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ORGAN TOXICITY AND MECHANISMS

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Inhibitory effect of esculetin on oxidative damage induced by *t*-butyl hydroperoxide in rat liver

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Abstract Increasing evidence regarding free radical-generating agents and inflammatory processes suggests that accumulation of reactive oxygen species can cause hepatotoxicity. A short-chain analog of lipid hydroperoxide, *t*-butyl hydroperoxide (t-BHP), can be metabolized to free radical intermediates by cytochrome P-450 in hepatocytes, which in turn can initiate lipid peroxidation, affect cell integrity and result in cell injury. In this study, we used t-BHP to induce hepatotoxicity in vitro and in vivo and determined the antioxidative bioactivity of esculetin, a coumarin compound. Our investigations showed that pretreatment with esculetin (5–20 μg/ml) significantly decreased the leakage of lactate dehydrogenase (LDH) and alanine transaminase (ALT), and also decreased the formation of malondialdehyde (MDA) in primary cultured rat hepatocytes induced by a 30-min treatment with t-BHP. An in vivo study in rats showed that pretreatment with esculetin (i.p.) at concentrations of 0.5 and 5 mg/kg for 5 days before a single i.p. dose of t-BHP (0.1 mmol/kg) significantly lowered the serum levels of the hepatic enzyme markers (ALT and AST) and reduced oxidative stress in the liver. Histopathological evaluation of the rat livers revealed that esculetin reduced the incidence of liver lesions induced by t-BHP, including hepatocyte swelling, leukocyte infiltration, and necrosis. Based on the results described above, we speculate that esculetin may play a chemopreventive role via reducing oxidative stress in living systems.

Key words Esculetin · *t*-Butyl hydroperoxide · Hepatotoxicity

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Introduction

The study of numerous compounds that could be useful as antioxidants including, for example, α-tocopherol and *β*-carotene and plant antioxidants such as flavones and tannins, has attracted increasing interest in the fields of food and medicine. Esculetin is a coumarin derivative present in many plants such as *Artemisia capillaris* (Compositae) and the leaves of *Citrus limonia* (Rutaceae) (Chang et al. 1996) which are used in herbal tea. It has shown multiple biological activities including the inhibition of xanthine oxidase activity (Egan et al. 1990), tumor cell proliferation (Matsunaga et al. 1998), and platelet aggregation (Okada et al. 1995). It also decreases the activity of ferric soybean lipoxygenase 1 (Kemal et al. 1987) and 5-lipoxygenase (Neichi et al. 1983). Due to its polyphenolic structure (Fig. 1), esculetin may show antioxidant activity in biological systems.

t-Butyl hydroperoxide (t-BHP) can be metabolized to free radical intermediates by cytochrome P-450 (hepatocytes) or hemoglobin (erythrocytes), which can subsequently initiate lipid peroxidation (Hogberg et al. 1975), affect cell integrity and form covalent bonds with cellular molecules, resulting in cell injury (Rush et al. 1985). Alternatively, t-BHP can be rapidly converted by glutathione peroxidase to *t*-butyl alcohol and glutathione disulfide (GSSG). GSSG is then converted to reduced glutathione (GSH) by GSSG reductase, resulting in pyridine nucleotide oxidation (NADP). Loss of GSH and oxidation of pyridine nucleotides are associated with altered Ca^{2+} homeostasis, which is considered to be a critical event in the formation of blebs on plasma

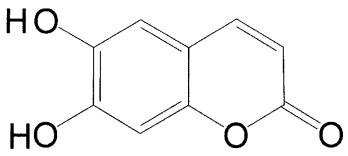

Fig. 1 Chemical structure of esculetin

membranes, an early sign of t-BHP-induced toxicity (Davies 1989; Thornalley et al. 1983). t-BHP is known to cause lactate dehydrogenase (LDH) and alanine aminotransferase (ALT) leakage, malondialdehyde (MDA) formation and GSH depletion in hepatocyte cultures (Joyeux et al. 1990; Tseng et al. 1996). It has also been shown to mediate DNA base damage in mammalian cells (Altman et al. 1994). These phenomena are similar to the oxidative stress occurring in cells and/or tissue. Oxidative stress is considered to play a prominent role in many inflammatory conditions such as cancer, and for example in aging (Breimer 1990; Cerutti 1985).

