eight fractions (E1-E8). Fraction E2 (980 mg) was chromatographed on silica gel column chromatography ($CHCl_3$-MeOH-H_2O, 60:10:1) to give eight subfractions (E21-E28). Subfraction E28 (150 mg) was further chromatographed on a Sephadex LH-20 column (MeOH) and purified on a JAI-GS310 cloumn (MeOH) to give compound 1 (13 mg). Fraction E3 (490 mg) was chromatographed on silica gel column chromatography ($CHCl_3$-MeOH-H_2O, 50:10:1) to give three subfractions (E31-E33). Subfraction E32 (190 mg) was further chromatographed on silica gel column chromatography ($CHCl_3$-MeOH-H_2O, 50:10:1) and purified on a JAI-GS310 cloumn (MeOH) to give compound 2 (15 mg). Fraction E4 (210 mg) was chromatographed on silica gel column chromatography ($CHCl_3$-MeOH-H_2O, 40:10:1) to give three subfractions (E41-E43). Subfraction E42 (42 mg) was further chromatographed on a JAI-GS310 cloumn (MeOH) and purified by Sephadex LH-20 (MeOH) to give compound 3 (6 mg). Fraction E5 (410 mg) was chromatographed on silica gel column chromatography ($CHCl_3$-MeOH-H_2O, 20:10:1) to give four subfractions (E51-E54). Subfraction E52 (42 mg) was further chromatographed on a JAI-GS310 cloumn (MeOH) to give compound 4 (8 mg). Subfraction E54 (104 mg) was chromatographed on a JAI-GS310 cloumn (MeOH) to give compound 5 (48 mg). Fraction E6 (230 mg) was chromatographed on silica gel column chromatography ($CHCl_3$-MeOH-H_2O, 30:10:1) to give six subfractions (E61-E66). Subfraction E62 (42 mg) was chromatographed on a JAI-GS310 cloumn (MeOH) to give compound 6 (5 mg). Subfraction E63 (48 mg) was chromatographed on a JAI-GS310 cloumn (MeOH) to give compound 7 (8 mg). Subfraction E65 (62 mg) was applied on a JAI-GS310 cloumn (MeOH) to give compound 8 (12 mg). Fraction E8 (50 mg) was chromatographed on a JAI-GS310 cloumn (MeOH) to give compound 9 (12 mg).

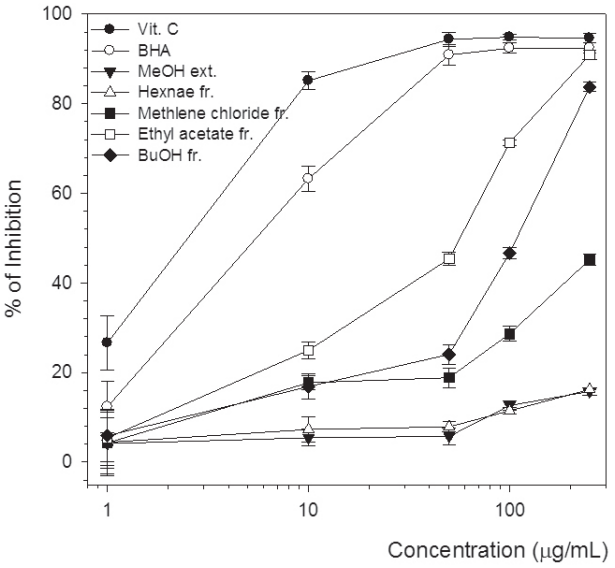


Fig. 1. Scavenging effects of methanol extract and its subsequent fractions from the aerial parts of *G. spurium* on 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical.

