Arch Pharm Res Vol 23, No 3, 237-239, 2000 Medicinal Chemistry & Natural Products

Isolation of Luteolin 7-O-rutinoside and Esculetin with Potential Antioxidant Activity from the Aerial Parts of *Artemisia montana*

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(Received March 30, 2000)

The antioxidant activity of *Artemisia montana* was determined by measuring the radical scavenging effect on 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical and inhibitory activity against free radical generation of hepatocytes (AC_2F). The methanol extract of *A. montana* showed strong radical scavenging activity at a concentration of 10.1 μg/ml, and thus fractionated by solvent extraction. Esculetin and luteolin 7-O-rutinoside (scolymoside) were isolated as the active principles from the EtOAc and Interphase fractions, respectively. The antioxidant activity of these compounds were comparable to that of L-ascorbic acid.

Key words: *Artemisia montana*, Esculetin, Luteolin 7-O-rutinoside, Antioxidant activity

INTRODUCTION

In a previous paper, we reported results of screening of many plants and marine algae in terms of their antioxidant effect on 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical (Choi *et al.*, 1993). Among those, the methanolic extract of the aerial parts of *Artemisia iwayomogi* exhibited a strong antioxidant activity on DPPH radical. From this methanolic extract, chlorogenic acid was isolated as one of the active principles (Kim *et al.*, 1997). In our continuous search for natural antioxidants from *Artemisia* species, we studied antioxidant principles of *A. montana*.

Artemisia montana Pamp. (Compositae) is a perennial herb growing in Korea and Japan. The aerial parts have been used in Chinese herbal medicine as an antipyretic, anti-diabetic, anti-hypertensive, anti-hepatotoxic, and anti-hemorrhoid (Kim, 1996). Previous workers reported the isolation of sesquiterpene lactones and scopoletin (Matsueda and Nagaki, 1984, Nagaki and Matsueda, 1989, Koreeda *et al.*, 1988) from this plant. The hemostatic principles from this plant have been identified as 3,5-, 3,4-, and 4,5-di-O-caffeoylquinic acids (Okuda *et al.*, 1986).

MATERIALS AND METHOD

The ^{1}H- and ^{13}C-NMR spectra were recorded at 300 MHz and 75.5 MHz, respectively on a Bruker AM 300 spectrometer with tetramethylsilane as internal standard. Multiplicities of ^{1}H- and ^{13}C-NMR signals are indicated as s (singlet), d (doublet) and t (triplet). The samples were run in DMSO-d_6, except for esculetin (**2**), which was run in D_2O. Electron impact mass spcetra (EIMS) were taken on a Jeol-SX102A GC/MS spectrometer operating at 70 eV.

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Plant materials

The aerial parts of *A. montana* were collected at UAm mountain in 1995, and authenticated by Prof. K. S. Lee of the College of Pharmacy, Chungbuk National University. A voucher specimen has been deposited in the herbarium of the College of Pharmacy, Chungbuk National University.

Isolation of compounds

The powdered aerial parts (5.0 kg) of *A. montana* were extracted with MeOH and concentrated *in vacuo* to give a dark residue (750 g), which was suspended in distilled H_2O and partitioned with dichloromethane (160 g), dichloromethane insoluble intermediated phase (Interphase, 20 g), ethyl acetate (25 g), *n*-butanol (95 g), and water (450 g) in sequence. Each extract was tested for its scavenging effect on DPPH radical. The EtOAc and Interphase extracts exhibited strong scavenging activity on DPPH radical, which were subjected to silica gel column chromatography with EtOAc/MeOH (gradient) solution, respectively. Compounds **1** (45 mg) and **2** (25 mg) were obtained from EtOAc extract and compound **3** (55 mg) was obtained from Interphase

extract, respectively.