To investigate the protective potential of esculetin in living systems, we studied its antioxidant action against t-BHP-induced oxidant damage in cultured rat hepatocytes and rat livers. The results showed that esculetin inhibited the injury induced by t-BHP in rat primary hepatocytes and rat livers probably by reducing oxidative stress.

Materials and methods

Chemicals

t-BHP, esculetin, DPPH (1,1-diphenyl-2-picrylhydrazyl), collagenase, thiobarbituric acid, EDTA, GSH, *o*-phthalaldehyde (OPT), and kits for LDH, aspartate aminotransferase (AST) and ALT were purchased from Sigma Chemical Co. (St. Louis, Mo.). Solvents were obtained from E. Merck (Darmstadt, Germany), protein assay kits from Bio-Rad Laboratories (Watford, UK), and medium for cell culture from GIBCO (Grand Island, N.Y.).

Determination of free radical quenching capacity

The free radical quenching capacity of esculetin was determined by a method involving the bleaching of stable DPPH (Ursini et al. 1994). A reaction mixture containing methanol, DPPH (10 m*M*, 30 μl) and 100 μl of solutions of various concentrations of esculetin (final concentrations 2 or 10 μg/ml) or the natural antioxidant quercetin (final concentration 10 μg/ml) was allowed to stand at room temperature for 30 min. After mixing with 1 ml redistilled water and 3 ml toluene, the solution was centrifuged, and the absorbance of the upper phase was read at 517 nm against a blank processed as above but without esculetin or quercetin.

Preparation of hepatocytes

Male Sprague-Dawley rats (250–300 g) (Taichung Veterans General Hospital Animal Center, Taiwan) were used for the experiments. Hepatocytes were prepared by the method of two-stage collagenase perfusion (Bonney et al. 1974) and cultured in Williams E medium supplemented with an antibiotic mixture of penicillin, streptomycin and neomycin (1%) and fetal calf serum (10%), and gassed with O_2/CO_2 (95%/5%). Cells were plated out at a density of 1×10^6 cells per 60-mm dish, and treated with chemicals as indicated in the following assay 3 h after attachment.

Microculture MTT assay

The range of nontoxic dosage levels of esculetin was established using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay with some modification (Alley et al. 1988). Briefly, to the primary cultured rat hepatocytes at a density of 5×10^4 cells/dish, esculetin was added at various concentrations in the range 10–100 μg/ml. After a 24-h incubation, the medium was replaced by one containing 20 μl MTT (5.0 mg/ml) with PBS washing in between. The cells were incubated for another 4 h, and then the blue crystals, which are the metabolized product of MTT, were extracted with isopropanol. Absorbance at 563 nm was determined and used for the measurement of the proportion of surviving cells.

Cytotoxicity assay of hepatocytes

After pretreatment with esculetin for 1 h at the final concentrations of 5, 10 and 20 μg/ml, hepatocytes were incubated with t-BHP (1.5 m*M*) for 0.5 h as previously described (Tseng et al. 1996). Next, 1 ml of the culture medium was removed, and the activities of LDH and ALT were determined following the procedures provided with the commercial kits (Sigma Chemical Co.). The cytotoxicity of hepatocytes was expressed in terms of the activity of LDH and ALT released from the treated cells.

Lipid peroxidation assay

Hepatocytes were pretreated with esculetin, and then with t-BHP as described above. The lipid peroxidation product, MDA, was assayed according to an improved thiobarbituric acid fluorometric method at 553 nm with excitation at 515 nm using 1,1,3,3-tetramethoxypropane as the standard (Yagi 1987). The protein concentration was determined using a standard commercial kit (Bio-Rad Laboratories) with bovine serum albumin as a standard.

