Arch Pharm Res Vol 28, No 1, 44-48, 2005

Archives of Pharmacal Research
http://apr.psk.or.kr

Hepatoprotective Constituents of *Cudrania tricuspidata*

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(Received September 2, 2004)

Phytochemical investigation of the MeOH extract of the root barks of *Cudrania tricuspidata* Bureau (Moraceae), as guided by hepatoprotective activity *in vitro*, furnished four isoprenylated xanthones, cudratricusxanthone A (**1**), cudraxanthone L (**2**), cudratricusxanthone E (**3**), and macluraxanthone B (**4**). All of these compounds showed the significant hepatoprotective effect on tacrine-induced cytotoxicity in human liver-derived Hep G2 cells. Compounds **1**, **2**, and **4** also exhibited the significant hepatoprotective effect on nitrofurantoin-induced cytotoxicity in human liver-derived Hep G2 cells.

Key words: *Cudrania tricuspidata*, Moraceae, Isoprenylated xanthone, Hepatoprotective, Hep G2 cells

INTRODUCTION

In the searching for the hepatoprotective agents from natural sources, it is important to use more relevant model system to human liver toxicosis to provide agents with therapeutic potential. Liver toxicity induced by the chemicals and drugs has been recognized as a toxicological problem for over 100 years. The amount of medicines consumed has increased greatly, resulting in dangers to the liver. Especially, some of drugs are given for prolonged period of time and in high doses lead to the serious clinical concern. Therefore, it is valuable to use the hepatotoxic agents that are more relevant to human liver toxicosis such as some drugs with liver toxic effect as their adverse effect. Tacrine (1,2,3,4-tetrahydro-9-aminoacridine hydrochloride) and nitrofurantoin [1-(5-nitro-2-furfurylideneamino)-hydantoin] belong to such category of drugs. Tacrine is an acetylcholinesterase inhibitor, and used for the treatment of Alzheimer's disease. Unfortunately, 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). Nitrofurantoin is a synthetic nitrofuran commonly used for the treatment and prophylaxis of urinary tract infections. It is reported that liver cirrhosis and fatal liver necrosis associated with the use of nitrofurantoin (Amit *et al.*, 2002; Edoute *et al.*, 2001). Therefore, studies for the constituents from natural medicines with protective effect on the tacrine- and/or nitrofurantoin-induced hepatotoxicity would be valuable as providing potential therapeutic use. In the present study, an immortalized human hepatoma cell line, Hep G2 was employed in the screening of hepatoprotective agents against tacrine-induced hepatotoxicity, since it retains many cellular functions often lost by primary hepatocytes such as expression of hepatocyte-specific cell surface receptors and synthesis and secretion of plasma proteins (Grant *et al.*, 1988).

In the course of screening for hepatoprotective constituents from plants, the $CHCl_3$ soluble fraction of MeOH extract of the root barks of *Cudrania tricuspidata* Bureau (Moraceae), which is one of the Chinese traditional medicine used for the treatment of lumbago, haemoptysis, and contusion (Jiang Su New Medical College (ed.), 1977), was found to have a promising hepatoprotective activity. This paper deals with the isolation and identification of hepatoprotective constituents of *C. tricuspidata* root barks.

MATERIALS AND METHODS

General experimental procedure

NMR spectra were taken on a JEOL JNM-ECP 500 (^1H, 500 MHz; ^{13}C, 125 MHz) spectrometer. ESI-MS spectra were obtained on an 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

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over silica gel 60 (Merck, particle size 230-400 mesh) and Sephadex LH-20 (Pharmacia, Sweden).

Plant material

The root barks of *Cudrania tricuspidata* were purchased in June 2004 at Kumsan Crude drug market, Chungnam Province, Korea, and identified by Dr. Kyu-Kwan Jang, Botanical garden, Wonkwang University. A voucher specimen (no. WP 527) was deposited at the Herbarium of the College of Pharmacy, Wonkwang University (Korea).

