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Antioxidant Activities of Isoflavones from the Rhizomes of *Belamcanda chinensis* on Carbon Tetrachloride-Induced Hepatic Injury in Rats

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The present study was carried out to clarify whether tectorigenin and tectoridin isolated from the rhizomes of *Belamcanda chinensis* (Iridaceae) inhibit hepatic damage induced by carbon tetrachloride (CCl_4)-intoxication in rats by the experimental methods *in vitro* and *in vivo*. Tectorigenin and tectoridin exhibited a significant decrease in serum transaminase activities elevated by hepatic damage induced by CCl_4-intoxication in rats, as well as in a lipid peroxidation causing a significant decrease in malondialdehyde (MDA) production by thiobarbituric acid (TBA)-reactant assay. Both compounds also showed strong increase in the antioxidant enzymes such as hepatic cytosolic superoxide dismutase (SOD), catalase and glutathione peroxidase (GSH-px) activities in CCl_4-intoxicated rats. These results suggested that tectorigenin and tectoridin isolated from the rhizomes of *B. chinensis* possess not only the antioxidative, but also the hepatoprotective activities in CCl_4-intoxicated rats.

Key words: *Belamcanda chinensis*, Iridaceae, Tectorigenin, Tectoridin, Carbon tetrachloride, Antioxidant activity

INTRODUCTION

There is extensive evidence to implicate free radicals in the development of degenerative diseases (Cross, 1987). It is suggested that free radical damage to cells leads to the pathological changes associated with aging (Beckman and Ames, 1998). Free radicals may also be a contributory factor in a progressive decline in the function of the immune system (Pike and Chandra, 1995). Cooperative defense systems that protect the body from free radical damage include the nutrients and antioxidant enzymes. The antioxidant enzymes include superoxide dismutase (SOD), catalase, glutathione peroxidase (GSH-px) and glutathione reductase. Their roles as protective enzymes are well established and have been investigated extensively both *in vivo* and *in vitro* model systems. The first three enzymes directly catalyze the transformation of peroxides and superoxides to nontoxic species. Glutathione reductase reduces oxidized glutathione to glutathione, a substrate for glutathione peroxidase.

The consequences of oxidative stress are serious, and in many cases are manifested by increased activities of enzymes involved in oxygen detoxification. Identification of new antioxidants remains a highly active research area because antioxidants may reduce the risk of various chronic diseases caused by free radicals.

Belamcanda chinensis is a perennial shrub growing on the hillsides in the East Asia including the Korean peninsula and has been used as Chinese traditional medicine for the treatment of throat ailment such as asthma and tonsillitis from this species. A number of isoflavonoids have been already isolated (Ito *et al.*, 2001).

It has been reported that tectorigenin isolated from the root of *Pueraria thunbergiana* has hypoglycemic, hypolipidemic and *in vitro* antioxidant effects (Lee *et al.*, 2000). It was also reported that tectorigenin has antimutagenic and anti-lipid peroxidative effect caused by bromoben-

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zene in rats (Park *et al.*, 2002).

In this present study, we report *in vivo* antioxidative effect of tectorigenin and tectoridin in the carbon tetrachloride (CCl_4)-intoxicated rats by investigating the activities of antioxidant enzyme.

MATERIALS AND METHODS

Plant materials

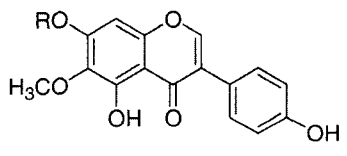
The rhizomes of *Belamcanda chinensis* were collected at the vicinity of Seoul, Korea and the voucher specimen (Voucher No. NPRI-97-037) was deposited at the Herbarium of Natural Products Research Institutes, Seoul National University, Korea.