Asperulosidic acid methyl ester (1)

Amorphous powder, ^{1}H-NMR (400 MHz, CD_3OD) δ: 7.61 (1H, s, H-3), 5.98 (1H, s, H-7), 5.02 (1H, d, *J*=8.8 Hz, H-1), 4.68 (1H, d, *J*=8.0 Hz, H-6), 3.74 (3H, s, -OC$\underline{H}_3$), 2.99 (1H, t, *J*=6.0 Hz, H-9), 2.60 (1H, t, *J*=8.4 Hz, H-5), 2.05 (3H, s, -C$\underline{H}_3$COO). ^{13}C-NMR, (100 MHz, CD_3OD) δ: 172.5 (-COO), 169.3 (C-11), 155.4 (C-3), 145.9 (C-8), 131.8 (C-7), 108.0 (C-4), 101.3 (C-1'), 100.6 (C-1), 78.5 (C-3'), 77.8 (C-5'), 75.3 (C-6), 74.9 (C-2'), 71.5 (C-4'), 63.7 (C-10), 62.9 (C-6'), 51.9 (O$\underline{C}H_3$), 46.2 (C-9), 42.4 (C-5), 20.8 (-$\underline{C}H_3$COO).

Asperuloside (2)

Amorphous powder, ^{1}H-NMR (400 MHz, CD_3OD) δ: 7.29 (1H, d, *J*=2.0 Hz, H-3), 5.96 (1H, m, H-7), 5.72 (1H, s, H-1), 5.57 (1H, d, *J*=7.2 Hz, H-6), 4.77 (2H, br s, H-10), 3.67 (1H, m, H-9), 3.37 (1H, m, H-5), 2.08 (3H, s, -$\underline{C}$H3). ^{13}C-NMR (100 MHz, CD_3OD) δ: 172.6 (-$\underline{C}$OO), 172.2 (C-11), 150.3 (C-3), 144.3 (C-8), 128.9 (C-7), 106.2 (C-4), 100.0 (C-1), 93.3 (C-1'), 86.3 (C-6), 78.3 (C-3'), 77.9 (C-5'), 74.6 (C-2'), 71.6 (C-4'), 62.8 (C-10), 61.9 (C-6'), 45.3 (C-9), 37.4 (C-5), 20.6 (-$\underline{C}H_3$COO).

Caffeic acid (3)

Yellowish amorphous solid, ^{1}H-NMR (400 MHz, CD_3OD) δ: 7.56 (1H, d, *J*=16.0 Hz, H-7), 7.04 (1H, d, *J*=2.0 Hz, H-2), 6.94 (1H, dd, *J*=8.4, 2.2 Hz, H-6), 6.77 (1H, d, *J*=7.6 Hz, H-5), 6.27 (1H, d, *J*=16.0 Hz, H-8). ^{13}C-NMR (100 MHz, CD_3OD) δ: 169.0 (C-9), 149.5 (C-4), 146.9 (C-7), 146.8 (C-3), 127.8 (C-1), 122.9 (C-6), 116.5 (C-5), 115.5 (C-8), 115.2 (C-2).

Kaempferol-3-*O*-L-rhamnopyranoside (4)

Yellowish powder, ^{1}H-NMR (400 MHz, CD_3OD) δ: 7.74 (2H, d, *J*=8.8 Hz, H-2', 6'), 6.91 (2H, d, *J*=8.8 Hz, H-3', 5'), 6.34 (1H, d, *J*=2.0 Hz, H-8), 6.17 (1H, d, *J*=2.0 Hz, H-6), 5.36 (1H, d, *J*=1.4 Hz, H-1"), 3.33 (1H, dd, *J*=5.2, 1.2 Hz, H-4") 0.92 (3H, d, *J*=5.6 Hz, H-6"). ^{13}C-NMR (100 MHz, CD_3OD): Table 1.

Quercetin-3-*O*-[α-L-rhamnopyranosyl (1→6)-β-D-glucopyranoside] (5)