Extraction and isolation

Dried and pulverized root barks of *C. cuspidata* (4 kg) were extracted twice with hot MeOH (2×20 L) for 2 h. The dried MeOH extract (168 g) was partitioned between equal volumes of *n*-hexane and 60% aqueous MeOH, and the aqueous MeOH layer extracted subsequently with $CHCl_3$. Finally, the 60% aqueous MeOH mixture was evaporated *in vacuo* and partitioned between *n*-BuOH and H_2O. A portion (10.8 g) of hepatoprotective $CHCl_3$ soluble fraction (53.3 g) was chromatographed by silica gel column with $CHCl_3$/MeOH/H_2O (9:1:0.1 → 4:1:0.1 → 6:4:1) to obtain 6 fractions (Fr. A-F). Fr. C (4.32 g) was subjected to silica gel column chromatography (eluent: $CHCl_3$/MeOH, 40:1 → 10:1) to afford 5 fractions (Fr. C1-C5). Fr. C3 (520 mg) was purified by Sephadex LH-20 column chromatography with $CHCl_3$/MeOH (15:1) to give cudratricusxanthone A (**1**, 93.1 mg). Fr. C4 (1.33 g) was further purified using Sephadex LH-20 column ($CHCl_3$/EtOAc, 8:1), followed by RP C-18 column (85% aqueous MeOH) to afford cudraxanthone L (**2**, 69.2 mg). Fr. C5 (1.15 g) was chromatographed by Sephadex LH-20 column with $CHCl_3$/MeOH (25:1) to yield 3 subfractions (Fr. C51- C53). Fr. C51 (400 mg) was purified with RP C-18 column chromatography (eluent: acetonitrile:H_2O, 7:3) to give cudratricusxanthone E (**3**, 11.8 mg). Fr. C52 (183 mg) was subjected to Sephadex LH-20 column chromatography using $CHCl_3$/MeOH (30:1) to yield macluraxanthone B (**4**, 101.2 mg). The structures of compounds **1** and **3** (Zou *et al.*, 2004), **2** (Hano *et al.*, 1991), and **4** (Groweiss *et al.*, 2000) were identified by comparison of their spectral data with those in the literature. Copies of the original spectra for compounds **1**-**4** are obtainable from the author of correspondence.

Assay for hepatoprotective activity on drug-induced cytotoxicity in Hep G2 cells

Details of hepatoprotective bioassays have been described elsewhere (Song *et al.*, 2001). Briefly, human hepatoma Hep G2 cells from American Type Culture Collection were maintained 2×10^5 cells/well in complete medium consisting of RPMI supplemented with 10% heat-inactivated FBS, penicillin G (100 IU/mL), streptomycin (100 μg/mL) and incubated at 37°C in a humidified atmosphere containing 5% CO_2 and 95% air. Cytotoxicity was assessed after 2-h incubation period in the corresponding medium containing tacrine (1.2 mM) or nitrofurantoin (1.6 mM) or without drugs (control), and evaluated by MTT assay. Four concentrations (10, 50, 100, 200 μg/mL for MeOH extract and fractions; 1, 5, 10, 20 μg/mL for compounds) were tested for each sample, and each experiment was performed in triplicate. The results were expressed protection ratio as the percentage of viability vs. control. Silybin (Sigma Chemical Co.) was tested as positive control. One-way ANOVA test was applied for detecting the significance of difference. $P<0.05$ was regarded as significant.

RESULTS AND DISCUSSION

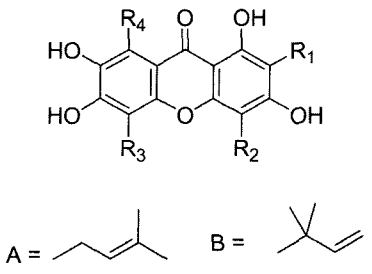
Solvent partition of the MeOH extract of *C. tricuspidata* root barks furnished *n*-hexane, $CHCl_3$, *n*-BuOH, and aqueous fractions. Protective effects of MeOH extract and its fractions on tacrine-induced cytotoxicity in Hep G2 cells have been evaluated (Table I). MeOH extract showed significant hepatoprotective effect *in vitro* when it compared to silybin. The $CHCl_3$ soluble fraction exhibited the most effective rate of cell viability among the tested samples. All of the samples, however, exhibited cytotoxicity over the concentration of 100 μg/mL. Activity-guided fractionation of the $CHCl_3$ soluble fraction afforded four isoprenylated xanthones, cudratricusxanthone A (**1**), cudraxanthone L (**2**), cudratricusxanthone E (**3**), and macluraxanthone B (**4**).

Tacrine is an acetylcholinesterase inhibitor approved for the treatment of Alzheimer's disease, but 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 evaluate the *in vitro* hepatoprotective effects of

Table I. Protective effects of the MeOH extract and its fractions of *C. tricuspidata* on tacrine-induced cytotoxicity in Hep G2 cells

Samples	Concentration (μg/mL)			
	10	50	100	200
MeOH extract	19.0	44.0	46.9	36.2
n-Hexane Fr.	9.4	34.1	49.1	38.1
$CHCl_3$ Fr.	38.2	77.0	90.8	18.5
n-BuOH Fr.	36.7	40.5	53.1	24.2
Aqueous Fr.	7.0	8.6	19.7	13.0
Silybin	-	46.5	-	-

Values represent the protective percentages of viability vs. control, which show the mean of 3 independent experiments.



