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In vitro Free Radical Scavenging and Hepatoprotective Compound from Sanguisorbae Radix

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Abstract – In the course of searching for hepatoprotective agents from natural products, four compounds were isolated from the MeOH extract of Sanguisorbae Radix, as guided by their DPPH free radical scavenging activity. The structures were determined as 4,5-dimethoxy-3-hydroxybenzoic acid methyl ester (**1**), (+)-gallocatechin (**2**), methyl 6-*O*-galloyl-β-D-glucopyranoside (**3**), and pomolic acid 3-*O*-[α-L-arabinopyranoside]-28-*O*-[β-D-glucopyranosyl] ester (ziyu-glycoside I) (**4**). Compounds **2** and **3** showed significant DPPH free radical scavenging effects, exhibiting IC_{50} values of 11.4 and 13.0 μM, respectively. L-Ascorbic acid was used as a positive control and exhibited the IC_{50} value of 50.3 μM. In evaluation of the hepatoprotective activity of the isolated compounds on drug-induced cytotoxicity, compound **2** showed the significant hepatoprotective effect with the EC_{50} value of 91.84 ± 11.0 μM on tacrine-induced cytotoxicity in Hep G2 cells, while silybin, a positive control, exhibited EC_{50} value of 122.4 ± 12.5 μM.

Keywords – Sanguisorbae Radix, (+)-gallocatechin, Hepatoprotective, Hep G2 cells, Free radical scavenger

Introduction

In the search for hepatoprotective agents from natural sources, it is important to employ a relevant model system to human liver toxicosis in order to provide therapeutically applicable agents. One of such approaches would be an *in vitro* cell-based hepatoprotective assay system involving hepatotoxic drugs. For a long time, liver toxicity induced by the chemicals and drugs has been recognized as a toxicological problem. Especially, certain drugs given for a prolonged period of time and in high doses are known to lead to serious clinical concern. Tacrine (1,2,3,4-tetrahydro-9-aminoacridine hydrochloride) is an acetylcholinesterase inhibitor, and one of the drugs approved for the treatment of Alzheimer's disease. However, administration of tacrine for the treatment of Alzheimer's disease results in a reversible hepatotoxicity in 30-50% of patients, consequently limiting clinical use (Watkins *et al.*, 1994). Therefore, searching for natural constituents, with protective effect on tacrine-induced hepatotoxicity, would be valuable in providing preparations of potential therapeutic use.

In addition, there is now increasing evidence that free radicals and active oxygen species are involved in a variety of pathological events (Halliwell, 1994). Free radical-mediated cell damage and free radical attack on polyunsaturated fatty acids result in the formation of lipid radicals. These lipid radicals react readily with molecular oxygen to produce peroxy radicals that are responsible for initiating lipid peroxidation. The peroxidation of cellular membrane lipid can lead to cell necrosis, which has been implicated in a number of pathophysiological conditions, as well as in the toxicity of many xenobiotics (Kappus, 1985).

In the course of our continuing search for hepatoprotective agents from medicinal plants, we employed the DPPH free radical scavenging assay as a primary screening system, since the antioxidative effect of certain compounds has been established as one of mechanisms to exert hepatoprotective activity (Oh *et al.*, 2004). The MeOH extract of Sanguisorbae Radix was found to exhibit a promising free radical scavenging effect on 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals. Sanguisorbae Radix has been used for the treatment of hemorrhage and inflammation in oriental traditional medicine (Chang and But, 1986). The reported biological activities of the roots of *Sanguisorba* species include anxiolytic activity (Lee and Chung, 1994), active-oxygen scavenging activity

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(Masaki *et al.*, 1995), inhibitory effect on ultraviolet-B-induced photodamage (Tsukahara *et al.*, 2001), antiviral activity (Kim *et al.*, 2001), hepatoprotective activity (Jeong *et al.*, 2003) in extract level, and inhibition of DNA topoisomerases (Bastow *et al.*, 1993), antiallergic activity (Park *et al.*, 2004) in its components level. This paper deals with the isolation and identification of the free radical scavenging constituents of Sanguisorbae Radix, and their protective effects on tacrine-induced cytotoxicity in Hep G2 cells.

