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Antioxidative Constituents of *Cyperus difformis* L.

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Abstract – In the course of screening for antioxidant compounds by measuring the DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging effect and superoxide quenching activity, methanol extract of *Cyperus difformis* (Cyperaceae) was found to show potent antioxidant activities. Subsequent activity-guided fractionation of the methanolic extract led to the isolation of three phenolic compounds, rosmarinic acid (**1**), luteolin (**2**) and caffeic acid (**3**). Their structures were elucidated by spectroscopic studies. Three compounds showed the significant antioxidative effects on DPPH and nitric oxide radical scavenging effects, riboflavin and xanthin originated superoxide quenching activities. Among them, compound **3** showed the most significant antioxidative effects on those four antioxidant tests. Compound **3** showed better antioxidative activities than vitamin C.
Keywords – *Cyperus difformis*, Cyperaceae, DPPH, Superoxide quenching activity, Caffeic acid

Introduction

Cyperus difformis L. (Cyperaceae) is widely distributed in Asia including Korea, Japan and China, and its whole plant has been used in folk medicine for bruise, gonorrhea, diuretic, and hematemesis (Lee, 1996; The dictionary of Chinese drugs, 1985). This plant grows widely in a dump area as a kind of weeds. But phytochemical and pharmacological studies of this plant has not been performed yet.

In the course of searching for antioxidants from natural plants in Korea by measuring the radical scavenging effect on DPPH (1,1-diphenyl-2-picrylhydrazyl), superoxide quenching activity and nitric oxide radical scavenging effect, a total extract of the whole plant of *Cyperus difformis* was found to show potent antioxidant activity. Subsequent activity-guided fractionation of the methanolic extract led to the isolation of three phenolic compounds from the active ethyl acetate fraction. Among them, compound **3** showed the significant antioxidative effects on DPPH and nitric oxide radical scavenging effects, riboflavin and xanthine originated superoxide quenching activities. This paper deals with the isolation and structural characterization of those three compounds and their scavenging activity of the stable DPPH free radical and nitric oxide radical scavenging effects, and riboflavin and xanthine originated superoxide quenching activities.

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Experimental

General experimental procedures – NMR spectra were determined on a JEOL JMN-EX 400 spectrometer. Sephadex LH-20 was used for column chromatography (Pharmacia, 25 - 100 μm). Low pressure liquid chromatography was performed over Merck LiChroprep Lobar-A Si 60 (240 × 10 mm) column. TLC was carried out on Merck 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 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 and BHA (butylated hydroxyanisole) were obtained from Sigma Chemical Co.

Plant materials – The whole plants of *C. difformis* were collected in August 2007 at Jinahn, 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-07-017).

Extraction and isolation – The shade dried and powdered the whole plants of *C. difformis* (630 g) was extracted three times with MeOH at 50 °C, and then

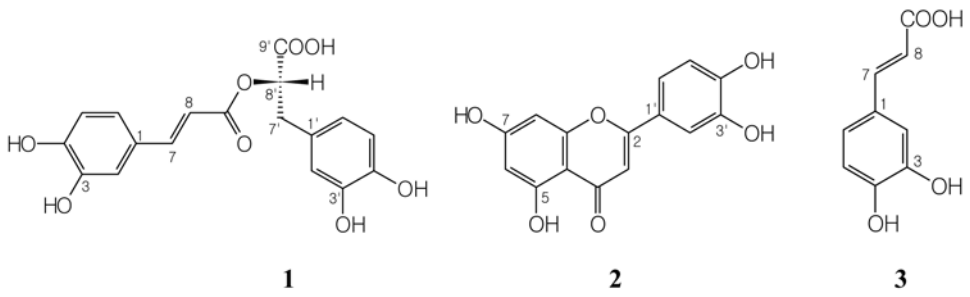

Fig. 1. Structures of compounds **1 - 3** isolated from *Cyperus difformis*.