Extraction and isolation

The air-dried powdered rhizomes (10 kg) were extracted three times with methanol (3 × 18 L). The extract thus obtained (1.8 kg) was partitioned between equal volumes of *n*-hexane and 10% methanol, and aqueous methanol layer was concentrated and was suspended in water and extracted subsequently with methylene chloride (180 g) and *n*-butanol (700 g). Repeated SiO_2 column chromatography (6 × 30 cm) of the methylene chloride fraction with a gradient of *n*-hexane : ethyl acetate (100 : 1 to 9 : 1) gave tectorigenin (5,7,4'-trihydroxy-6-methoxyisoflavone, 120.0 mg, 0.0012% from the dry rhizomes), and repeated SiO_2 column chromatography (12 × 65 cm) of the *n*-butanol fraction with a gradient of methylene chloride : methanol (100 : 0 to 9 : 1) gave tectoridin (5,4'-dihydroxy-6-methoxy-7-*O*-β-D-glucopyranosylisoflavone, 20.0 g, 0.2% from the dry rhizomes). Their chemical structures are shown in Fig. 1.

Chemicals

Sodium azide, ethylenediamine tetraacetic acid (EDTA), β-nicotinamide adenine dinucleotide phosphate, reduced form (NADPH), cumene hydroperoxide, glutathione reductase, DL-α-tocopherol, DL-α-tocopherol acetate (TA), CCl_4, xanthine, potassium cyanide (KCN), sodium dodecylsulfate, trichloroacetic acid (TCA), cytochrome *C*, thiobarbituric acid (TBA), *n*-butanol, pyridine and 1,1-diphenyl-2-picrylhydrazyl (DPPH) were purchased from Sigma Chem. Co. (St. Louis, MO). All other chemicals and reagents were analytical grade.



Tectorigenin: R = H

Tectoridin: R = β-D-glucopyranosyl

Fig. 1. Chemical structures of tectorigenin (5,7,4'-trihydroxy-6-methoxy-isoflavone) and tectoridin (5,4'-dihydroxy-6-methoxy-7-*O*-β-D-glucopyranosyl-isoflavone)

Animals

Animal study was carried out in a pathogen-free barrier zone at Seoul National University Hospital in accordance with the procedure outlined in the Guide for the Care and Use of Laboratory Animals. Male Sprague-Dawley rats weighing 200-250 g were used in all experiments. Animals were maintained on 12 h light/dark cycle at approximately 22°C and allowed food and water *ad libitum*.

Hepatic cytosolic extract preparation

All treatments were conducted between 9:00 and 10:00 AM to minimize variations in animal response due to circadian rhythm. Rats were injected i.p. with a mixture of CCl_4 in olive oil (1:1) at a dose of 0.6 mL/kg to induce hepatotoxicity. Control animals were given the vehicle alone. Rats were pretreated once with TA or test samples were given intraperitoneally (i.p.) at a dose of 100 mg/kg/day for seven consecutive days prior to the administration of CCl_4. Animals were sacrificed 24 h after CCl_4 administration and blood was collected by decapitation for the determination of serum transaminases.

Hepatic tissues were carefully excised and homogenized in cold 1.15% KCl-10 mM phosphate buffer with EDTA (pH 7.4) and centrifuged at 10,000 rpm for 10 min. The supernatant was further centrifuged at 40,000 rpm for 60 min to obtain cytosolic extract for the measurement of liver cytosolic SOD, catalase, GSH-px activities and malondialdehyde (MDA) content. The protein content was measured by the method of Lowry *et al.* (1951) with bovine serum albumin as a standard.

Determination of serum AST and ALT activities

Hepatocellular damage was estimated by measuring aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities (Reitman and Frankel, 1957). Blood was collected from the abdominal aorta of each rat. The blood was centrifuged at 3,000 rpm at 4°C for 10 min to separate the serum.

