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An Isocoumarin with Hepatoprotective Activity in Hep G2 and Primary Hepatocytes from *Agrimonia pilosa*

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Phytochemical investigation of the aqueous extract of the roots of *Agrimonia pilosa* Ledeb. (Rosaceae), as guided by hepatoprotective activity *in vitro*, furnished two isocoumarins, agrimonolide (**1**) and agrimonolide 6-*O*-β-D-glucoside (**3**), and (+)-catechin (**2**). Compound **1** showed hepatoprotective effects on both tacrine-induced cytotoxicity in human liver-derived Hep G2 cells and *tert*-butyl hydroperoxide-induced cytotoxicity in rat primary hepatocytes with EC_{50} values of 88.2 ± 2.8 and 37.7 ± 1.6 μM, respectively.

Key words: *Agrimonia pilosa*, Rosaceae, Isocoumarin, Agrimonolide, Hepatoprotective

INTRODUCTION

In connection with our studies on the isolation of hepatoprotective constituents from natural products, we have recently reported hepatoprotective compounds including phenolic bakuchiol (Cho *et al.*, 2001), diarylheptanoids (Song *et al.*, 2001), furocoumarins (Oh *et al.*, 2002). In the course of continuing efforts, the aqueous extract of the roots of *Agrimonia pilosa* Ledeb. (Rosaceae) was found to exhibit promising hepatoprotective activity. *A. pilosa* is a perennial herb distributed throughout South Korea, and its roots have been used as the hemostatic, antimalarial, and antidysenteric agent in oriental medicine (Jiangsu New Medical College, 1977). In this paper, we describe the isolation of hepatoprotective component on the basis of its viability against tacrine-induced cytotoxicity in human hepatoma Hep G2 cells as well as the evaluation of protective effect on *tert*-butyl hydroperoxide(*t*-BHP)-induced cytotoxicity in rat primary hepatocytes.

MATERIALS AND METHODS

General experimental procedure

NMR spectra were taken on a JEOL JNM-ECP 500 (^{1}H, 500 MHz; ^{13}C, 125 MHz) spectrometer. ESI-MS spectra were obtained on a API-2000 spectrometer. TLC was carried out on silica gel 60 F_{254} and RP-18 F_{254} plates (Merck, Germany). Column chromatography was performed over silica gel 60 (Merck, particle size 230-400 mesh) and Sephadex LH-20 (Pharmacia, Sweden).

Plant material

The roots of *Agrimonia pilosa* were collected in June 2001 at Mt. Hamra, Jeonbuk Province, Korea, and identified by Dr. Kyu-Gwan Jang, Botanical garden, Wonkwang University. A voucher specimen (no. WP 523) was deposited at the Herbarium of the College of Pharmacy, Wonkwang University (Korea).

Extraction and isolation

Dried and pulverized roots of *A. pilosa* (290 g) were extracted with hot water (500 mL) for 2 h. Filtrated aqueous solution was partitioned with EtOAc (400 mL × 2) and *n*-BuOH (400 mL × 2), successively. The EtOAc soluble fraction (1.83 g) was chromatographed by silica gel (100 g) column with CH_2Cl_2/MeOH/H_2O (3:1:1, 600 mL) to obtain 5 fractions (Fr. A-E). Fr. B (between 120 and 200 mL, 240 mg) was subjected to silica gel (20 g) column chromatography (eluent: *n*-hexane/EtOAc, 3:1) to afford agrimonloide (**1**, between 85 and 125 mL, 44 mg, 0.015 w/w%). The BuOH soluble fraction (3.0 g) was chromatographed by MPLC on reversed-phase C-18 (2.6 × 30 cm) with a gradient of MeOH/H_2O from 2:3 to 1:0.

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Each gradient step (volume: 160 mL) was increased in MeOH by 20%. The 40% aqueous MeOH eluted fraction (240 mg) was subjected to silica gel (30 g) column chromatography using CH_2Cl_2/MeOH (8:1) to yield (+)-catechin (**2**, between 30 and 60 mL, 77 mg, 0.027 w/w%). The MeOH-eluted fraction (200 mg) was separated by silica gel (30 g) column chromatography (eluent: CH_2Cl_2/MeOH, 9:1) to afford agrimonolide 6-*O*-β-D-glucopyranoside (**3**, between 70 and 120 mL, 149 mg, 0.051 w/w). The structures of compounds **1** (Yamato and Hashigaki, 1976), **2** (Kim and Shin, 1998), and **3** (Pei *et al.*, 1989) were identified by comparison of their spectral data with those in the literature. Copies of the original spectra for compounds **1-3** are obtainable from the author of correspondence.