Compound 1 (apigenin): ^{1}H-NMR (300 MHz, DMSO-d_6) δ; 10.5(1H, OH), 13.0(1H, OH), 6.20(1H, d, J=1.8Hz, H-6), 6.48(1H, d, J=1.8Hz, H-8), 6.78 (2H, d. J=9.0Hz, H-2′ and 6′), 6.93(2H, d, J=9.0Hz, H-3′ and 5′), ^{13}C-NMR (75.5 MHz, DMSO-d_6) δ; 93.87(C-8), 98.73(C-6), 103.13(C-3), 103.59 (C-10), 115.85(C-3′ and 5′), 121.09(C-1′), 128.38 (C-2′ & 6′), 157.22(C-9), 161.05(C-4′), 161.36(C-5), 161.64(C-7), 164.05 (C-2), 181.64 (C-4)

Compound 2 (esculetin): Mp 270-1°C, MS (*m/z*, %); 178(M^+, 100), 150([M-CO]$^+$, 62), ^{1}H-NMR (300MHz, D_2O) δ; 6.20(1H, d, J=9.4, H-3), 6.75(1H, s, H-8), 6.93 (1H, s, H-5), 7.78(1H, d, J=9.4, H-4)

Compound 3 (luteolin 7-O-rutinoside, scolymoside): ^{1}H-NMR (300MHz, DMSO-d_6) δ; 12.09(1H, OH), 7.55 (1H, d, J=2.0, H-2′), 7.42(1H, d, J=8.0 and 2.0, H-6′), 6.92(1H, d, J=8.0, H-5′), 6.73(1H, d, J=1.8, H-8), 6.45 (1H, d, J=2.0, H-6), 5.07(1H, d, J=7.2, anomeric H of Glc), 4.55(1H, s, anomeric H of Rha), 1.07(3H, d, J=6.2, Rha-CH_3) ^{13}C-NMR (75.5MHz, DMSO-d_6) δ; 181.85 (C-4), 164.55 (C-7), 162.85(C-2), 161.20(C-5), 156.89(C-9), 149.99 (C-4′), 145.76(C-3′) 121.30(C-1′), 119.19(C-6′), 116.06 (C-5′), 113.55 (C-2′), 105.36(C-10), 103.13(C-3), 99.88(C-6), 94.73(C-8); Glc-100.51(Glc-1), 73.08(Glc-2), 76.25(Glc-3), 69.75(Glc-4), 75.55 (Glc-5), 66.02(Glc-6); Rha-99.88 (Rha-1), 70.28(Rha-2), 70.28 (Rha-2), 70.71(Rha-3), 72.01(Rha-4), 68.31 (Rha-5), 17.78(Rha-6)

DPPH radical scavenging effect

The DPPH radical scavenging effect was evaluated according to the method first employed by Blois (Blois, 1958). Four milliliters of MeOH solution of varing sample concentrations (0.25-160 μg/ml) was added to 10 ml DPPH methanol solution (1.5×10^{-4} M). After mixing gently and leaving for 30 min at room temperature, the optical density was measured at 520 nm using a spectrophotometer. The antioxi-dant activity of each fraction and sample was expressed in terms of IC_{50} microgram per ml concentration required to inhibit DPPH radical formation by 50%) and calculated from the log-dose inhibition curve.

Assay for the free radical generation

Liver cells (AC_2F) were incubated for 24 h. in serum free media in CO_2 incubator at 37°C until confluent, and the cells were transferred to multiwell plates with about 10^5 cells/well and cultured with or without a suspension of test samples (0.5 mg/ml), then incubated with 12.5 μM DCFH-DA at 37°C for 30 min. Fluorescence was monitored on a spectrofluorometer with excitation and emission wavelengths of 480 nm and 530 nm, respectively.

RESULTS AND DISCUSSION

1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical is a dyed free radical. Due to its odd electron, DPPH radical gives a strong absorption band at 520 nm (deep violet color). The change in absorbance produced by reduced DPPH was used to evaluate the ability of test samples to act as free radical scavenger. The DPPH radical scavenging effect for the methanol extract, its fractions and isolated compounds from *A. montana* are shown in Table I. The IC_{50} values of the methanol extract, and EXOAc, interphase, and *n*-BuOH fractions obtained from the methanol extract were shown at concentrations of 10.1, 1.3, 5.0, and 5.1 mg/ml, respectively, while the CH_2Cl_2- and H_2O-soluble fractions showed no activity (>40 μg/ml).