Yellowish powder, ^{1}H-NMR (400 MHz, CD_3OD) δ: 7.66 (1H,

Table 1. ^{13}C-NMR spectral data of compounds 4-9

C	4[a]	5[a]	6[a]	7[a]	8[b]	9[b]
2	158.5	158.5	158.7	149.7	154.4	147.6
3	136.2	135.6	135.3	136.2	133.4	135.7
4	179.5	179.4	179.4	179.5	177.5	175.8
5	163.1	163.1	163.1	163.0	160.8	160.7
6	99.8	100.0	99.9	99.9	98.8	98.1
7	165.8	166.1	166.0	165.7	164.1	163.8
8	94.8	94.8	94.7	94.7	94.0	93.3
9	159.2	159.4	158.5	158.4	156.9	156.1
10	105.9	105.7	105.8	105.8	104.1	103.0
1'	122.6	123.1	123.1	122.9	121.2	121.9
2'	131.9	116.1	114.3	117.0	131.1	115.0
3'	116.5	145.9	148.4	146.3	115.3	146.7
4'	161.5	149.8	150.9	159.2	159.7	145.0
5'	116.5	117.7	116.0	116.3	115.3	115.5
6'	131.9	123.6	123.8	122.8	131.1	119.9
1"	103.5	104.7	103.6	103.5	101.4	
2"	72.0	75.7	75.9	71.9	74.4	
3"	72.1	78.2	78.6	72.1	76.5	
4"	73.2	71.4	71.5	73.2	70.7	
5"	71.9	77.2	78.1	72.0	75.8	
6"	17.6	68.6	62.5	17.6	67.0	
1"'		102.4			101.0	
2"'		72.1			70.5	
3"'		72.2			70.0	
4"'		73.9			71.6	
5"'		69.7			68.5	
6"'		17.9			17.8	
OCH_3			56.8			

[a]Recorded at 100 MHz in CD_3OD, [b]Recorded at 100 MHz in DMSO-d_6

d, *J*=1.6 Hz, H-2'), 7.61 (1H, dd, *J*=8.4, 2.0 Hz, H-6'), 6.86 (1H, d, *J*=8.0 Hz, H-5'), 6.39 (1H, d, *J*=2.0, H-8), 6.20 (1H, d, *J*=2.0 Hz, H-6), 5.09 (1H, d, *J*=7.6 Hz, H-1"), 4.51 (1H, br s, H-1"'), 1.11 (3H, d, *J*=6.0 Hz, H-6"'). ^{13}C-NMR (100 MHz, CD_3OD): Table 1.

Isorhamnetin-3-*O*-glucopyranoside (6)

Yellowish amorphous powder, ^{1}H-NMR (400 MHz, CD_3OD) δ: 7.83 (1H, d, *J*=2.0 H-2'), 7.49 (1H, dd, *J*=28.4, 2.0 Hz, H-6'), 6.79 (1H, d, *J*=28.4 Hz, H-5'), 6.30 (1H, d, *J*=22.0, H-8), 6.11 (1H, d, *J*=22.0 Hz, H-6), 5.32 (1H, d, *J*=27.6Hz, glc anomeric H), 3.85 (3H, s, -OCH_3). 13C-NMR (100 MHz, CD_3OD): Table 1.

Quercetin-3-*O*-α-L-rhamnopyranoside (7)

Yellow amorphous powder, ^{1}H-NMR (400 MHz, CD_3OD) δ: 7.29 (1H, s, H-2'), 7.25 (1H, d, *J*=7.6 Hz, H-6'), 6.86 (1H, d, *J*=8.4 Hz, H-5'), 6.29 (1H, d, *J*=1.8 Hz, H-8), 6.13 (1H, d, *J*=1.8 Hz, H-6), 5.30 (1H, d, *J*=1.0 H-1"), 4.19 (1H, s, H-2"), 3.72 (1H, d, *J*=6.7 Hz, H-3"), 3.36 (1H, m, H-5"), 3.34 (1H, d, *J*=9.2 Hz, H-4"), 0.90 (3H, d, *J*=6.1 Hz, H-6"). ^{13}C-NMR (100 MHz, CD_3OD): Table 1.

Kaempferol-3-*O*-[α-L-rhamnopyranosyl (1→6)-β-D-glucopyranoside) (8)

Yellowish powder, ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 7.97 (2H, d, *J*=8.8 Hz, H-2', 6'), 6.79 (2H, d, *J*=8.8 Hz, H-3', 5'), 6.43 (1H, d, *J*=2.0 Hz, H-8), 6.24 (1H, d, *J*=2.0 Hz, H-6), 5.00 (1H, d, *J*= 7.0 Hz, H-1"), 4.41 (1H, brs, H-1"'). ^{13}C-NMR (100 MHz, DMSO-d_6): Table 1.