filtered. The extracts were combined and evaporated *in vacuo* at 40 °C. The resultant methanolic extract (103 g) was successively partitioned as *n*-hexane (11.3 g), methylene chloride (4 g), ethyl acetate (2.4 g), *n*-butanol (10.7 g) and water soluble fractions. Each fraction was tested for the radical scavenging effect on DPPH free radical and nitric oxide radical scavenging effects, and riboflavin and xanthine originated superoxide quenching activities. Among these fractions, the ethyl acetate soluble fraction showed the most significant free radical scavenging effect on four tests. This fraction was subjected to chromatography on a Sephadex LH-20 column (MeOH), and give ten fractions (E1-E10). Fraction E3 was chromatographed on silica gel column chromatography ($CHCl_3$-MeOH-H_2O, 120 : 20 : 1) as an eluent to give eight subfractions (E31-E38). Subfraction E35 (260 mg) was further purified on a Sephadex LH-20 column (MeOH) to give compound **1** (25 mg). Fraction E6 was chromatographed on silica gel column chromatography ($CHCl_3$-EtOAc-MeOH, 6 : 3 : 1) as an eluent to give eleven subfractions (E61-E611). Subfraction E65 (20 mg) was further purified on a Sephadex LH-20 column (MeOH) to give compound **2** (17 mg). Fraction E8 was chromatographed on silica gel column chromatography ($CHCl_3$-MeOH-H_2O, 20 : 20 : 1) as an eluent to give eleven subfractions (E81-E86). Subfraction E82 was further purified on a Sephadex LH-20 column (MeOH) to give compound **3** (8 mg).

Rosmarinic acid (1) – greenish white powder, ^{1}H-NMR (400 MHz, CD_3OD) δ: 7.43 (1H, d, *J* = 16.0 Hz, H-7), 6.93 (1H, d, *J* = 2.0 Hz, H-2), 6.84 (1H, dd, *J* = 8.0, 2.0 Hz, H-6), 6.67 (2H, d, *J* = 8.0 Hz, H-5), 6.66 (1H, d, *J* = 2.0 Hz, H-2'), 6.58 (1H, d, *J* = 8.0 Hz, H-5'), 6.51 (1H, dd, *J* = 8.0, 2.0 Hz, H-6'), 6.16 (1H, d, *J* = 16.0 Hz, H-8), 5.04 (1H, dd, *J* = 10.0, 4.0 Hz, H-8'), 3.00 (1H, dd, *J* = 14.0, 4.0 Hz, H-7'a), 2.83 (1H, dd, *J* = 14.0, 10.0 Hz, H-7'b), ^{13}C-NMR (100 MHz, CD_3OD) δ: 175.6 (C-9'), 169.8 (C-9), 149.4 (C-4), 147.2 (C-7), 146.6 (C-3), 145.9 (C-3'), 144.9 (C-4'), 130.2 (C-1'), 127.8 (C-1), 123.0 (C-6), 121.8 (C-6'), 117.6 (C-2'), 116.5 (C-5), 116.3 (C-5'), 115.2 (C-8), 115.0 (C-2), 76.2 (C-8'), 38.3 (C-7')

Luteolin (2) – yellow needles, mp 330 - 332 °C; ^{1}H-NMR (400 MHz, CD_3OD) δ: 7.25 (1H, dd, *J* = 8.8, 2.4 Hz, H-6'), 7.25 (1H, d, *J* = 2.4 Hz, H-2'), 6.78 (1H, d, *J* = 8.8 Hz, H-5'), 6.41 (1H, s, H-3), 6.32 (1H, d, *J* = 2.4 Hz, H-8), 6.09 (1H, *J* = 2.4 Hz, H-6), ^{13}C-NMR (100 MHz, CD_3OD) δ: 183.8 (C-4), 166.3 (C-7), 166.0 (C-2), 163.2 (C-9), 159.4 (C-5), 150.9 (C-4'), 147.0 (C-3'), 123.7 (C-6'), 120.3 (C-1'), 116.8 (C-5'), 114.2 (C-2'), 105.3 (C-10), 103.8 (C-3), 100.1 (C-6), 95.0 (C-8)