Recently, 2′, 7′-dichlorofluorescin diacetate (DCFH-DA) has been used as a probe of reactive oxygen species (ROS) such as $\cdot O_2^-$ and H_2O_2. Liposoluble DCFH-DA becomes water-soluble dichlorofluorescin (DCFH) as a result of the activities of mitochondrial esterase or hydrolysis, then it is oxygenized to dichlorofluorescein (DCF) which has strong fluorescence. Therefore, this is useful method to measure changes of ROS (Label and Bondy, 1990). As shown in Table II, the fractions obtained from the MeOH extract of

Table I. Radical scavenging effects on DPPH radical of the methanol extract, its subsequent fractions, and isolated compounds from *A. montana*

Samples	IC_{50}(mg/ml)[a]
MeOH extract	10.1
Interphase fraction	5.0
CH_2Cl_2 fraction	> 40.0
EtOAc fraction	1.3
BuOH fraction	5.1
H_2O fraction	> 40.0
L-ascorbic acid	2.1
Apigenin(**1**)	>120.0
Esculetin(**2**)	1.1
Luteolin 7-O-rutinoside(**3**)	3.4

[a] Amount required for reduction of DPPH radical after 30 min. Values are means of three experiments.

Table II. The effect of the MeOH extract and its fractions on free radical generation of hepatocyte (AC_2F)[a]

Samples	Fluorescence intensity(dF/min)	% of control
control	15.1 ± 0.99	100
CH_2Cl_2[a]	41.9 ± 2.26	242.2
EtOAca	4.0 ± 0.50***	23.1
n-BuOH[a]	9.8 ± 0.71*	56.6
H_2O[a]	7.8 ± 0.07**	45.1
Interphase[a]	9.3 ± 0.28*	53.8

[a] Hepatocytes were incubated in serum free media and prepared at concentration of 0.5 mg/ml. After preincubation for 1 h., 12.5 μM DCFH-DA were added and change in fluorescence was measured. Values are means ± S.E. of three experiments. Statistcal significance: *$p<0.05$, **$p<0.01$, ***$p<0.001$ *vs* control group.

A. montana except for CH_2Cl_2 fraction showed a significant inhibitory activity on free radical generation at a concentration of 0.5 mg/ml. Among them, the EtOAc fraction exhibited greater inhibitory action than the others on the generation of free radicals of hepatocyte. Whereas, CH_2Cl_2 fraction was found to enhance generating effect at the same concentration. The results suggest that the methanol extract and the EtOAc and interphase fractions of *A. montana* are effective radical scavengers. Therefore, the EtOAc- and Interphase fractions were subjected to column chromatographic separation to afford three compounds (**1**~**3**). These compounds were readily elucidated as apigenin, esculetin, and luteolin 7-*O*-rutinoside (scolymoside), respectively, by comparison of reported spectroscopic data, and finally confirmed by comparison with authentic samples (Choi and Woo, 1984, Hirai *et al.*, 1984). A report on their occurrence from the areial parts of *A. montana* has been unprecedented.

The radical scavenging effect of three components obtained from *A. montana* was also shown in Table I. Among three isolated compounds, the *ortho*-dihydroxylated aromatic components esculetin, and luteolin 7-*O*-rutinoside exhibited higher scavenging activity on DPPH with IC_{50} values of 1.1 and 3.4 μg/ml, respectively. However, apigenin without a catechol group showed no activity. In particular, the antioxidiant activity of esculetin was higher than that of L-ascorbic acid, which is a well known antioxidant. These results suggest that the radical scavenging effect in the original methanol extract of *A. montana* was partially attributable to esculetin and luteolin 7-*O*-rutinoside.

Esculetin and luteolin derivatives having catechol moiety, naturally occurring phenolic compounds are reported as antioxidant agent on reactive oxygen species (Hoult and Paya, 1996, Yoshida *et al.*, 1989). Radical scavenging effect of phenolic compounds isolated from natural sources has been widely studied. These compounds react with the free radicals formed during autoxidation, and generate a new radical which is stabilized by the resonance effect of the aromatic nucleus (Cuvelier *et al.*, 1992). The high radical scavenging property of esculetin and luteolin 7-*O*-rutinoside are probably due to a superior stability of radical derived from catechol moiety compared to that of phenoxyl radical (Ruiz-Larrea *et al.*, 1994). The present work indicate that esculetin and luteolin 7-*O*-rutinoside may be useful for the treatment of diseases associated with oxidative damage. Investigation of further antioxidant principles are now in progress.

ACKNOWLEDGEMENTS

This work was supported by Pukyong National University (1998).

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