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Superoxide Quenching Activity of Phenolic Compounds from the Whole Plant of *Galium verum* var. *asiaticum*

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Abstract – During the process of screening for antioxidative effects of natural plants in Korea, by measuring the superoxide quenching activity, methanol extract of the whole plant, *Galium verum* var. *asiaticum* (Rubiaceae), was found to show potent antioxidant activity. Subsequent activity-guided fractionation of methanol extract of *Galium verum* var. *asiaticum* led to the isolation of five phenolic compounds. Using spectroscopic techniques, the chemical structures were elucidated as: caffeic acid (**1**), narcissin (**2**), rutin (**3**), luteolin-7-*O*-α-L-rhamnopyranosyl (1 → 2)-β-D-glucopyranoside (**4**), and luteolin-7-*O*-β-D-glucopyranoside (**5**). These compounds were isolated for the first time from this plant. Among them, compound **1** showed the most significant riboflavin-originated superoxide and xanthine-originated superoxide quenching activities. Compounds **3** and **4** exhibited mild superoxide quenching effects compared with vitamine C.
Keywords – *Galium verum* var. *asiaticum*, Rubiaceae, superoxide quenching activity

Introduction

It is well known that overproduction of reactive oxygen species (ROS) such as superoxide anion ($\cdot O_2^-$), hydrogen peroxide (H_2O_2) and hydroxyl anion ($OH\cdot$) beyond the ability of antioxidant defense systems to scavenge them may bring about oxidative stress (Er *et al*., 2007). The ROS can subsequently peroxidize unsaturated bonds of membrane lipids, denature proteins and attack nucleic acids. Oxidative stress in the human body is thought to be due to excess ROS. Among these, superoxide anion radicals are the best known. It is considered that the dismutation activity for superoxide anion radicals is the most important indicator of antioxidant effects (Hanaoka *et al*., 2004).

During the process of screening for antioxidant effect from natural plants in Korea, by measuring the superoxide quenching activity, a methanol extract of the whole plant of *G. verum* var. *asiaticum* was found to show potent antioxidant activity. *Galium verum* var. *asiaticum* (Rubiaceae) is a perennial plant, widely distributed in Korea (Lee, 1996; Lee, 2003). Previous phytochemical investigations of the genus *Galium* resulted in the isolation of iridoid glycosides, anthraquinones, and triterpenes (Böjthe-Horváth *et al*., 1980; Handjieva *et al*., 1996; de Rosa *et al.*, 2000; Morimoto *et al*., 2002; El-Gamal *et al*., 1995; Uesato *et al*., 1984). However, phytochemical and pharmacological studies of *G. verum* var. *asiaticum* have not been performed yet. This paper presents information on the isolation, structure elucidation, and identification of compounds **1 - 5** and their superoxide quenching activities.

Experimental

General experimental procedures – NMR spectra were recorded on a JEOL JMN-EX 400 NMR spectrometer. TLC was performed using Merck precoated Silica gel 60 F_{254} plates. Detection of constituents was performed by spraying with 10% H_2SO_4 in ethanol followed by heating at 100 - 120 ºC for 2 min. Silica gel column chromatography was performed on Kiesel gel 60 (230 - 400 mesh, Merck). The molecular sieve column chromatography was used Sephadex LH-20 (25 - 100 μm, Pharmacia, Sweden). The column used for LPLC was Lobar-A (Si 60, 240 × 10 mm, Merck LiChroprep). HPLC was performed using a Jai L-6000 pump with a Jai UV detector and Jaigel GS310 column (20 × 500 mm, Japan). All other chemicals and solvents were of analytical grade and used without further purification. Vitamin C and BHA were obtained from Sigma Chemical Co. Absorbance of the resulting solution was measured on a microplate reader (GENios, Tecan, Austria).

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Plant materials – The whole plants *of G. verum* var. *asiaticum* were collected in August 2009 at Wanju, Jeonbuk, Korea, and identified by Dae Keun Kim, College of pharmacy, Woosuk University. A voucher specimen was deposited in the herbarium of the College of Pharmacy, Woosuk University (WSU-09-003).