Animal treatment

Male Sprague-Dawley rats (220 ± 10 g) were used for the experiments. The rats were provided with food and water ad libitum and divided into five groups (six rats per group). To study the protective effect against the t-BHP-induced hepatotoxicity, esculetin (0.5 and 5 mg/kg) was given daily by intraperitoneal (i.p.) injection to the animals for five consecutive days. On the 5th day, t-BHP (0.1 mmol/kg) was injected (i.p.) into each animal, and 18 h later the rats were killed by decapitation and blood samples were collected for assay of ALT and AST. The livers were excised from the animals and assayed for MDA formation (as described above), GSH level and pathological histology according to the procedures described below.

Hepatotoxicity assessment

The hepatic enzymes AST and ALT were used as biochemical markers for early acute hepatic damage. The serum activities of AST and ALT were determined by the colorimetric method of Reitman and Frankel (1957).

Glutathione assay

A small portion of rat liver was removed for the GSH assay by the method of Hissin and Hilf (1976). In brief, the tissue was homogenized with phosphate buffer containing 25% meta-phosphoric acid. After centrifugation (100,000 *g*, 30 min), OPT was added to the supernatant followed by incubation for 15 min at room temperature and pH 8. Fluorescence at 420 nm was determined, the excitation being at 350 nm. The results are expressed as micrograms per gram liver.

Pathological histology

Immediately after removal from the animals, hepatic tissues were fixed in 10% buffered formaldehyde, processed for histological examination according to conventional methods and stained with hematoxylin and eosin. The morphology of any lesions observed was classified and recorded.

Statistical analysis

The data are reported as means ± standard deviations, and analyzed using Dunnett's *t*-test. Differences were considered statistically significant for *P*-values < 0.05.

Results

Free radical quenching capacity of esculetin

For the determination of the free radical quenching capacity of esculetin, the bleaching of DPPH by esculetin was measured and compared with that of quercetin, a flavonoid that possesses strong antioxidant activity. The results, summarized in Table 1, show that esculetin was able to quench 52% of DPPH free radicals at a concentration of 2 μg/ml and 92% at 10 μg/ml, and quercetin was able to quench 68% of the free radicals at 10 μg/ml. From the results of our previous study and the study reported here, we defined the dose effect curve and found that the EC_{50} of DPPH bleaching for esculetin and quercetin is similar (about 25 μ*M*).

Effect of esculetin on t-BHP-induced cytotoxicity

The MTT assay showed that after 24 h treatment with up to 50 μg/ml esculetin, primary cultures of hepatocytes showed over 90% cell viability (Table 2). Therefore, 5, 10 and 20 μg/ml of esculetin were used for the subsequent cell culture experiments. Adding esculetin to the primary cultured hepatocytes partially protected the cells from the cytotoxicity induced by t-BHP as expressed by leakage of LDH and ALT (Fig. 2). The leakage induced by treatment of hepatocytes with 1.5 m*M* t-BHP for 30 min was partially decreased following pretreatment with esculetin.

Table 1 Effect of esculetin on DPPH bleaching. Esculetin or quercetin was mixed with DPPH (10 m*M*, 30 μl) in methanol (3 ml). The reaction mixtures were then colored by the addition of toluene, and read at 517 nm against a blank without esculetin or quercetin. The degree of DPPH bleaching is expressed as a percentage in relation to the absorbance of the control. Each value is the mean from three independent experiments

	DPPH bleaching (%)
Control	0
Esculetin (2 μg/ml)	52 ± 1
Esculetin (10 μg/ml)	92 ± 2
Quercetin (10 μg/ml)	68 ± 2

Table 2 Cytotoxicity of esculetin in hepatocytes as determined by a microculture tetrazolium assay. Hepatocyte cultures were treated with various concentrations of esculetin for 24 h. The values are means ± SD ($n = 3$)