Experimental

Plant material and isolation – Sanguisorbae Radix was purchased from the University Oriental herbal drugstore, Iksan, Korea, in October 2004, and the voucher specimen (No. WP 04-023) was deposited at the Herbarium of the College of Pharmacy, Wonkwang University (Korea). Dried and pulverized Sanguisorbae Radix (1.2 kg) was extracted twice with MeOH (5 L) under the ultrasonic condition for 3 h. The MeOH extract (99 g) was suspended in H_2O and partitioned successively with $CHCl_3$, EtOAc, and *n*-BuOH. A portion (28.5 g) of the EtOAc-soluble extract (48.3 g) was subjected to silica gel column chromatography eluting with CH_2Cl_2-MeOH-H_2O (4 : 1 : 0.1) followed by stepwise addition of MeOH and H_2O to yield three fractions (Fr. A-C). Fraction C (7.06 g) was further fractionated by silica gel column chromatography using CH_2Cl_2-MeOH-H_2O (7 : 3 : 0.3) to afford four fractions (Fr. C1-C4). Fraction C3 (707 mg) was subjected to Sephadex LH-20 (CH_2Cl_2-MeOH, 3:1) chromatography and purified by reversed-phase MPLC (column: ODS-S-50 B, 120 Å, 50 μm) eluting with 20% MeOH in H_2O to give compounds **1** (163.8 mg), **2** (84.4 mg), and **3** (36.0 mg). Fraction C4 (2.46 g) was chromatographed on a reversed-phase (YMC Gel ODS-A, S-75 μm) column eluted with 70% MeOH in H_2O to yield compound **4** (1.5 g).

Compound 1 – White powder; (–)-ESI-MS *m/z* 211 $[M-H]^-$, ^{1}H-NMR (CD_3OD, 500 MHz) δ : 3.83 (3H, s, 5-OCH_3), 3.85 (3H, s, $COOCH_3$), 3.86 (3H, s, 4-OCH_3), 7.14 (1H, d, *J* = 1.8 Hz, H-2), 7.15 (1H, d, *J* = 2.3 Hz, H-6); ^{13}C-NMR (CD_3OD, 125 MHz) δ : 51.3 ($COO\underline{C}H_3$), 55.2 (5-OCH_3), 59.7 (4-OCH_3), 104.7 (C-2), 110.7 (C-6), 125.2 (C-1), 140.8 (C-4), 150.3 (C-5), 153.1 (C-3), 167.0 (C = O).

Compound 2 – Pale yellow amorphous powder, $[\alpha]_D$ = +150° (CH_3OH); (–)-ESI-MS *m/z* 305 $[M-H]^-$, ^{1}H-NMR (CD_3OD, 500 MHz) δ : 2.47-2.82 (2H, m, H-4), 3.96 (1H, m, H-3), 4.52 (1H, d, *J* = 7.3 Hz, H-2), 5.85 (1H, d, *J* = 2.2 Hz, H-8), 5.91 (1H, d, *J* = 2.2 Hz, H-6), 6.39 (2H, s, H-2", 6"); ^{13}C-NMR (CD_3OD, 125 MHz) δ : 26.8 (C-4), 67.4 (C-3), 81.5 (C-2), 94.2 (C-8), 94.9 (C-6), 99.4 (C-4a), 105.9 (C-2", 6"), 130.3 (C-1"), 132.7 (C-4"), 145.5 (C-3", 5"), 155.5 (C-5), 156.3 (C-7), 156.5 (C-8a).