Extraction and isolation – The shade dried plant material (600 g) was extracted three times with methanol at 50 °C and filtered. The extracts were combined and evaporated *in vacuo* at 50 °C. The resultant methanolic extract (40 g) was successively partitioned as *n*-hexane (5.2 g), methylene chloride (2.4 g), *n*-BuOH (13.4 g), and H_2O soluble fractions. Each fraction was tested for the superoxide quenching activity. Among these fractions, the *n*-BuOH soluble fraction showed the most significant the riboflavin-originated superoxide quenching activity (Fig. 2). Sephadex LH-20 (MeOH) column chromatography of *n*-BuOH soluble (3 g) extract gave six fractions (B1-B6). Fraction B4 (3 g) was chromatographed by HPLC (Acetonitril-MeOH, 1 : 1) to give five subfractions (B41-B45). Subfraction B41 was purified by Sephadex LH-20 (MeOH) column chromatography to give compound 1 (25 mg). Subfraction B42 was purified by Lobar-A column ($CHCl_3$-MeOH-H_2O, 20 : 10 : 1) to give compound 2 (7 mg). Fraction B43 was purified by Lobar-A column ($CHCl_3$-MeOH-H_2O, 20 : 10 : 1) to give compound 3 (40 mg). Fraction B44 was chromatographed by a silica gel column chromatography ($CHCl_3$-MeOH-H_2O, 20 : 10 : 1), and purified by Sephadex LH-20 (MeOH) column chromatography to give compound 4 (20 mg). Subfraction B45 was purified by Sephadex LH-20 (MeOH) column chromatography to give compound 5 (4 mg).

Caffeic acid (1) – Yellow powder; ^{1}H-NMR (400 MHz, CD_3OD) 7.55 (1H, d, J = 15.6 Hz, H-β), 7.04 (1H, d, J = 2.0 Hz, H-2), 6.94 (1H, dd, J = 8.8, 2.0 Hz, H-6), 6.77 (1H, d, J = 8.8 Hz, H-5), 6.27 (1H, d, J = 15.6 Hz, H-α). ^{13}C-NMR (100 MHz, CD_3OD) 169.0 (COO), 149.5 (C-4), 146.9 (C-β), 146.8 (C-3), 127.8 (C-1), 122.9 (C-6), 116.5 (C-5), 115.5 (C-α), 115.2 (C-2).

Narcissin (2) – Yellowish powder; ^{1}H-NMR (400 MHz, CD_3OD) δ: 7.93 (1H, d, J = 1.6 Hz, H-2'), 7.61 (1H, dd, J = 8.8, 1.6 Hz, H-6'), 6.89 (1H, d, J = 8.8 Hz, H-5'), 6.38 (1H, d, J = 2.0 Hz, H-8), 6.19 (1H, d, J = 2.0 Hz, H-6), 5.22 (1H, d, J = 8.0 Hz, H-1"), 4.52 (1H, br s, H-1'"), 3.94 (3H, s, OCH_3), 1.08 (3H, d, J = 6.4 Hz, H-6'"). ^{13}C-NMR (100 MHz, CD_3OD): See Table 1.

Rutin (3) – Yellowish solid; ^{1}H-NMR (400 MHz, CD_3OD) δ: 7.65 (1H, d, J=2.0 Hz, H-2'), 7.60 (1H, dd, J = 8.4, 2.0 Hz, H-6'), 6.85 (1H, d, J = 8.4 Hz, H-5'), 6.35 (1H, d, J = 1.6 Hz, H-8), 6.16 (1H, d, J = 1.6 Hz, H-6), 5.08 (1H, d, J = 7.6 Hz, H-1"), 4.52 (1H, br s, H-1'"), 1.11 (3H, d, J = 6.4, H-6'"). ^{13}C-NMR (100 MHz, CD_3OD): See Table 1.