1 $R_1 = R_3 = H$, $R_2 = B$, $R_4 = A$
2 $R_1 = B$, $R_2 = R_4 = H$, $R_3 = A$
3 $R_1 = R_2 = A$, $R_3 = R_4 = H$
4 $R_1 = B$, $R_2 = A$, $R_3 = R_4 = H$

Fig. 1. Chemical structures of compounds **1-4** isolated from *C. tricuspidata*

isolated compounds from *C. tricuspidata*, protective effects on tacrine-induced cytotoxicity in Hep G2 cells were tested. Compounds **1-4** showed concentration-dependent protective effects up to the concentration of 10 μg/mL, and exhibited the maximal hepatoprotective effects at 10 μg/mL with the protection ratio of 63.1 ± 3.2, 69.0 ± 3.7, 86.4 ± 6.4, and 90.3 ± 2.0%, respectively (Fig. 2). Silybin as positive control showed the protection ratio of 40.2 ± 2.9% at the concentration of 20 μg/mL, which concentration having the maximal protection effect of silybin. All of these compounds, however, revealed the decreased hepatoprotective effects at the concentration of 20 μg/mL. It is suggested that these decreasing hepatoprotective effects are related to their cytotoxicity in corresponding concentration. In connection with this phenomenon, the cytotoxic effect of compounds **1** and **3** had been reported (Zou *et al.*, 2004).

These promising results encouraged us to survey the protective effects of the isolated four xanthones (**1-4**) on nitrofurantoin-induced cytotoxicity in Hep G2 cells. Nitrofurantoin is a synthetic nitrofuran commonly used for the treatment and prophylaxis of urinary tract infections. However, it is established that nitrofurantion associated with liver cirrhosis and fatal liver necrosis in some cases (Amit *et al.*, 2002; Edoute *et al.*, 2001). Oxidative stress is involved in the liver toxicity of nitrofurantoin (Klee *et al.*, 1994), and it complexed to an endogenous peptide, which is presented by the class I HLA antigen on the hepatocyte cell membrane, inducing cytotoxic T cell activation and subsequently, hepatocyte death (Kelly *et al.*, 1998). Because of similar cytotoxic mechanisms between tacrine and nitrofurantoin, it was expected that compounds **1-4** might have protective effects on nitrofurantoin-induced cytotoxicity in Hep G2 cells as in the case of tacrine-induced