Hepatocyte isolation, culture and hepatotoxicity assays

Hepatocytes were isolated from livers of male Sprague-Dawley rats (200-220 g) fed *ad libitum* with a standard diet by a collagenase perfusion technique and isolated hepatocytes were cultured and treated with *t*-BHP as described previously (Park *et al.*, 2003). Details of tacrine-induced cytotoxicity assay have been described elsewhere (Song *et al.*, 2001). All experimental data were expressed as mean ± S.D. of 3 independent experiments.

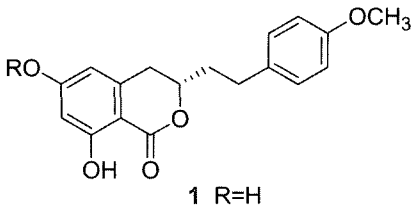
RESULTS AND DISCUSSION

The subsequent assay-guided fractionation of the aqueous extract of this plant led to the isolation of three compounds, agrimonolide (**1**), (+)-catechin (**2**), and agrimonolide 6-*O*-β-D-glucopyranoside (**3**). Compound **1** showed protective effect with the EC_{50} value of 88.2 ± 2.8 μM on tacrine-induced cytotoxicity in human liver-derived Hep G2 cells (Table I). It is revealed that compound **1** possesses the moderate hepatoprotective effect when it compares with that of silybin (EC_{50} = 69.0 ± 3.4 μM). On the other hand, glucoside of **1** (**3**) did not showed hepatoprotective effect in our assay system. The viability of Hep G2 cells was not altered in the presence (1-100 μM) or absence of

Table I. Protective activities of 1-3 on tacrine-induced cytotoxicity in Hep G2 cells and *t*-BHP-induced cytotoxicity in primary rat hepatocytes

Compound	EC_{50} value (μM)	
	Hep G2 cells	Primary rat hepatocytes
1	88.2 ± 2.8	37.7 ± 1.6
2	>100	>100
3	>100	>100
Silybin	69.0 ± 3.4	67.2 ± 3.5

Values represent the mean ± S.D. of 3 independent experiments.



1 R=H
3 R=glc

Fig. 1. Chemical structures of compounds **1** and **3** isolated from *A. pilosa*

1, as determined by MTT assay (data not shown). Compound **1** had been previously isolated from the same plant (Yamato and Hashigaki, 1976), however, no biological activity has been reported.

Tacrine(1,2,3,4-tetrahydro-9-aminoacridine hydrochloride) is an acetylcholinesterase inhibitor approved for the treatment of Alzheimers disease, and reversible hepatotoxicity as a side effect of this pharmaceutical reagent had been reported (Watkins *et al.*, 1994). It was also suggested that oxidative stress is one of the mechanisms involved in tacrine cytotoxicity (Osseeni *et al.*, 1999). To verify this assumption, evaluation of compounds **1-3** on *tert*-butyl hydroperoxide(*t*-BHP)-induced cytotoxicity in rat primary hepatocytes was also conducted.

t-BHP is an organic hydroperoxidant that can be metabolized to free radical intermediates, and by exposing primary cultures of rat hepatocytes to *t*-BHP it is possible to mimic several aspects related to liver pathology characterized by increased lipid peroxidation and cytotoxicity due to oxidative stress (Yamamoto and Farber, 1992). Among the tested compounds, **1** showed significant inhibition of the cellular leakage of lactate dehydrogenase (LDH) on the treatment with 1.5 mM *t*-BHP (Table I). It is revealed that compound **1** (EC_{50} = 37.7 ± 1.6 μM) possesses the hepatoprotective effect when it compares with that of silybin (EC_{50} = 67.2 ± 3.5 μM). From these results, it is suggested that the hepatoprotective activity of compound **1** is related to its free radical scavenging effect.

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