Table 1. ^{13}C-NMR spectral data of compounds **2 - 5**

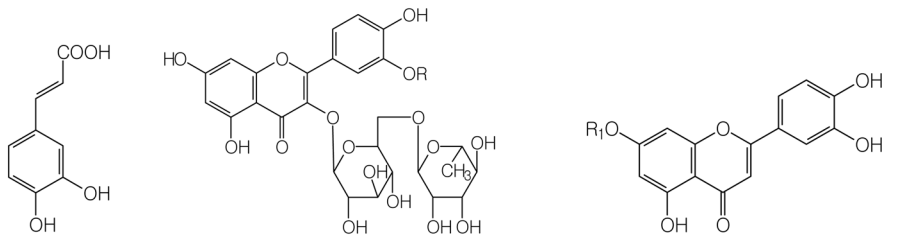
compound position	**2**[a] δc	**3**[a] δc	**4**[a] δc	**5**[b] δc
2	158.8	158.4	166.8	164.5
3	135.5	135.6	104.2	103.1
4	179.3	179.3	183.9	181.9
5	163.0	162.8	162.9	161.1
6	100.0	99.9	99.8	99.5
7	166.1	165.9	164.3	162.9
8	94.9	94.9	95.9	94.7
9	158.5	159.2	158.9	156.9
10	105.7	105.6	107.0	105.3
1'	123.0	123.6	123.4	121.3
2'	114.6	116.0	114.3	113.6
3'	150.8	145.7	147.0	145.8
4'	148.3	149.7	151.1	150.0
5'	116.1	117.7	116.8	116.0
6'	124.0	123.1	120.5	119.1
1"	104.4	104.8	100.9	99.9
2"	75.9	75.7	79.0	73.1
3"	78.2	78.1	79.1	76.4
4"	72.3	71.3	71.4	69.5
5"	77.4	77.1	78.3	77.2
6"	68.5	68.5	62.4	60.6
1'"	102.5	102.4	102.5	
2'"	71.6	72.0	72.2	
3'"	72.1	72.2	72.2	
4'"	73.8	73.9	74.0	
5'"	69.8	69.7	70.0	
6'"	17.9	17.9	18.3	
OCH_3	56.8			

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

Luteolin-7-*O*-α-L-rhamnopyranosyl(1 → 2)-β-D-glucopyranoside (4) – Yellowish solid; ^{1}H-NMR (400 MHz, CD_3OD) δ: 7.37 (1H, dd, J = 8.8, 2.4 Hz, H-6'), 7.35 (1H, d, J = 2.4 Hz, H-2'), 6.87 (1H, d, J = 8.8 Hz, H-5'), 6.70 (1H, d, J = 2.0 Hz, H-8), 6.55 (1H, s, H-3), 6.42 (1H, d, J = 2.0 Hz, H-6), 5.28 (1H, br s, H-1'"), 5.17 (1H, d, J = 7.6 Hz, H-1"), 1.32 (3H, d, J = 6.4 Hz, H-6'"). ^{13}C-NMR (100 MHz, CD_3OD): See Table 1.

Luteolin-7-*O*-β-D-glucopyranoside (5) – Yellowish solid; ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 12.99 (1H, s, 5-OH), 7.45 (1H, d, J = 2.2 Hz, H-2'), 7.45 (1H, dd, J = 8.8, 2.2 Hz, H-6'), 6.93 (1H, d, J = 8.8 Hz, H-5'), 6.80 (1H, d, J = 2.4 Hz, H-8), 6.75 (1H, s, H-3), 6.45 (1H, d, J = 2.4 Hz, H-6), 5.08 (1H, d, J = 7.6 Hz, H-1"). ^{13}C-NMR (100 MHz, DMSO-d_6): See Table 1.

Riboflavin-originated superoxide quenching activity – The superoxide quenching activities of test samples were photochemically measured using an assay system consisting of methionine, riboflavin, and nitrobluetetra-



1 **2** R = OCH_3 **4** R_1 = α-L-Rha (1→2)-β-D-Glc

3 R = H **5** R_1 = β-D-Glc

Fig. 1. Structures of compounds **1** - **5** isolated from *G. verum* var. *asiaticum.*

zolium (NBT) (Ginnopolitis *et al*., 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. During the light illumination, the temperature within the light storage box was 20 ± 1 °C. The light intensity at the sample level was 5,500 lux. During the light illumination, NBT was reduced to blue formazan formation that was measured by the absorbance at 560 nm. The inhibition of blue formazan formation was taken as a superoxide quenching activity.