Determination of hepatic antioxidative enzyme activities

SOD was assayed by the method of McCord and Fridovich (1969). The reaction mixture containing 0.5 mM xanthine as substrate (300 μL), 0.05 mM KCN (100 μL), 1% sodium deoxycholate (100 μL), xanthine oxidase (20 μL), cytosolic extract (20 μL) and 0.1 mM cytochrome C (300 μL) was placed in 1 cm cuvette and the rate of increase in absorbance at 550 nm was recorded for 5 min. SOD activity was expressed as unit/mg protein.

Catalase was assayed by the method of Rigo and Rotilio (1977). The cytosolic extract of liver (40 μL) diluted 10 times was added with 0.13 mM phosphate buffer (pH 7.0, 500 μL), distilled water (660 μL) and 15 mM H_2O_2 (1800 μL), and thoroughly mixed. The rate of changes in the absorbance at 240 nm for 5 min was recorded. Catalase activity was expressed as unit/mg protein.

GSH-px was assayed by the method of Burk *et al.* (1978). The reaction mixture containing 0.3 mM phosphate buffer with 4.0 mM EDTA (pH 7.2, 1000 μL), 26.56 mM sodium azide (500 μL), 294.37 mM GSH (60 μL), 8.4 mM NADPH (110 μL), 1 mM cumene hydroperoxide (320 μL), glutathione reductase (5 μL) and cytosolic solution (30 μL) was placed in 1 cm cuvette and the rate of changes in absorbance was recorded at 340 nm for 5 min. GSH-px activity was expressed as unit/mg protein.

Determination of lipid peroxidation

Lipid peroxidation in rat liver microsomes was evaluated by the TBA method (Buege and Aust, 1978). The reaction mixture of liver microsome, 8.1% sodium dodecylsulfate (0.2 mL) and TCA-thiobarbituric acid in 20% acetate buffer (2 mL, pH 3.5) was heated for 1 h in boiling water bath. After cooling, *n*-butanol:pyridine (15:1) solution was added and centrifuged to obtain *n*-butanol:pyridine layer. The absorbance of the sample was determined at 532 nm. The level of lipid peroxides is expressed as MDA nmol/mg protein.

Statistical analysis

The data are shown as the mean ± S.E.M. Significant difference was calculated by Student's *t*-test. Significance was accepted at $p < 0.05$.

RESULTS AND DISCUSSION

Tectorigenin and tectoridin isolated from the rhizomes of *B. chinensis* were tested for their free radical scavenging effects and the effects on lipid peroxidation as well as on hepatocellular damage in CCl_4-intoxicated rats.

The free radical scavenging potency of the tectorigenin and tectoridin were measured as IC_{50} of 275.0 and 813.3 μM, respectively. The value was lower than that of the D,L-α-tocopherol (IC_{50}=10.4 μM), as a positive reference compound (data not shown). Lipid peroxidation was assessed by determining the production of MDA in rat liver microsome. The effects of tectorigenin and tectoridin to prevent lipid peroxidation are shown in Fig. 2. These compounds, when administered *i.p.* with a dosage of 100 mg/kg/day for seven consecutive days prior to the administration of CCl_4, were shown to exhibit significant inhibition of MDA production. In the CCl_4-intoxicated rats, the MDA content (7.57 ± 0.65 nmol/mg protein) was much higher than in the normal rats (4.19 ± 0.49 nmol/mg protein). In the CCl_4-intoxicated rats with the tectorigenin, tectoridin or TA, the MDA content was significantly reduced by 83.5, 69.7, and 74.0%, respectively.

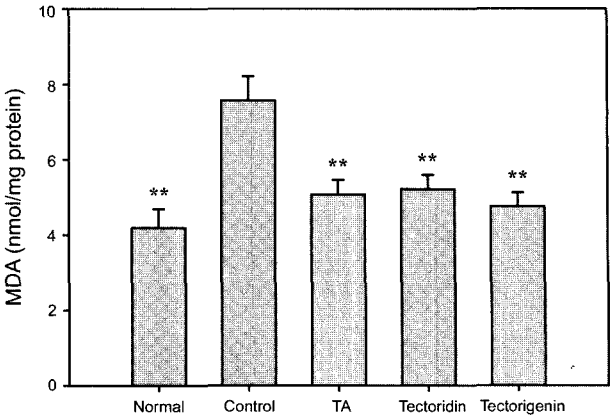


Fig. 2. Effects of tectorigenin and tectoridin on the hepatic MDA production. Each value represents the mean ± S.E.M. Significantly different from the control; $^{**}p<0.01$. TA (D,L-α-tocopherol acetate) was used as positive control.