Treatment (μg/ml esculetin)	$OD_{563\ nm}$	Absorbance (% of control)
Control	0.82 ± 0.08	100
10	0.80 ± 0.08	97
20	0.79 ± 0.15	96
50	0.74 ± 0.06	90
100	0.73 ± 0.07	89

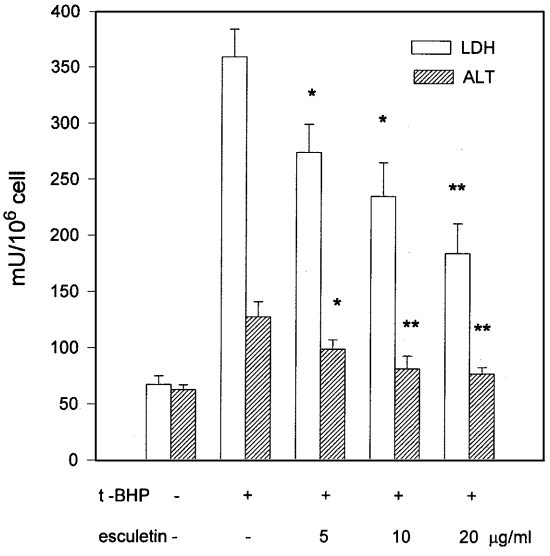

Fig. 2 Effect of esculetin on the leakage of LDH and ALT from primary cultured rat hepatocytes treated with 1.5 m*M* t-BHP for 30 min. Each column represents the mean value and bars the SD ($n = 3$). *$P < 0.05$, ** $P < 0.01$, vs treatment with t-BHP alone

Effect of esculetin on lipid peroxidation of hepatocytes

Lipid peroxidation has been recognized as a potential mechanism of cell injury. The MDA concentration, an index of lipid peroxidation, was increased in hepatocytes treated with 1.5 m*M* t-BHP for 30 min alone. Pretreatment with esculetin (5–20 μg/ml) significantly decreased the formation of MDA ($P < 0.05$ and $P < 0.01$; Fig. 3) indicating that esculetin effectively inhibited t-BHP-induced lipid peroxidation.

Effects of esculetin on t-BHP-induced rat hepatotoxicity

The hepatic enzymes AST and ALT were used as biochemical markers for early acute hepatic damage. A single dose of t-BHP given to rats by i.p. injection after 18 h caused elevations of serum AST and ALT and increased levels of MDA (Table 3) and also led to a significant depletion of GSH in the liver compared with the control group. Esculetin pretreatment attenuated the effect of t-BHP indicating that esculetin has the potential to reduce the hepatotoxicity of t-BHP (Table 3).

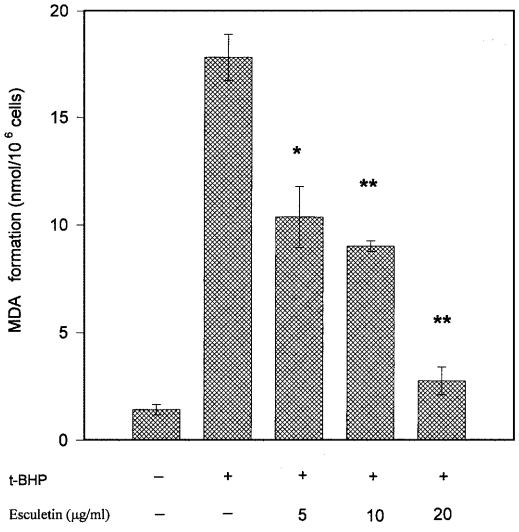

Fig. 3 Effect of esculetin on the t-BHP-induced lipid peroxidation in primary cultured rat hepatocytes. Each column represents the mean value and bars the SD ($n = 3$). *$P < 0.05$, **$P < 0.01$, vs treatment with t-BHP alone

Pathological histology of the liver

Treatment of rats with t-BHP led to neutrophil infiltration into the liver, and swelling of the liver cells and necrosis (Fig. 4B). Histological examination showed that the livers of rats pretreated with 0.5 mg/kg esculetin showed only slight neutrophil infiltration and swelling of cells (Fig. 4C). Furthermore, the rats pretreated with 5 mg/ml esculetin showed no hepatotoxicity (Fig. 4D).