Compound 3 – White powder, (–)-ESI-MS *m/z* 345 $[M-H]^-$, ^{1}H-NMR (CD_3OD, 500 MHz) δ : 3.18-3.56 (4H, m, H-2, 3, 4, 5), 3.49 (3H, s, 1-OCH_3), 4.21 (1H, d, *J* = 7.8 Hz, H-1), 4.38, 4.53 (each 1H, m, H-6), 7.08 (2H, s, galloyl-2, 6); ^{13}C-NMR (CD_3OD, 125 MHz) δ : 57.4 (1-OCH_3), 64.9 (C-6), 71.8 (C-4), 75.2 (C-2), 75.6 (C-5), 78.0 (C-3), 105.6 (C-1), 110.3 (galloyl-2, 6), 121.5 (galloyl-1), 140.0 (galloyl-4), 146.6 (galloyl-3, 5), 168.5 (-COO-).

Compound 4 – Colorless powder; (–)-ESI-MS *m/z* 765 $[M-H]^-$, ^{1}H-NMR (Pyridine-d_5, 500 MHz) δ : 0.91, 0.96, 1.19, 1.26, 1.39 (each 3H, s, 5×CH_3), 1.06 (1H, d, *J* = 6.4 Hz, H-30), 1.70 (1H, s, H-29), 2.94 (1H, s, H-18), 3.32 (1H, dd, *J* = 11.4, 4.1 Hz, H-3), 3.82 (1H, d, *J* = 11.0 Hz, ara-5), 4.15 (1H, dd, *J* = 8.7, 3.4 Hz, ara-3), 4.24 (1H, t, *J* = 8.5 Hz, glc-2), 4.49 (1H, dd, *J* = 11.7, 2.1 Hz, glc-6), 4.76 (1H, d, *J* = 6.9 Hz, ara-1), 5.55 (1H, br. s, H-12), 6.31 (1H, d, *J* = 8.2 Hz, glc-1); ^{13}C-NMR (Pyridine-d_5, 125 MHz) δ : 15.5 (C-25), 16.6 (C-30), 16.8 (C-26), 17.3 (C-24), 18.6 (C-6), 23.9 (C-11), 24.5 (C-27), 26.0 (C-16), 26.6 (C-21), 26.6 (C-2), 26.9 (C-29), 28.1 (C-23), 29.1 (C-15), 33.4 (C-7), 36.9 (C-10), 37.6 (C-22), 38.8 (C-1), 39.5 (C-4), 40.4 (C-8), 42.0 (C-20), 42.0 (C-14), 47.6 (C-9), 48.5 (C-17), 54.3 (C-18), 55.8 (C-5), 62.2 (glc-6), 66.7 (ara-5), 69.4 (ara-4), 71.1 (glc-4), 72.5 (C-19), 72.8 (ara-2), 74.0 (glc-2), 74.5 (ara-3), 78.9 (glc-3), 79.2 (glc-5), 88.7 (C-3), 95.7 (glc-1), 107.4 (ara-1), 128.3 (C-12), 139.2 (C-13), 176.8 (C-28).

***In vitro* free radical scavenging and hepatoprotective assays** – The scavenging action of DPPH radical was measured as follows. The reaction mixture contained 1 mL of 0.1 mM DPPH-ethanol solution, 1 mL of ethanol, 0.95 mL of 0.05 M Tris-HCl buffer (pH 7.4) and 50 μL of either test samples or deionized water (control). Reduction of the DPPH free radical was measured by reading the absorbance at 517 nm exactly 30 sec after adding the samples. L-Ascorbic acid was used as a positive control. The absorbance of the sample alone was subtracted as the blank from that of the reaction mixture. DPPH radical scavenging activity of the sample was expressed as the percent decrease in the absorbance compared with the control. Interaction with DPPH using four different concentrations of the compounds (1, 10, 50, and 100 μM) was carried out.