The effects of both compounds on serum transaminases in CCl_4-intoxicated rats were estimated and the results are shown in Fig. 3. In the CCl_4-intoxicated group, the AST and ALT levels were increased significantly when compared with the normal group. In the CCl_4-intoxicated rats with the tectorigenin, tectoridin or TA, the AST and ALT level were significantly inhibited by 47.4, 42.8 and 48.2%, respectively and by 39.8, 36.1, and 56.7% respectively.

Fig. 4 depicts the effect of treatment of rats with CCl_4 and pretreatment with tectorigenin, tectoridin or TA, on the activities of SOD, catalase, and GSH-px enzyme in liver homogenate. Treatment of rats with a single dose of CCl_4 at 0.6 mL/kg significantly reduces the activities of SOD, catalase, and GSH-px enzyme. However, pretreatment of the rats with tectorigenin and tectoridin restored the activities of SOD, catalase, and GSH-px by 75.6, 63.8, and 70.4% and by 94.9, 60.2, and 63.4%, respectively. Restoration of SOD activity indicates that both compounds can help in cellular defense mechanisms by preventing cell membrane oxidation. Increase in the catalase activity with respect to CCl_4 treatment indicates that tectorigenin and tectoridin can play an important role in scavenging hydrogen peroxide. Similarly, an increase in glutathione peroxidase activity indicates that both compounds also help in the restoration of vital molecules such as NAD, cytochromes, and glutathione. It has been reported that chronic dietary administration of genistein, which resembles in chemical structure with tectorigenin, significantly elevated the activities of catalase, SOD, glutathione peroxidase, and glutathione reductase in murine skin and small

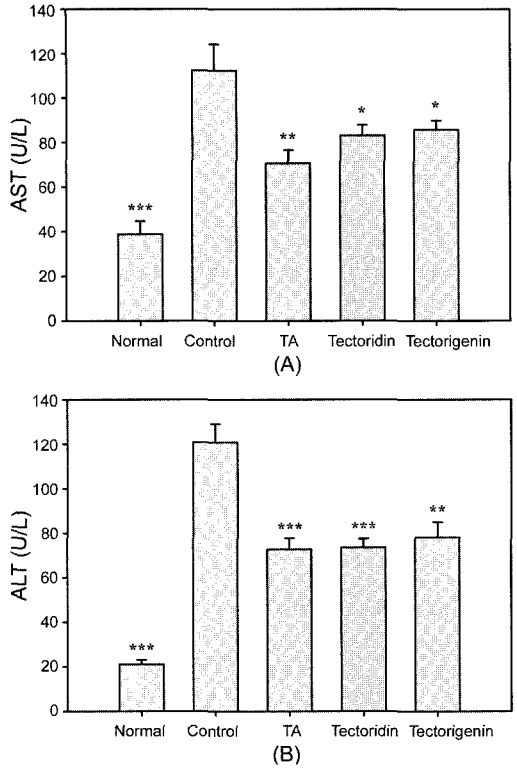


Fig. 3. Effects of tectorigenin and tectoridin on the AST (A) and ALT (B) activities. Each value represents the mean ± S.E.M. Significantly different from the control; *p<0.05, **p<0.01, ***p<0.001. TA (D,L-α-tocopherol acetate) was used as positive control.