Quercetin (9)

Yellowish powder, ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 7.68 (1H, d, *J*=2.4 Hz, H-2'), 7.54 (1H, dd, *J*=8.0, 2.0 Hz, H-6'), 6.89 (1H, d, *J*=8.4 Hz, H-5'), 6.41 (1H, d, *J*=2.0 Hz, H-8), 6.19 (1H, d, *J*=2.0 Hz, H-6). ^{13}C-NMR (100 MHz, DMSO-d_6): Table 1.

DPPH radical scavenging effect

Ethanol solutions of test samples at various concentrations (0.1-100 μg/ml) were added to a solution of DPPH in ethanol (1.5×10^{-4} M) in 96 well plates. After storing these mixtures for 30 minutes at room temperature, the remaining amounts of DPPH were determined by colorimetry at 520 nm on a microplate reader (Yoshida *et al.*, 1989). And the radical scavenging activity of each compound was expressed by the ratio of the lowering of the DPPH solution in the absence of compounds. The mean values were obtained from triplicate experiments.

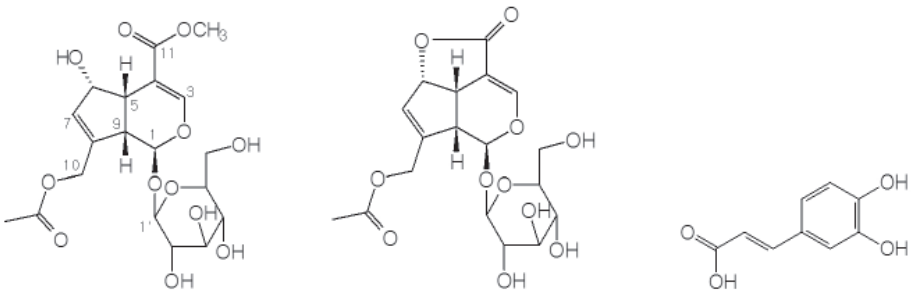
Superoxide quenching activity

Superoxide quenching activities of test samples were measured photochemically, using an assay system consisting of methionine, riboflavin, and nitrobluetetrazolium (NBT) (Ginnopolitis and Ries, 1977; Choi *et al.*, 2001). The reaction mixture was composed of 2.6 μM riboflavin, 13 mM methionine, 75 μM NBT, 0.1 mM EDTA, 0.05 M sodium phosphate (pH 7.8), and various concentrations of test samples. The sample was randomly placed in a light storage box and replaced randomly every 5 min for 15 min. The temperature within the light storage box was 20 ± 1°C during the light illumination. The light intensity at the sample level was 5,500 lux. NBT was reduced to blue formazan formation during the light illumination, and that was measured by the absorbance at 560 nm. The inhibition of blue formazan formation was taken as superoxide quenching activity.

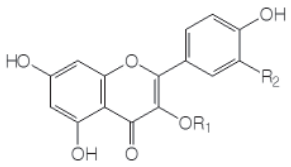
RESULTS AND DISCUSSION

In the course of our screening for antioxidative components from Korean natural plants, the ethyl acetate soluble fraction of methanolic extract of the aerial parts of *G. spurium* was found to show scavenging activity on DPPH radical (Fig. 1). Subsequent activity-guided fractionation of the ethyl acetate soluble fraction led to the isolation of two iridoid glycosides, six flavonoids and a phenylpropanoid (Fig. 2).