Caffeic acid (3) – yellowish amorphous solid; mp 193 - 194 °C; ^{1}H-NMR (400 MHz, CD_3OD) δ: 7.50 (1H, d, *J* = 15.6 Hz, H-7), 7.03 (1H, d, *J* = 2.2 Hz, H-2), 6.92 (1H, dd, *J* = 8.4, 2.2 Hz, H-6), 6.76 (1H, d, *J* = 8.4 Hz, H-5), 6.21 (1H, d, *J* = 15.6 Hz, H-8), ^{13}C-NMR (100 MHz, CD_3OD) δ: 168.3 (C-9), 149.3 (C-4), 146.7 (C-7), 146.5 (C-3), 128.0 (C-1), 122.7 (C-6), 116.5 (C-5), 115.7 (C-8), 115.1 (C-2)

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 methanol (0.2 mM) 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.

Riboflavin and superoxide quenching activity – Superoxide quenching activities of test samples were measured photochemically, using an assay system consisting of methionine, riboflavin, and nitrobluetetrazolium (NBT) (Choi *et al*., 2001; Ginnopolitis *et al*., 1977). The reaction mixture was composed of 0.13 μM riboflavin, 13 mM methionine, 75 μM NBT, 0.1 mM

EDTA, PBS buffer (pH 7.4), 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. During the light illumination, NBT was reduced to blue formazan formation was measured by the absorbance at 560 nm. The inhibition of blue formazane formation was taken as superoxide quenching activity.

Xanthine and superoxide scavenging assay – 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.

Nitric oxide radical scavenging activity – The scavenging effect on nitric oxide was basically measured according to the method of Sreejayan and Rao, 1997. 5 μL of extract solution at various concentrations were added in the tubes to 495 μL of 10 mM sodium nitroprusside solution. After storing these mixtures for 2 hours at room temperature, 100 μL of Griess reaction (naphthyl ethylene diamine dihydrochloride 0.2% in 2% sulfanilamide and 5% phosphorylic acid) was added to 100 μL reacted samples. The absorbance was immediately read at 570 nm and referred to the absorbance of standard solutions of sodium nitrite salt treated in the same way with Griess reagent. The inhibition of nitric oxide radical was taken as scavenging activity.

Rseults and Discussion

In the course of our screening for antioxidative components from natural plants, methanolic extract of the whole plants of *C. differmis* was found to show scavenging activity on DPPH radical (Fig. 2), and to show riboflavin and xanthin originated superoxide quenching activities (Fig. 3 and 4). Subsequent activity-guided fractionation of the ethyl acetate soluble fraction led to the isolation of three phenolic compounds.

Compound **1** was isolated as a greenish white powder and positive to $FeCl_3$ reagent test. In ^{1}H-NMR, two olefinic protons having *trans*-configuration were observed at δ 7.43 (1H, d, J = 16.0 Hz, H-7) and 6.16 (1H, d,

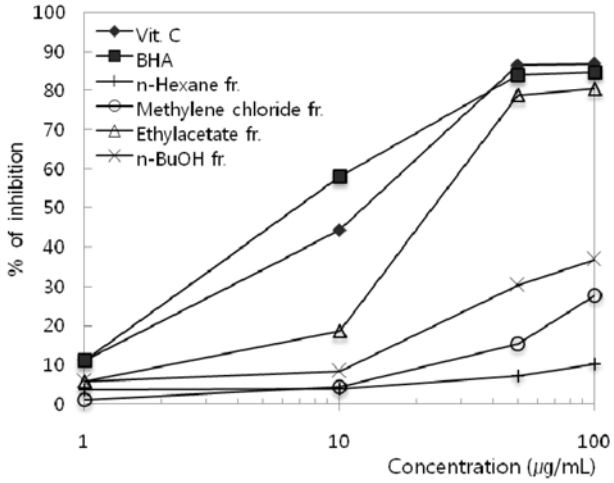

Fig. 2. Radical scavenging effects on DPPH radical of the fractions from *Cyperus differmis*.

