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Antioxidative Constituents from the Seeds of *Cuscuta chinensis*

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Abstract – MeOH extract of *Cuscuta chinensis* seeds was fractionated with *n*-hexane, EtOAc and BuOH successively, and antioxidant activities were tested for all fractions using DPPH free radical scavenging method. In the tested fractions, EtOAc fraction showed high antioxidant activity(EC_{50}, 50 μg) From the EtOAc fraction, five compounds have been isolated. On the basis of spectral data, these compounds were identified as β-sitosterol, methyl 4-hydroxy-3,5-dimethoxycinnamate, β-sitosterol-3-*O*-β-D-glucopyranoside, caffeic acid, quercetin, kaempferol and calycopteretin. Among these compounds, β-sitosterol and β-sitosterol-3-*O*-β-D-glucopyranoside showed no antioxidant activity. EC_{50} values of methyl 4-hydroxy-3,5-dimethoxycinnamate, caffeic acid, quercetin, kaempferol and calycopteretin were 0.6, 8, 19, 17 and 12 μg, respectively.

Key words – *Cuscuta chinensis*; Convolvulaceae; sitosterols; phenyl propanoids; methyl 4-hydroxy-3,5-dimethoxycinnamate; flavonoids; calycopteretin; antioxidants; DPPH free radical scavenging method

Introduction

Cuscuta chinensis has been used traditional chinese medicine as a tonic(Kim *et al.*, 1997). Studies on the chemical constituents of this plant have been carried out by some investigators, and various constituents have been found(Yahara, *et al.*, 1994; Du, *et al.*, 1998; Jin, *et al.*, 1992).

In the course of a screening program to evaluate antioxidative constituents from medicinal plants, we found that EtOAc soluble fraction from the seeds of *C. chinensis* exhibited a potent antioxidant activity in DPPH (1,1-diphenyl-2-picrylhydrazyl) free radical scavenging assay (Cho *et al.*, 1998). Repeated column chromatography of the EtOAc soluble fraction led to the isolation of seven compounds.

This paper deals with the structure elucidation of these compounds and their antioxidant activities.

Experimental

Plant materials – DPPH, α-tocopherol and BHA (butylated hydroxyanisole) were of reagent grade, purchased from Sigma. The seeds of *C. chinesis* were purchased from Kyungdong market in March, 1999. The voucher specimen has been deposited in the herbarium of the College of Pharmacy, Kangwon National University.

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Reagents and Instruments – For column packing materials Kiselgel 60(70-230 mesh ASTM, Merck, Art 7734), Kiselgel 63(230-400 mesh ASTM, Merck, Art 9385), and ODS(70-230 mesh, ODS-A, YMC-gel) were used. For TLC, Kieselgel $60F_{254}$(precoated, Merck, Art 5715) was employed.

The melting points were taken on a Fisher/Johns melting point appratus(uncorrected). UV absorption spectra were measured by Hitachi U-2000 UV-VIS spectrophotometer. FT-IR spectra were taken on Bio-Rad FTS-7 spectrophotometer using KBr disc method. FAB-MS was taken with a Micromass units. ^{1}H- and ^{13}C-NMR were recorded with a Varian Gemini 200.

Extraction, fractionation and isolation – The air dried seeds of *C. chinensis* (3 kg), were extracted repeatedly 3 times refluxing with MeOH for 4 hours. The total filtrate was concentrated to dryness *in vacuo* at 40°C to obtain MeOH extract (330 g) which was partitioned with *n*-hexane (32 g), EtOAc (6 g) and BuOH (28 g), successively.

The EtOAc soluble fraction (6 g) was subjected to stepwise silica gel column chromatography by eluting with $CHCl_3$-MeOH (9:1)→MeOH (100) to obtain 6 subfractions. Subsequently, the subfraction 1 was further chromatographed on silica gel eluting with benzene:EtOAc (4:1) to obtain compounds I (197 mg) and II (255 mg). The subfractions 3 and 4 were further chromatographed on silica gel and ODS column eluting with $CHCl_3$-MeOH (9:1) and MeOH-H_2O (50:50) to obtain compound IV (34

mg), compound V (17 mg), compound VI (111 mg) and compound VII (34 mg). The subfraction 6 was further chromatographed on ODS eluting with MeOH-H_2O (40:60) to obtain compound III (127 mg).