The ^{1}H-NMR spectrum of compound 1 showed a methoxy signal at a δ 3.74 (3H, s), doublet at δ 7.61 (1H, s) which was assigned to the enol ether proton at C-3 and the triplet at δ 2.60 (1H, t, *J*=8.4 Hz) and 2.99 (1H, t, *J*=6.0 Hz) were assigned to the protons at C-5 and C-9. In the ^{13}C-NMR spectrum, 19 carbon signals were observed, which included two carbonyl groups at δ 172.5 and 169.3, four olefinic carbons at δ 155.4, 145.9, 131.8 and 108.0, six sugar carbons at δ 101.3, 78.5, 77.8, 74.9, 71.5 and 62.9, and a methyl group at δ 20.8. From these results, compound 1 was indicated to be a iridoid



1 **2** **3**



	R_1	R_2
4	Rhamnose	H
5	Rutinose	OH
6	Glucose	OCH_3
7	Rhamnose	OH
8	Rutinose	H
9	H	OH

Fig. 2. Structures of compounds 1-9 isolated from *G. spurium.*

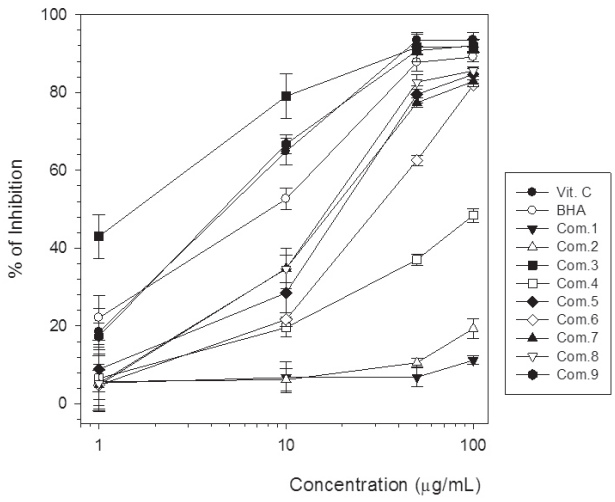


Fig. 3. Scavenging effects of compounds 1-9 from the ethyl acetate soluble fraction of *G. spurium* on 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical.

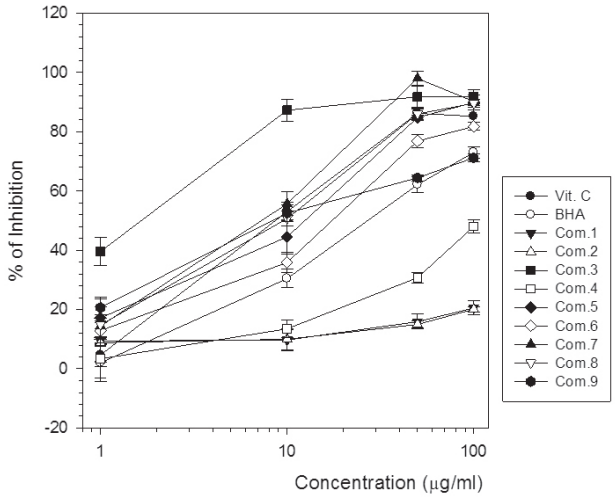


Fig. 4. Superoxide quenching activities of compounds 1-9 from the ethyl acetate soluble fraction of *G. spurium.*

glycoside. The structure of 1 was determined to be asperulosidic acid methyl ester on the basis of the above evidences, together with a comparison of the above data with those published in the literature (El-Naggar and Beal, 1980; Takeda *et al.*, 2002). The ^{1}H-NMR spectrum of 2 showed a doublet at δ 7.29 (1H, d, *J*=2.0 Hz) which was assigned to the enol ether proton at C-3, and the multiplet at δ 3.37 (1H, m) and 3.67 (1H, m), were assigned to the protons at C-5 and C-9. In the ^{13}C-NMR spectrum, 18 carbon signals were observed, which included two carbonyl groups at δ 172.6 and 172.2, four olefinic carbons at δ 150.3, 144.3, 128.9 and 106.2, six sugar carbons at δ 93.3, 78.3 77.9, 74.6, 71.6 and 61.9, and a methyl group at δ 20.6. From these results, compound 2 was indicated to be