In vitro hepatoprotective assays used have been

described elsewhere (Song *et al.*, 2001). Briefly, human hepatoma Hep G2 cells, from the American Type Culture Collection, were maintained at 2×10^5 cells/well in complete medium, consisting of RPMI supplemented with 10% heat-inactivated FBS, penicillin G (100 IU/mL) and streptomycin (100 μg/mL), and incubated at 37°C in a humidified 5% CO_2 and 95% air atmosphere. The cytotoxicity was assessed after a 2-h incubation period in media containing tacrine (1.2 mM) or without tacrine (control), and evaluated using the MTT assay. Each sample was tested in triplicate at four different concentrations (10, 30, 50, and 100 μM). All experimental data were calculated from the protective percentages of viability versus control cells, and expressed as the mean ± S.D. of three independent experiments. One-way ANOVA test was applied for detecting the significance. P value < 0.05 was considered statistically significant.

Results and Discussion

In the present study aiming at the identification of antioxidative and/or hepatoprotective components from traditional medicine, the MeOH extract of Sanguisorbae Radix was investigated. Among the partitioned fractions of the MeOH extract, the EtOAc-soluble extract showed the promising DPPH free radical scavenging effect at the concentration of 100 μg/mL. The subsequent assay-guided fractionation of the extract led to the isolation of four compounds (**1-4**). The structures of **1-4** were identified as 4,5-dimethoxy-3-hydroxybenzoic acid methyl ester (Wipf and Weiner, 1999), (+)-gallocatechin (Foo *et al.*, 2000), methyl 6-*O*-galloyl-β-D-glucopyranoside (Tanaka *et al.*, 1984), and pomolic acid 3-*O*-[α-L-arabinopyranoside]-28-*O*-[β-D-glucopyranosyl] ester (ziyu-glycoside I) (Cheng and Cao, 1992; Wenjuan *et al.*, 1986), respectively, on the basis of comparisons of $[\alpha]_D$, MS, ^{1}H-NMR and ^{13}C-NMR data with those reported in the literature (Fig. 1). The free radical scavenging effects of the isolated compounds were tested on DPPH radicals. Two phenolic compounds, which possess a galloyl moiety in each molecule, **2** and **3**, showed significant DPPH free radical scavenging effects with the IC_{50} values of 11.4 and 13.0 μM, respectively. On the other hand, the methylated gallic acid derivative (compound **1**) and the triterpenoidal bisglycoside (compound **4**) did not show the DPPH free radical scavenging activity up to the concentration of 100 μM. L-Ascorbic acid was used as a positive control and exhibited the IC_{50} value of 50.3 μM (Table 1).

Tacrine is an acetylcholinesterase inhibitor approved for the treatment of Alzheimer's disease, but reversible hepatotoxicity has been reported as a side effect of this pharmaceutical reagent (Watkins *et al.*, 1994). Oxidative stress has also been suggested as one of the mechanisms involved in tacrine cytotoxicity (Osseni *et al.*, 1999). These suggest that antioxidative compounds may have protective effects against tacrine-induced cytotoxicity. Therefore, the isolated antioxidative compounds were investigated to test whether the hepatoprotective effects were mediated through their antioxidative effects. Among the isolated compounds, (+)-gallocatechin (**2**) exhibited in a concentration-dependent hepatoprotective effect with EC_{50} value of 91.84 ± 11.0 μM, which is comparable with that (EC_{50} = 122.4 ± 12.5 μM) of a positive control, silybin, on tacrine-induced cytotoxicity in Hep G2 cells (Fig. 2). The viability of Hep G2 cells was not altered in the presence (10-100 μM) or absence of compound **2**. These results suggested that hepatoprotective effect of **2** was mediated through, at least in part, its free radical scavenging activity. On the other hand, compound **3** showed no hepatoprotective effect up to 100 μM level although it possesses free radical scavenging effect.