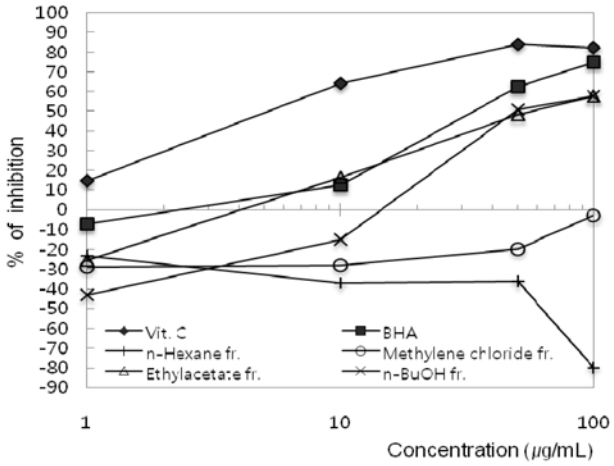

Fig. 3. Riboflavin originated superoxide quenching activities of the fractions from *Cyperus differmis*.

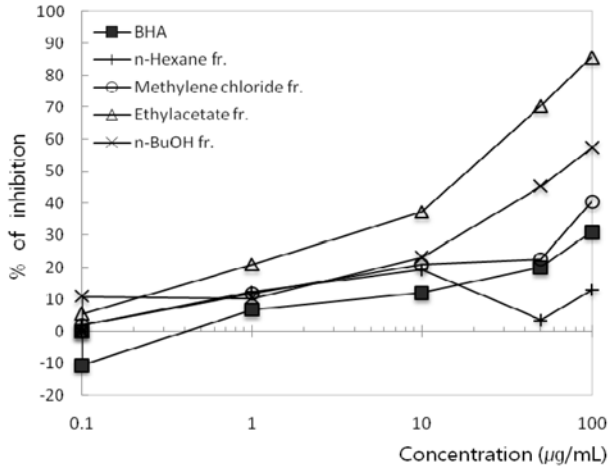

Fig. 4. Xanthine originated superoxide scavenging effects of the fractions from *Cyperus differmis*.

J = 16.0 Hz, H-8). Two sets of typical signals for 1,3,4-trisubsituted benzene were detected at δ 6.93 (1H, d, J = 2.0 Hz, H-2), 6.67 (1H, d, J = 8.0 Hz, H-5), 6.84 (1H, dd, J = 8.0 and 2.0 Hz, H-6), 6.66 (1H, d, J = 2.0 Hz, H-2'), 6.58 (1H, d, J = 8.0 Hz, H-5'), and 6.51 (1H, dd, J = 8.0 and 2.0 Hz, H-6'). These were good accordance with the NMR data in a reference (Kang and Kim, 2004; Lee *et al*., 1998). Compound **2** was positive to $FeCl_3$ reagent suggesting that it had phenol groups. In ^{1}H-NMR, two *meta*-coupled aromatic signal were detected at δ 6.32 (1H, d, J = 2.4 Hz, H-8) and 6.09 (1H, d, J = 2.4 Hz, H-6). And, typical 1,3,4-trisubstituted benzene signals appeared at δ 7.25 (1H, d, J = 8.8, 2.4 Hz, H-6'), 7.25 (1H, d, J = 2.4 Hz, H-2'), and 6.78 (1H, d, J = 8.5 Hz, H-5'). The signal at δ 6.41 (1H, s, H-3) was characteristic to the C-3 position of flavone skeleton. In ^{13}C-NMR, 15 carbons were detected including a carbonyl carbon at ä 183.8. On the basis of these observation and the comparison of the data with those previously published (Kim and Kim, 2006; Choi *et al*., 2008), the structure of compound **2** was identified as luteolin.