Xanthine-originated superoxide quenching activity – Superoxide radicals were generated by xanthine/xanthine oxidase and measured by previously reported method (Thuong *et al*., 2007). In brief, test samples were mixed with 20 mM phosphate buffer (pH 7.8) containing 0.48 mM NBT and 1.6 mM xanthine. After 5-min, xanthine oxidase (0.05 U/mL) 100 μL was added. The absorbance of reaction mixture was read at 570 nm after 30-min incubation at 37 °C. Superoxide radical scavenging activity was expressed by the degree of NBT reduction of a test group in comparison to that of control.

Results and Discussion

In the course of screening for antioxidant components from Korean natural plants (Lee *et al*., 2008; Kim 2009; Lee *et al*., 2009; Lee *et al*., 2010), the *n*-BuOH soluble fraction of methanolic extract of the whole plant of *G. verum* var. *asiaticum* was found to show potent riboflavin-originated superoxide quenching activity (Fig. 2). Subsequent activity-guided chromatography of *n*-BuOH soluble fraction led to the isolation of five compounds.

Compound **1** was isolated as a yellow amorphous powder and positive to $FeCl_3$ reagent test. In the ^{1}H-NMR spectrum, two olefinic protons having trans-configuration were observed at δ 7.55 (1H, d, *J* = 15.6 Hz, H-β) and 6.27 (1H, d, *J* = 16.0 Hz, H-α). 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* = 8.8, 2.0 Hz, H-6) and 6.77 (1H, d, *J* = 8.8 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 **1** was identified as caffeic acid (Wu *et al.,* 2008).

Compound **2** was positive to the $FeCl_3$ reagent, suggesting that this compound contained phenol groups. The ^{1}H-NMR spectrum in CD_3OD showed a methoxy signal at δ 3.94, two *meta*-coupled doublets of one proton each at δ 6.19 (1H, d, *J* = 2.0 Hz, H-6) and 6.38 (1H, d, *J* = 2.0 Hz, H-8), one *ortho*-coupled doublet of one proton at δ 6.89 (1H, d, *J* = 8.8 Hz, H-5'), a double doublet of one proton at δ 7.61 (1H, dd, *J* = 8.8, 1.6 Hz, H-6'), a *meta*-coupled doublet of one proton at δ 7.93 (1H, d, *J* = 1.6 Hz, H-2'), and two anomeric proton signals at δ 4.52 (1H, br s, H-1''') and 5.22 (1H, d, *J*=7.6 Hz, H-1''). These data indicated that compound **2** was a 3,5,7,3',4'-oxgenated flavonoid with two sugars. The appearance of the H-2' signal at lower field than the H-6' signal suggested the presence of a 3'-methoxy-4'-hydroxy moiety in the B ring (Mabry *et al*., 1970). The moiety of two sugars was deduced from its ^{13}C-NMR. The characteristic methyl signal of rhamnose appeared at the low field at δ 1.08 (3H, d, *J* = 6.4 Hz, H-6'''), indicating that the disaccharide should be rutinose. On the basis of the evidence presented above, together with a direct comparison of the data published in the literature, the structure of compound **2** was determined to be isorhamnetin-3-*O*-α-

L-rhamnopyranosyl(1 → 6)-β-D-glucopyranoside (narcissin) (Mabry *et al.,* 1970; Choi and Woo, 1989).

Compound **3** was positive to $FeCl_3$ reagent suggesting, this compound contained phenol groups. In the ^{1}H-NMR spectrum, the peaks, based on quercetin, were observed at δ 6.16 (1H, d, *J* = 1.6 Hz, H-6) and 6.35 (1H, d, *J* = 1.6 Hz, H-8) due to the A-ring, δ 6.85 (1H, d, *J* = 8.4 Hz, H-5'), 7.60 (1H, dd, *J* = 8.4, 2.0 Hz, H-6') and 7.65 (1H, d, *J* = 2.0 Hz, H-2') due to the B-ring. The two anomeric protons of sugars were at δ 5.08 (1H, d, *J* = 7.6 Hz, H-1'') and 4.52 (1H, br s, H-1'''), and the rhamnosyl CH_3 was found at δ 1.11 (3H, d, *J* = 6.4, H-1'''). In the ^{13}C-NMR spectrum, the two peaks at δ 104.8 and 102.4 were attributable to the two anomeric carbons of D-glucose and L-rhamnose. The NMR spectral patterns of sugar region of compound **3** were similar to those of compound **2**, suggesting the presence of the same sugars. On the basis of the evidence presented above, together with a direct comparison of the data published in the literature, the structure of compound **3** was determined to be quercetin-3-*O*-α-L-rhamnopyranosyl(1 → 6)-β-D-glucopyranoside (rutin) (Lim *et al.,* 2006).