Measurment of antoxidant activity – A 4 ml of MeOH solution of test extracts at various concentrations (2.5-120 μg/ml) was added to a solution of DPPH (1.5×10^{-4}M) in MeOH (1 ml), and the reaction mixture was shaken vigorously. After storage at room temperature for 30 min. in air, remaining DPPH determined by spectrophotometry at 517 nm, and the radical scavenging activity of each sample was expressed by the ratio of lowering of the absorption of DPPH (%), relative to the absorption (100%) of DPPH solution in the absence of test sample (control). The mean values were obtained from triplicate experiments.

Compound I – mp 137-139; IR, ν_{max}(KBr) 3400 (-OH), 1540, 1450(C=C) cm^{-1}; ^{1}H-NMR(200 MHz, $CDCl_3$) δ: 0.68(3H, s, 18-CH_3), 1.01(3H, s, 19-CH_3), 3.52(1H, m, H-3), 5.36(1H, br s, H-6); ^{13}C-NMR (50MHz, $CDCl_3$): 140.77(C-5), 121.7(C-6), 71.82(C-3), 56.78(C-14), 56.07(C-17), 50.15(C-9), 45.85(C-24), 42.34(C-4), 42.31(C-13), 39.79(C-12), 37.27(C-1), 36.52(C-10), 36.16(C-20), 33.96(C-22), 31.92(C-2), 31.67(C-7), 31.67(C-8), 29.16(C-25), 28.26(C-16), 26.09(C-23), 24.32(C-15), 23.08(C-28), 21.10 (C-11), 19.83(C-26), 19.41(C-19), 19.05(C-27), 18.79 (C-21), 11.87(C-18), 12.00(C-29)

Compound II – mp 84-85(lit. 91-92, Fujita *et al.*, 1984); IR, ν_{max}(KBr) 3400(-OH), 1720(C=O), 1540, 1450(C=C)cm^{-1}; UV: λ_{max}(MeOH), 252, 369 nm; ^{1}H-NMR (200 MHz, $CDCl_3$) δ: 7.61(1H, d, *J*=15.8 Hz, H-7), 6.77(2H, s, H-2 and H-6), 6.31(1H, d, *J*=15.8 Hz, H-8), 3.92(6H, s, -OCH_3×2), 3.80(3H, s, -OCH_3), ^{13}C-NMR (50 MHz, $CDCl_3$) δ: 167.07(C=O), 147.31 (C-3 and C-5), 145.29(C-7), 137.20(C-4), 125.97 (C-1), 115.64(C-8), 105.09(C-2 and C-6), 56.40(OCH_3×2), 51.75($COOCH_3$)

Compound III – mp 283-284°C; IR, ν_{max}(KBr) 3400 (OH), 2958(CH_2), 1271(C-O) cm^{-1}; ^{1}H-NMR (200 MHz, pyridine-d_5) δ: 4.98(1H, d, *J*=7.4Hz, Glc. anomeric H), 0.90(3H, d, *J*=6 Hz, H-21), 0.85(3H, s, H-19), 0.79(3H, d, *J*=6.4 Hz, H-26), 0.78(6H, d, *J*= 6.8 Hz, H-27 and H-28), 0.57(3H, s, H-18); ^{13}C-NMR(50 MHz, pyridine-d_5) δ: 140.83(C-5), 121.84(C-6), 102.46 (C-1'), 78.48(C-5') 78.36(C-3'), 78.01(C-3), 75.21(C-2'), 71.56(C-4'), 62.69(C-6'), 56.71(C-14), 56.12(C-17), 50.21(C-9), 45.90(C-24), 42.34(C-13), 39.81(C-4), 39.20(C-12) 37.34(C-1), 36.79(C-10), 36.26(C-20), 34.07(C-22) 32.04(C-7), 31.92(C-8), 29.83(C-2), 29.31(C-25), 28.40(C-16), 26.23(C-23), 24.37(C-15), 23.24(C-28 21.14(C-11), 19.85(C-27), 19.28(C-19), 19.06(C-26), 18.87(C-21), 12.02 (C-29), 11.83 (C-18); positive FAB-MS, *m/z* 599 $[M+Na]^+$.