Compound **3** was isolated as a yellowish amorphous solid and positive to $FeCl_3$ reagent test. In ^{1}H-NMR, two olefinic protons having *trans*-configuration were observed at δ 7.50 (1H, dd, J = 15.6 Hz, H-7) and 6.21 (1H, d, J = 15.6 Hz, H-8). Typical signals for 1,3,4-trisubsituted benzene were detected at δ 7.03 (1H, d, J = 2.2 Hz, H-2), 6.92 (1H, dd, J = 8.4, 2.2 Hz, H-6), 6.76 (1H, d, J = 8.4 Hz, H-5). In ^{13}C-NMR, 9 carbons were detected including a carbonyl carbon at δ 168.3. On the basis of these observation and the comparison of the data with those previously published (Wu *et al*., 1999), the structure of compound **3** was identified as caffeic acid.

The radical scavenging effects of three compounds obtained from *C. difformis* were shown in Fig. 5. The positive control vitamin C showed the DPPH radical scavenging effect with the IC_{50} value of 4.1 μg/mL. Compound **3** exhibited the highest scavenging activity dose-dependently on DPPH with IC_{50} value of 2.9 μg/mL. Compounds **1** and **2** showed similar activities in comparison with reference antioxidants such as ascorbic acid and BHA. Fig. **6** and **7** show the superoxide quenching activities of the isolated compounds **1 - 3**, as measured by the riboflavin-NBT-light and xanthine-NBT-xanthine oxidase systems. Compound **3** was found to be a potent scavenger of superoxide radical generated in two systems. In riboflavin-NBT-light system, Compound **3** exhibited the formation of the blue formazan in a dose-dependent manner with IC_{50} value of 2.8 μg/mL (vitamin C, positive control, IC_{50} value, 7.1 μg/mL) (Fig. 6). In

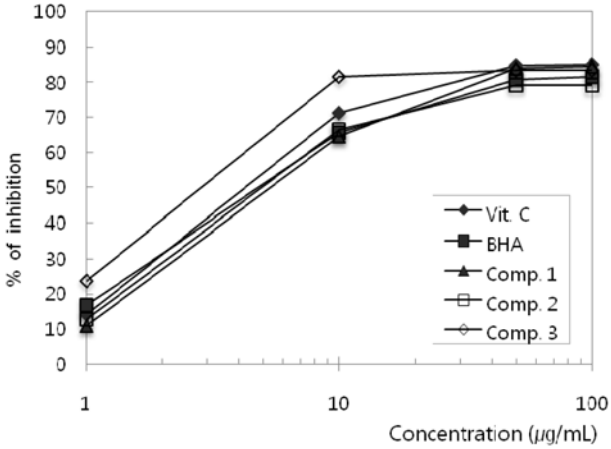

Fig. 5. Radical scavenging effects on DPPH radical of the isolated compounds from *Cyperus difformis*.

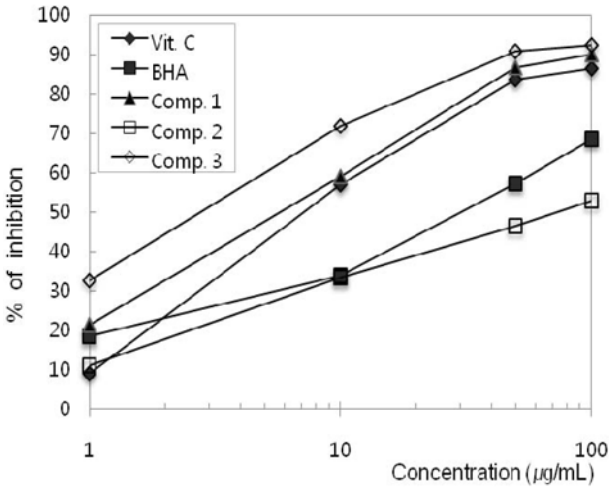

Fig. 6. Riboflavin originated superoxide quenching activities of the isolated compounds from *Cyperus difformis*.