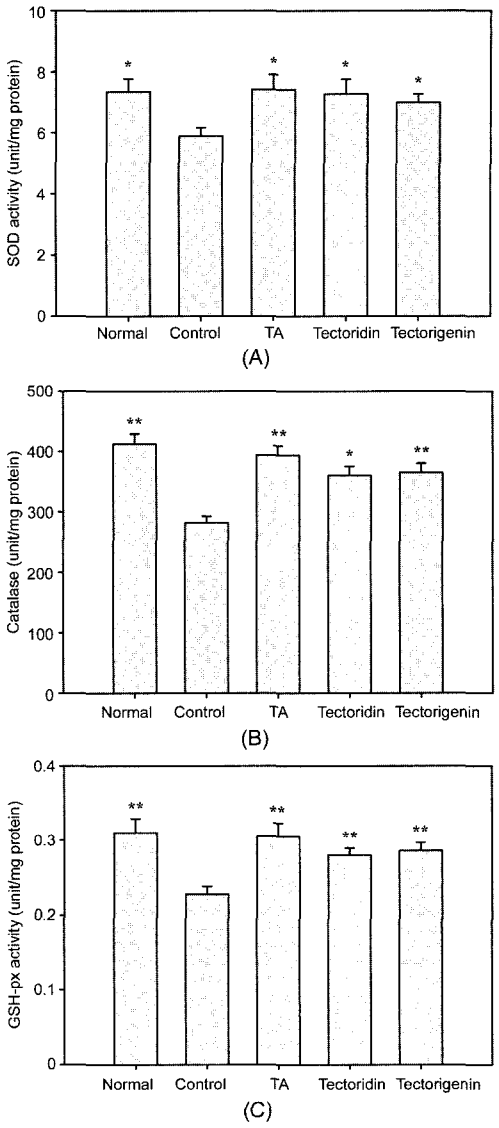


Fig. 4. Effects of tectorigenin and tectoridin on the liver cytosolic SOD (A), catalase (B), and GSH-px (C) activities. Each value represents the mean ± S.E.M. Significantly different from the control; *p<0.05, **p<0.001. TA (D,L-α-tocopherol acetate) was used as positive control.

intestine (Wei *et al.*, 1995). These findings strongly support our results that the antioxidant activity of tectorigenin and tectoridin might be due to their ability to enhance the activities of antioxidant enzymes.

We previously demonstrated that tectorigenin and tectoridin was capable of inhibiting 12-O-tetradecanoyl phorbol-13-acetate (TPA)-mediated inflammatory responses in macrophages (Kim *et al.*, 1999), and suppress angiogenesis and tumor progression *in vitro* and *in vivo* (Jung *et al.*, 2003). Oxidants induce a number of oncogenes associated with the tumor process (Kovacic and Jacintho, 2001). Oxidants induce a number of growth factors that are related to cell growth and angiogenesis. Induction of VEGF by oxidants has been reported recently. A wide variety of metabolic pathways, including the activation of protein kinases, NF-κB, other transcription factors, and cyclins have been implicated in the stimulation of cell growth by oxidants (Brar *et al.*, 2002). Since reactive oxygen species and oxidative damage to DNA have been implicated in the carcinogenic process, inhibition of MDA and stimulation of antioxidant enzymes by tectorigenin and tectoridin may, at least in part, explain the mechanisms of anti-tumorigenic action of both compounds.

In conclusion, the results of the present studies indicate that tectorigenin and tectoridin isolated from the rhizomes of *Belamcanda chinensis* is capable of protecting the hepatic enzymes, which play important roles in combating the reactive oxygen species. Both compounds also provide a hepatoprotection against CCl_4 toxicity as indicated by serum transaminase level. Tectorigenin and tectoridin did not show potent free radical scavenging activity. However, both compounds showed antioxidative activity by inducing hepatic antioxidant enzymes such as SOD, catalase and GSH-px as well as by inhibiting lipid peroxidation. Further studies may be undertaken to elucidate the mechanisms involved in the enhancement of enzyme activity and protective activity on lipid peroxidation.

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