Compound **4** was positive to $FeCl_3$ reagent suggesting, this compound contained phenol groups. In the ^{1}H-NMR spectrum, the peaks, based on luteolin, were observed at δ 6.42 (1H, d, *J* = 2.0 Hz, H-6) and 6.70 (1H, d, *J* = 2.0 Hz, H-8) due to the A-ring, and δ 6.87 (1H, d, *J* = 8.8 Hz, H-5'), 7.37 (1H, dd, *J* = 8.8, 2.4 Hz, H-6') and 7.35 (1H, d, *J* = 2.4 Hz, H-2') due to the B-ring, and δ 6.55 (1H, s, H-3) due to the C-ring. The two anomeric protons of sugars were at δ 5.17 (1H, d, *J* = 7.6 Hz, H-1'') and 5.28 (1H, br s, H-1'''), and the rhamnosyl CH_3 was found at δ 1.32 (3H, d, *J* = 6.4, H-1'''). In the ^{13}C-NMR spectrum, the two peaks at δ 100.9 and 102.5 were attributable to the two anomeric carbons of D-glucose and L-rhamnose. The NMR spectral patterns of sugar region of compound **4** were similar to those of compound **3** except chemical shifts of 2'' and 6''. On the basis of the evidence presented above, together with a direct comparison of the data published in the literature, the structure of compound **4** was determined to be luteolin-7-*O*-α-L-rhamnopyranosyl (1 → 2)-β-D-glucopyranoside (Coleta *et al.,* 2006).

Compound **5** was positive to $FeCl_3$ reagent suggesting, that this compound contained phenol groups. Compound **5** had very similar structural signals compared to compound **4**. The main differences was the absence of rhamnose signals. On the basis of the evidence presented above, together with a direct comparison of the data published in the literature, the structure of compound **5** was determined to be luteolin-7-*O*-β-D-glucopyranoside

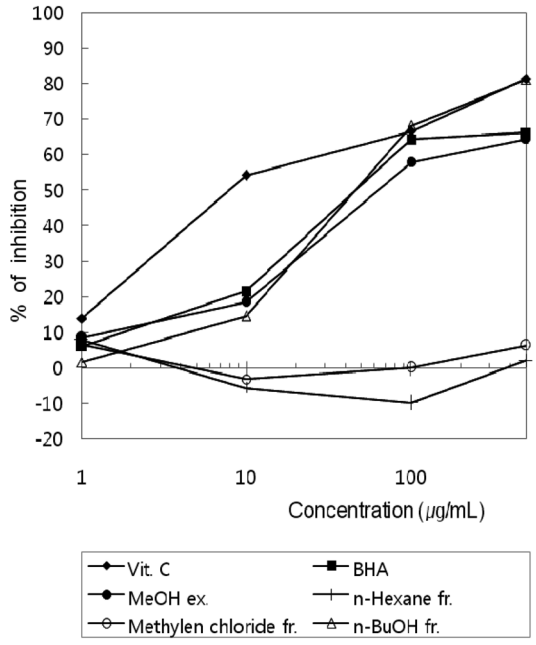


Fig. 2. Riboflavin-originated superoxide quenching activities of the fractions from the whole plant of *G. verum* var. *asiaticum*.

(Lee *et al.,* 2011).