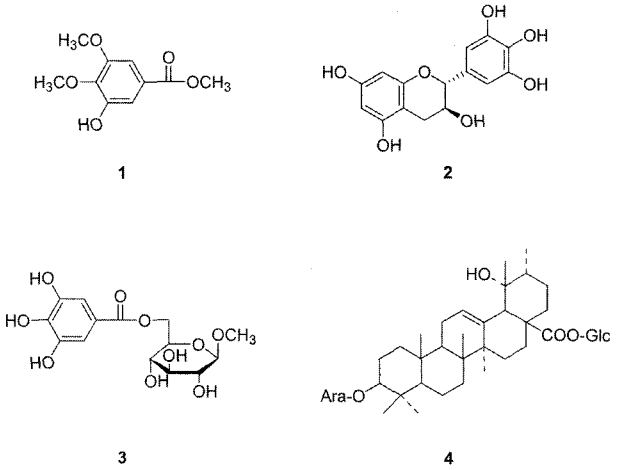

Fig. 1. Chemical structures of compounds **1-4**.

Table 1. DPPH radical scavenging effects of the MeOH extract of Sanguisorbae Radix and its isolated compounds **1-4**

Sample	DPPH radical scavenging effects	
	IC_{50} value (μg/mL)	IC_{50} value (μM)
MeOH extract	29.3	–
1	–	>100
2	3.5	11.4
3	4.5	13.0
4	–	>100
L-Ascorbic acid	–	50.3

Values represent the mean of three independent experiments.

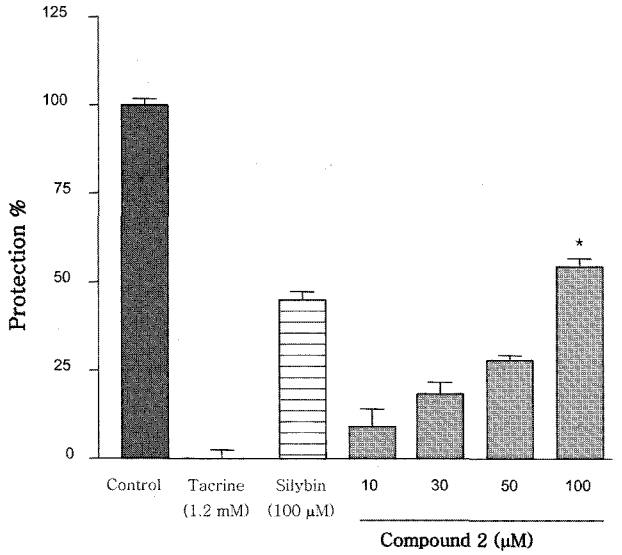

Fig. 2. Hepatoprotective effect of compound **2** on tacrine-induced cytotoxicity in Hep G2 cells. Cytototoxicity was assessed after 2-h incubation period with 1.2 mM of tacrine in RPMI medium. Each value represents the mean ± S.D. of three experiments. Significantly different from the control; $^{*}: p < 0.05$. Silybin was used as a positive control.

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References

Bastow, K.F., Bori, I.D., Fukushima, Y., Kashiwada, Y., Tanaka, T., Nonaka, G., Nishioka, I., and Lee, K.H., Inhibition of DNA topoisomerases by sanguiin H-6, a cytotoxic dimeric ellagitannin from *Sanguisorba dfficinalis*. *Planta Med.*, **59**, 240-245 (1993).

Chang, H.-M. and But, P.P.-H., Pharmacology and Applications of Chinese Materia Medica, World Scientific, Singapore, pp. 468-473 (1986).

Cheng, D. and Cao, X., Pomolic acid derivatives from the root of *Sanguisorba officinalis*. *Phytochemistry*, **31**, 1317-1320 (1992).

Foo, L.Y., Lu, Y., Molan, A.L., Woodfield, D.R., and McNabb, W.C., The phenols and prodelphinidins of white clover flowers. *Phytochemistry*, **54**, 539-548 (2000).