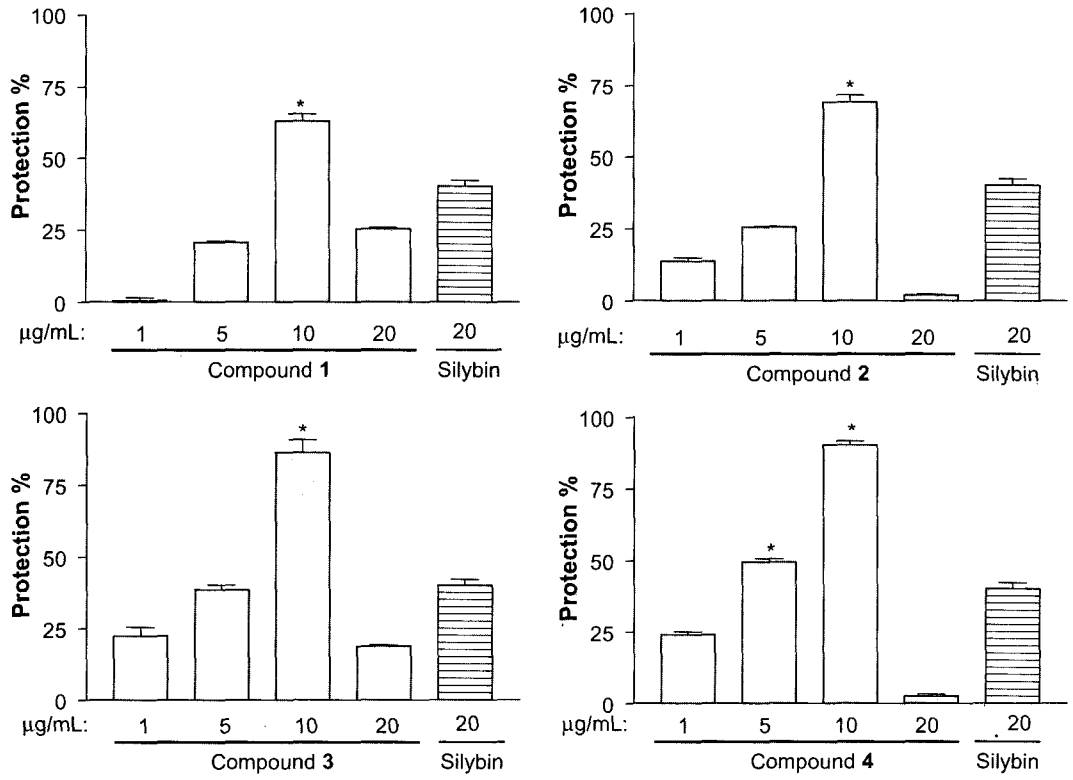


Fig. 2. Protective effects of compounds **1-4** on tacrine-induced cytotoxicity in Hep G2 cells. Cytotoxicity was assessed after 2-h incubation period with 1.2 mM of tacrine in RPMI medium. Each value represents the mean±S.D. of three experiments. Significantly different from the control; *$p<0.05$. Silybin was used as positive control.

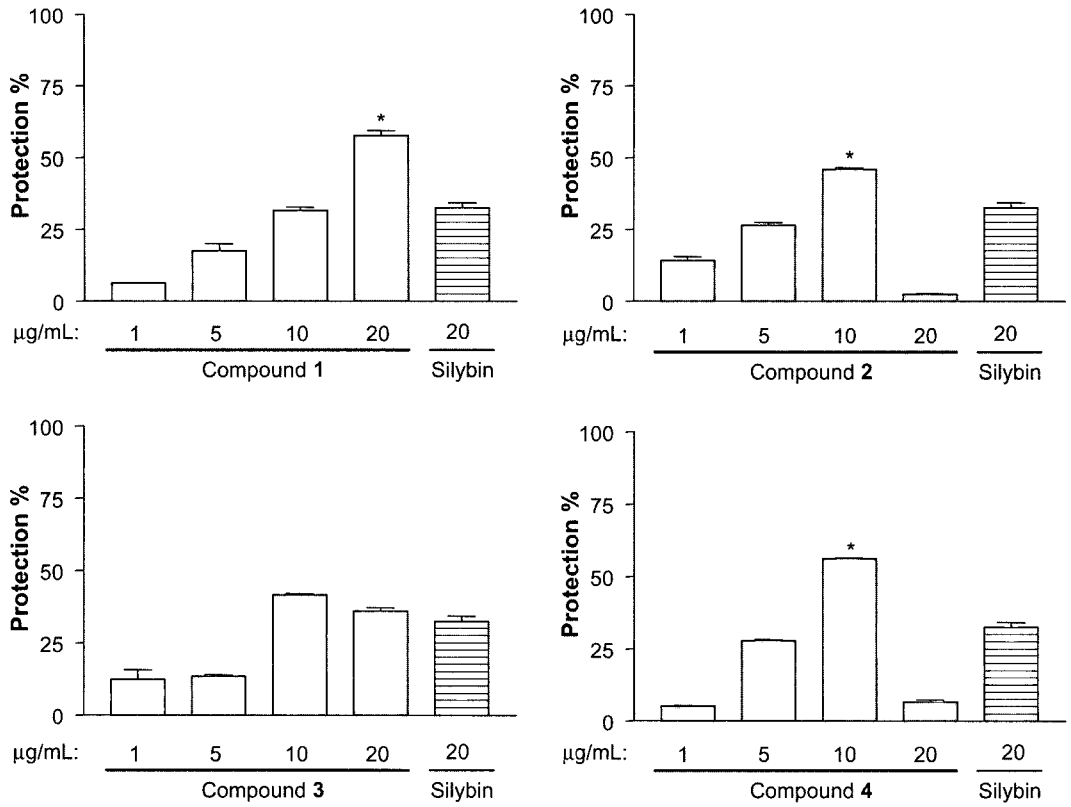


Fig. 3. Protective effects of compounds **1-4** on nitrofurantoin-induced cytotoxicity in Hep G2 cells. Cytotoxicity was assessed after 2-h incubation period with 1.6 mM of nitrofurantoin in RPMI medium. Each value represents the mean±S.D. of three experiments. Significantly different from the control; *$p<0.05$. Silybin was used as positive control.

cytotoxicity. As shown in Fig. 3, compounds **1-4** showed concentration-dependent protective effects. Compound **1** did not show the cytotoxicity up to the concentration of 20 μg/mL, which is the concentration for the maximal hepatoprotective effect with the protection ratio of 57.7±2.5%. Silybin as positive control showed the protection ratio of 32.6±2.4% at the concentration of 20 μg/mL.

In conclusion, four isoprenylated xanthones from *C. tricuspidata* showed significant hepatoprotective effects *in vitro*, and this suggests that these compounds can be valuable source of potential liver protective agents.

ACKNOWLEDGEMENT

This work was supported by Wonkwang University in 2003.

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