Superoxide ($\cdot O_2^-$), which is one of reactive oxygen species, is toxic because of its capacity to induce various oxidative damages in human body. The superoxide quenching activity assay is principally designed to evaluate the superoxide quenching ability by sample. The superoxide quenching activities of each solvent partitioned fraction from *G. verum* var. *asiaticum* are shown in Fig. 2. *n*-BuOH soluble fraction showed the most significant effects. Five compounds were isolated from this fraction by using several chromatographic methods. Four isolated compounds except compound **5** were tried to assay the superoxide quenching activities, because compound **5** was not enough to test the effect. The riboflavin-originated superoxide quenching activities of the four compounds isolated from the *n*-BuOH soluble fraction of this plant are shown in Fig. 3. Among these compounds, compound **1** (IC_{50} value, 4.2 μg/mL) exhibited stronger quenching effects on riboflavin-originated superoxide than vitamin C (IC_{50} value, 6.4 μg/mL). Compound **2** showed no activity, and compounds **3** (IC_{50} value, 17.5 μg/mL) and **4** (IC_{50} value, 25.7 μg/mL) showed less quenching effects on superoxide than positive control, vitamin C.

To verify superoxide quenching effect of isolated compounds, xanthine-originated superoxide quenching activities were measured. The xanthine-originated superoxide quenching activities of the four compounds are

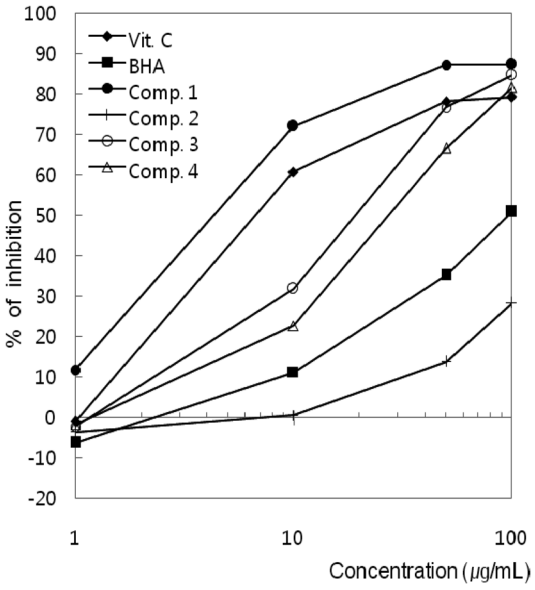


Fig. 3. Riboflavin-originated supeoxide quenching activities of the isolated compounds from the whole plant of *G. verum* var. *asiaticum*.

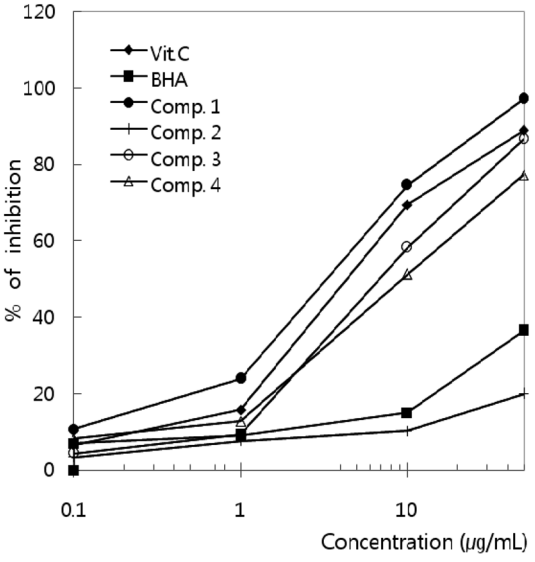


Fig. 4. Xanthine-originated supeoxide quenching activities of the isolated compounds from the whole plant of *G. verum* var. *asiaticum*.

shown in Fig. 4. The quenching effects of four compounds appeared similar to that of riboflavin-originated superoxide. Compound **1** (IC_{50} value, 3.3 μg/mL) exhibited stronger quenching activities on xanthine-originated superoxide than vitamin C (IC_{50} value, 4.2 μg/mL). Compound **2** showed no activity, and compounds **3** (IC_{50} value, 6.9 μg/mL) and **4** (IC_{50} value, 9.3 μg/mL) showed mild quenching effects compared with vitamin C.

In this study, these five compounds were isolated for the first time from this plant. Among these compounds, compound 1 exhibited stronger superoxide quenching effects than vitamin C, and compound 3 and 4 showed mild quenching activities against both the riboflavin-originated superoxide and the xanthine-originated superoxide. Consequently, isolated compounds 1, 3 and 4 from the *n*-BuOH soluble fraction of *G. verum* var. *asiaticum* may be useful to treat oxidative damages of superoxide.

Acknowledgments

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