an iridoid glycoside. The structure of 2 was determined to be asperuloside on the basis of the above evidences, together with a comparison of the above data with those published in the literature (El-Naggar and Beal, 1980; Lee *et al.*, 2004). Compound 3 was isolated as a yellowish amorphous solid and positive to FeCl3 reagent test. In the ^{1}H-NMR spectrum, two olefinic protons having trans-configuration were observed at δ 7.56 (1H, d, *J*=16.0 Hz, H-7) and 6.27 (1H, d, *J*=16.0 Hz, H-8). Typical signals for 1,3,4-trisubsituted benzene were detected at δ 7.04 (1H, d, *J*=2.0 Hz, H-2), 6.94 (1H, dd, *J*=8.4, 2.2 Hz, H-6) and 6.77 (1H, d, *J*=7.6 Hz, H-5). In the ^{13}C-NMR spectrum, 9 carbons were detected including a carbonyl carbon at δ 169.0. On the basis of these observations and the comparision of the data with those previously published, the structure of compound 3 was identified as caffeic acid (Wu *et al.*, 1999).

Compounds 4-9 showed the positive reaction on Mg-HCl tests, and have similar patterns in their NMR spectra. Compounds 4-9 were deduced to be flavonoids. Structure characterization of these compounds was carried out by interpretation of their spectral data and comparison with the data reported in the literature. Compounds 4-9 were identified as kaempferol-3-*O*-L-rhamnopyranoside (4) [Hur *et al.*, 2001; Jang *et al.*, 2002], quercetin-3-*O*-[α-L-rhamnopyranosyl(1→6)-β-D-glucopyranoside] (5) [Kim *et al.*, 1992; Hasan *et al.*, 1995], isorhamnetin-3-*O*-glucopyranoside (6) [Kim *et al.*, 1998], quercetin-3-*O*-α-L-rhamnopyranoside (7) [Lee *et al.*, 2007], kaempferol-3-*O*-[α-L-rhamnopyranosyl(1→6)-β-D-glucopyranoside] (8) [Hong *et al.*, 2008], and quercetin (9) [Ternai and Markham 1976; Lee *et al.*, 1997; Lee *et al.*, 2007], respectively.

The DPPH radical scavenging effect of the methanolic extract and its solvent partitioned fractions from *G. spurium* are shown in Fig. 1. The positive control vitamin C showed the DPPH radical scavenging effect with the IC_{50} value of 4.7 μg/ml. Compounds 3 and 9 exhibited scavenging activities dose-dependently on DPPH with IC_{50} values of 1.5 and 4.6 μg/ml, respectively (Fig. 3). However, compounds 1 and 2 showed no activities in comparison with reference antioxidants such as ascorbic acid and BHA. Fig. 4 shows the superoxide quenching activities of the isolated compounds 1-9, as measured by the riboflavin-NBT-light system. The positive control vitamin C showed the superoxide quenching activity with the IC_{50} value of 7.8 μg/ml. Whereas, compounds 3 and 7 exhibited superoxide quenching activities dose-dependently on the riboflavin-NBT-light system with IC_{50} values of 1.7 and 6.9 μg/ml, respectively. Compounds 1 and 2 showed no activities in comparison with reference antioxidants (Fig. 4).

It was reported that compound 3, caffeic acid has protective effects on chemical-induced hepatotoxicity in rodents, β-amyloid-induced neurotoxicity and neuronal damage in mouse brain (Janbaz *et al.*, 2004; Zhang *et al.*, 2007; Sul *et al.*, 2009). Recently, Compound 7, quercetin-3-*O*-α-L-rhamnopyranoside was reported the protective effect against hydrogen peroxide-induced dysfunction in osteoblastic MC3T3-E1 cells (Choi, 2010), and lipid peroxidation (Wagner *et al.*, 2006). It was reported that compound 3, quercetin has protective effects on rat liver against lead-induced oxidative stress and apoptosis, and kidney against oxidative stress-mediated DNA damage and apoptosis induced by lead (Liu *et al.*, 2010a; 2010b). The results from free radical scavenging effects and superoxide quenching activities revealed that the ethyl acetate soluble fraction of *G. spurium*, and isolated compounds 3, 7 and 9 may be useful for the treatment of various oxidative damage.

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