Halliwell, B., Free radicals, antioxidants and human disease: curiosity, cause or consequence? *Lancet*, **344**, 721-724 (1994).

Jeong, C.S., Suh, I.O., Hyun, J.E., and Lee, E.B., Screening of hepatoprotective activity of medicinal plant extracts on carbon tetrachloride-induced hepatotoxicity in rats. *Nat. Prod. Sci.*, **9**, 87-90 (2003).

Kappus, H., Lipid peroxidation: mechanisms, enzymology & biological relevance. In Sies II (ed.). Oxidative Stress. Academic Press, London, pp. 273-310 (1985).

Kim, T.G., Kang, S.Y., Jung, K.K., Kang, J.H., Lee, E., Han, H.M., and Kim, S.H., Antiviral activities of extracts isolated from *Terminalis chebula* Retz., *Sanguisorba officinalis* L., *Rubus coreanus* Miq. and *Rheum palmatum* L. against hepatitis B virus. *Phytother. Res.*, **15**, 718-720 (2001).

Lee, S.Y. and Chung, S.H., Anxiolytic activities of Sanguisorbae Radix. *Yakhak Hoeji*, **38**, 733-741 (1994).

Masaki, H., Sakaki, S., Atsumi, T., and Sakurai, H., Active-oxygen scavenging activity of plant extracts. *Biol. Pharm. Bull.*, **18**, 162-166 (1995).

Oh, H., Kim, D.H., Cho, J.H., and Kim, Y.C., Hepatoprotective and free radical scavenging activities of phenolic petrosins and flavonoids isolated from *Equisetum arvense*. *J. Ethnopharmacol.*, **95**, 421-424 (2004).

Osseni, R.A., Debbasch, C., Christen, M.-O., Rat, P., and Warnet, J.-M., Tacrine-induced reactive oxygen species in a human liver cell line: the role of anethole dithiolethione as a scavenger. *Toxicol. In Vitro*, **13**, 683-688 (1999).

Park, K.H., Koh, D., Kim, K., Park, J., and Lim, Y., Antiallergic activity of a disaccharide isolated from *Sanguisorba officinalis*., **18**, 658-662 (2004).

Song, E.K., Cho, H., Kim, J.S., Kim, N.Y., An, N.H., Kim, J.A., Lee, S.H., and Kim, Y.C., Diarylheptanoids with free radical scavenging and hepatoprotective activity *in vitro* from *Curcuma longa*. *Planta Med.*, **67**, 876-877 (2001).

Tanaka, T., Nonaka, G., and Nishioka, I., Tannins and related compounds. XVI. Isolation and characterization of six methyl glucoside gallates and a gallic acid glucoside gallate from *Sanguisorba officinalis* L. *Chem. Pharm. Bull.*, **32**, 117-121 (1984).

Tsukahara, K., Moriwaki, S., Fujimura, T., and Takema, Y., Inhibitory effect of an extract of *Sanguisorba officinalis* L. on ultraviolet-B-induced photodamage of rat skin. *Biol. Pharm. Bull.*, **24**, 998-1003 (2001).

Watkins, P.B., Zimmerman, H.J., Knapp, M.J., Gracon, S.I., and Lewis, K.W., Hepatotoxic effects of tacrine administration in patients with Alzheimer's disease. *J. Am. Med. Assoc.*, **271**, 992-998 (1994).

Wenjuan, Q., Xiue, W., Junjie, Z., Fukuyama, Y., Yamada, T., and Nakagawa, K., Triterpenoid glycosides from leaves of *Ilex cornuta*. *Phytochemistry*, **25**, 913-916 (1986).

Wipf, P., and Weiner, W.S., Enantioselective synthesis and photoracemization studies of (+)-2-cyclopropyl-7,8-dimethoxy-2*H*-chromene-5-carboxylic acid methyl ester, an advanced intermediate of a dihydrofolate reductase inhibitor. *J. Org. Chem.*, **64**, 5321-5324 (1999).

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