Compound IV – mp 223-224°C; UV, λ_{max}(MeOH) 213, 253, 369 nm; ^{1}H-NMR (200 MHz, DMSO-d_6) δ: 7.42(1H, d, *J*=15.8 Hz, H-7), 7.03(1H, d, *J*=1.6 Hz, H-2), 6.98(1H, dd, *J*=1.6, 8.2 Hz, H-6), 6.76(1H, d, *J*=8.2 Hz, H-5), 6.18(1H, d, *J*=15.8 Hz, H-8); ^{13}C-NMR (50 MHz, DMSO-d_6) δ: 168.68(C=O), 148.92 (C-4), 146.34(C-7), 145.34(C-3), 126.48(C-1), 121.89 (C-6), 116.52(C-5), 115.91(C-2), 115.34(C-8).

Compound V – mp >133-134°C; UV, λ_{max}(MeOH) 259, 379 nm; λ_{max}(MeOH+NaOH) 274, 420 nm; λ_{max} (MeOH+NaOAc) 274, 431 nm; λ_{max}(MeOH+$AlCl_3$) 230, 440 nm; λ_{max}(MeOH+$AlCl_3$+HCl) 230, 430 nm; ^{1}H-NMR(200 MHz, DMSO-d_6) δ: 7.68(1H, d, *J*=2.2 Hz, H-2'), 7.57(1H, dd, *J*=8.6, 2.2 Hz, H-6'), 6.89 (1H, d, *J*=8.6 Hz, H-5'), 6.41(1H, d, *J*=1.6 Hz, H-6), 6.19(1H, d, *J*=1.6 Hz, H-8); ^{13}C-NMR (50 MHz, DMSO-d_6) δ: 176.60(C-4), 164.61(C-7), 161.48(C-5), 156.89(C-9), 148.40(C-4'), 147.53(C-2), 145.77(C-3'), 136.49(C-3), 122.69(C-1'), 120.73(C-6'), 116.34 (C-5'), 115.76(C-2'), 103.75(C-10), 98.89(C-6), 94.08 (C-8).

Compound VI – mp >300°C; UV, λ_{max}(MeOH) 266, 324, 362.5 nm; λ_{max}(MeOH+NaOH) 275, 319, 401.5 nm; λ_{max}(MeOH+NaOAc) 272.5, 305.5, 379.5 nm; λ_{max}(MeOH+NaOAc+H_3BO_3) 267, 364 nm; λ_{max} (MeOH+$AlCl_3$) 256, 285, 345, 420.5 nm; λ_{max} (MeOH+$AlCl_3$+HCl) 256, 286, 347, 420.5 nm; ^{1}H-NMR (200 MHz, Acetone-d_6) δ: 8.18(2H, d, *J*=8.8 Hz, H-2'and H-6'), 7.04(2H, d, *J*=8.8 Hz, H-3' and H-5'), 6.55(1H, d, *J*=2.2Hz, H-6), 6.29(1H, d, *J*=2.2 Hz, H-8); ^{13}C-NMR (50MHz, Acetone-d_6) δ: 174.97 (C-4), 163.31(C-7), 160.69(C-5), 158.52(C-4'), 156.13 (C-5), 145.37(C-2), 135.02(C-3), 128.84(C-2' and C-6'), 121.69(C-1'), 114.67(C-3' and C-5'), 102.51(C-10), 97.49(C-6), 92.84(C-8).

Compound VII – mp 276-278°C; UV, λ_{max}(MeOH) 248, 281, 363.5 nm; λ_{max}(MeOH+NaOH) 261, 290, 404.5 nm; λ_{max}(MeOH+NaOAc) 255.5, 282.5, 379.5 nm; λ_{max}(MeOH+NaOAc+H_3BO_3) 257, 282.5, 365 nm; λ_{max}(MeOH+$AlCl_3$) 267, 343, 422 nm; λ_{max} (MeOH+$AlCl_3$+HCl) 261, 345.5, 422.5 nm; ^{1}H-NMR (200 MHz, Acetone-d_6) δ: 7.95(2H, d, *J*=8.8 Hz, H-2' and H-6'), 6.95(2H, d, *J*=8.8 Hz, H-3' and H-5'); ^{13}C-NMR (50 MHz, Acetone-d_6): 174.97(C-4), 165.95

(C-7), 163.30(C-5),1 60.96(C-4'), 158.52(C-5), 145.84 (C-2), 135.06(C-3), 132.51(C-6), 131.11(C-2' and C-6'), 127.00(C-8), 120.97(C-1'), 114.32(C-3' and C-5'), 102.51(C-10).