xanthine-NBT-xanthine oxidase system, compound **3** also exhibited the formation of the blue formazan in a dose-dependent manner with IC_{50} value of 2.4 μg/mL (vitamin C, positive control, IC_{50} value, 16.8 μg/mL) (Fig. 7). Two kinds of superoxide quenching activities of compound **3** were more potent than vitamin C, used as a positive control. Suppression of nitric oxide radical release might be attributed to a direct nitric oxide radical scavenging effects as compounds **1 - 3** decreased the amount of sodium nitroprusside as shown in Fig. 8. The positive control vitamin C showed the nitric oxide radical scavenging effect with the IC_{50} value of 180.7 μg/mL. Compounds **1** and **3** exhibited similar nitric oxide radical scavenging activities with IC_{50} values of 18.7 and 16.4 μg/mL, respectively.

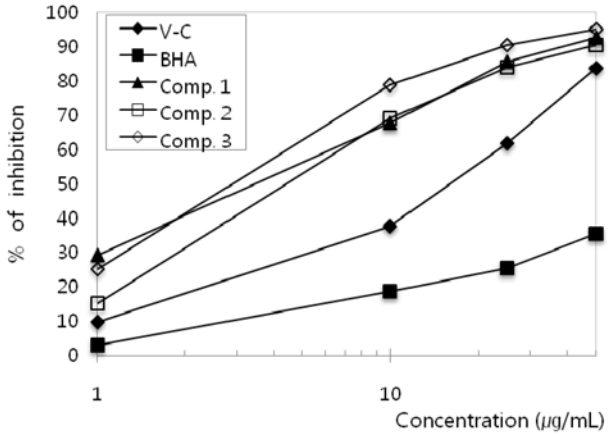

Fig. 7. Xanthine originated superoxide scavenging effects of the isolated compounds from *Cyperus difformis*.

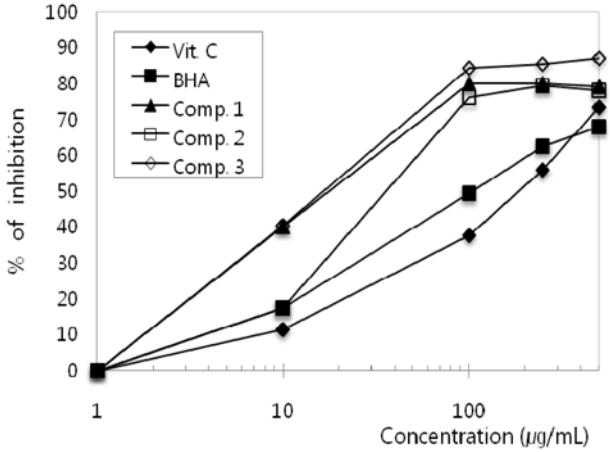

Fig. 8. Nitric oxide radical scavenging effects of the isolated compounds from *Cyperus difformis*.

Free radicals like superoxide anion and hydroxyl radicals are highly reactive molecules with an unpaired electron and are produced by radiation or as byproducts of metabolic processes (Devi *et al*., 2008). Superoxide radical is known to be very harmful to cellular components as a precursor of more reactive oxygen species, and the harmful action of the free radicals can be blocked by antioxidant substances, which scavenge the free radicals and detoxify the organism (Kumaran and Karunakaran, 2006). The results from free radical scavenging systems revealed that the ethyl acetate soluble fraction of the whole plants of *C. difformis,* and compounds **1** - **3** had significant antioxidant activities. Among them, compound **3** showed the most significant antioxidative effects on those four antioxidant tests. Compound **3** showed better antioxidative activities than vitamin C. Therefore compound **3** including **1** and **2** may be useful for the treatment of various oxidative damages.

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