Results and Discussion

Repeated column chromatography of the EtOAc soluble fractions from the seeds of *C. chinensis* led to the isolation of seven compounds.

Compounds I, III, IV, V and VI were identified as β-sitosterol (Kim and Kang, 1986), β-sitosterol-3-*O*-β-D-glucopyranoside (Iribarren and Pomolio, 1983), caffeic acid (Kim *et al.*, 1999), quercetin (Wenkert and Gottlieb, 1977), kaempferol (Okuyama *et al.*, 1978) and calycopteretin (Fang *et al.*, 1986), respectively, by comparison of their physical and spectroscopic data in the literature (IR, UV, FAB-MS, ^{1}H- and ^{13}C-NMR).

Compound II displayed hydroxyl (3400 cm^{-1}), ester (1720 cm^{-1}), aromatic (1540, 1450 cm^{-1}) absorptions in its IR spectrum. Its ^{1}H-NMR spectrum showed signals due to two aromtic protons at 6.77 (2H, s), two olefinic protons at 7.61(1H, d, *J*=15.8 Hz), 6.31 (1H, d, *J*=15.8 Hz), and three methoxyl groups at 3.92(6H, s) and 3.80(3H, s). This means that compound II should be a cinnamic acid derivative. In the ^{13}C-NMR spectrum (Table 1), twelve carbon signals observed due to three oxygenated carbons, one quaternary carbon, two carbons bearing an aromatic proton and one carbonyl carbon along with three methoxyl groups. From the evidence of ^{13}C-NMR spectrum, compound II was deduced to be a 3,4,5-trisubstituted cinnamic acid derivative. A singlet of ^{1}H-NMR spectrum at δ3.80 and a signal of ^{13}C-NMR spectrum at δ51.75 suggested that compound II has a methyl ester. Therefore, compound II was identified as methyl 4-hydroxy-3,5-dimethoxycinnamate.

Table 1. ^{13}C-NMR chemical shifts of compound II and related compounds

Carbon No.	Compound II[a)]	3,4,5-trimethoxy cinnamic acid[b)]	methyl 3,4,5-trimethoxy cinnamate[b)]
1	125.97	130.2	129.7
2	105.09	106.0	105.9
3	147.31	153.4	153.2
4	137.20	139.8	139.7
5	147.31	153.4	153.2
6	105.09	106.0	105.9
7	145.29	144.5	144.8
8	115.64	118.7	117.0
C=O	167.07	168.2	166.9
OMe	56.40	56.1, 60.4	56.0, 60.1
COOMe	51.75		51.3

[a)]50 MHz, $CDCl_3$

[b)]25.05 MHz, DMSO-d_6 (Fujita *et al.*, 1984)

Table 2. DPPH free radical scavenging activities of n-hexane fraction, EtOAc fraction, BuOH fraction, β-sitosterol, methyl 4-hydroxy-3, 5-dimethoxycinnamate, β-sitosterol-3-*O*-β-D-glucopyranoside, caffeic acid, quercetin, kaempferol and calycopteretin from the seeds of *Cuscuta chinensis*

Test samples	EC_{50} (μg)[a]
MeOH ex. of the seeds of *Cuscuta chinensis*	170
n-hexane fraction	150
EtOAc fraction	50
BuOH fraction	100
β-sitosterol	–
methyl 4-hydroxy-3,5-dimethoxy-cinnamate	0.6
β-sitosterol-3-*O*-β-D-glucopyranoside	–
caffeic acid	8
quercetin	19
kaempferol	17
calycopteretin	12
BHA	14
α-tocopherol	12

* '–' means that substance has no activity.

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

All of the isolated compounds were examined the antioxidant activities using DPPH scavenging activity (Table 2). In the tested samples, methyl 4-hydroxy-3,5-dimethoxycinnamate was showed significantly high antioxidant activity (EC_{50}, 0.6 μg) compared with BHA (EC_{50}, 14 μg) and α-tocopherol (EC_{50}, 12 μg).

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