Discussion

Recently, much attention has been focused on the protective biochemical function of naturally occurring antioxidants in biological systems, and on the mechanisms of their action. Phenolic compounds, which are widely distributed in plants, are considered to play an important role as dietary antioxidants in the prevention of oxidative damage in living systems (Block 1992; Hertog and Feskens 1993). Esculetin is a phenolic compound, and its antioxidative effects in living system were determined in this study. Consistent with the results of a previous studies (Chang et al. 1996; Miguel et al. 1992), in vitro experiments demonstrated that esculetin has a strong capacity to quench DPPH free radicals. Further evaluation in hepatocytes showed that esculetin decreased t-BHP-induced MDA formation at nontoxic concentrations. However, we cannot rule out the possibility that esculetin may have inhibited the cell damage caused by t-BHP by interacting directly with the t-BHP in the medium.

In in vivo experiments, we used t-BHP to induce hepatic oxidative damage and investigated the protective effects of esculetin. Pretreatment with esculetin (5 mg/kg) partially protected rats from hepatotoxicity and liver inflammation caused by t-BHP, as indicated by the reduced serum leakage of LDH and ALT and decreased formation of MDA, neutrophil infiltration, necrosis and cell swelling in the liver. LDH and ALT are known to be general indices of hepatic cytotoxicity. MDA, on the other hand, is the major oxidative degradation product of membrane unsaturated fatty acid, and has been shown to be biologically active with hepatotoxic and genotoxic properties (Husain et al. 1987). In addition, it has been proposed that reactive oxygen species such as superoxide anion, hydrogen peroxide and hydroxyl radicals generated in inflamed tissue can cause injury to target cells (Shimoda et al. 1994). We therefore suggested that esculetin could inhibit t-BHP-induced oxidative damage in the liver by blocking the cytotoxicity induced by t-BHP and decreasing progressive damage during inflammation. Furthermore, esculetin may partially inhibit the arachidonic cascade induced by t-BHP in liver tissue subsequently alleviating the inflammation caused by t-BHP (Neichi et al. 1983).

GSH acts as an essential intracellular reducing agent for maintenance of thiol groups on intracellular proteins and for antioxidant molecules. It is well established that GSH, the most important biomolecule protecting against chemically induced cytotoxicity, can participate in the elimination of reactive intermediates by conjugation (Habig et al. 1974) and hydroperoxide reduction, or of free radicals by direct quenching. A relationship between GSH concentration and the extent of liver damage has been demonstrated in experiments in which the

Table 3 Effect of esculetin on serum enzymes and hepatic MDA and GSH in rats treated with t-BHP. Animals were pretreated i.p. with various concentrations of esculetin for five consecutive days before administration of t-BHP, and were killed 18 h later. Serum ALT and AST and hepatic MDA and GSH were then determined

Treatment	AST (U/l)	ALT (U/l)	MDA (nmol/g liver)	GSH (μg/g liver)
Control	121 ± 16	50 ± 9	100 ± 14	1606 ± 157
Esculetin (5 mg/kg)	128 ± 28	57 ± 12	106 ± 12	1628 ± 257
t-BHP (0.1 mmol/kg)	605 ± 81	204 ± 48	198 ± 18	1329 ± 229$^{\#}$
Esculetin (0.5 mg/kg) plus t-BHP	394 ± 55**	141 ± 26*	134 ± 15*	1570 ± 253*
Esculetin (5 mg/kg) plus t-BHP	255 ± 30**	109 ± 28**	118 ± 16**	1726 ± 244**

*$P < 0.05$ vs group treated with t-BHP alone ($n = 6$); **$P < 0.01$ vs group treated with t-BHP alone ($n = 6$); $^{\#}P < 0.05$ vs control group ($n = 6$)

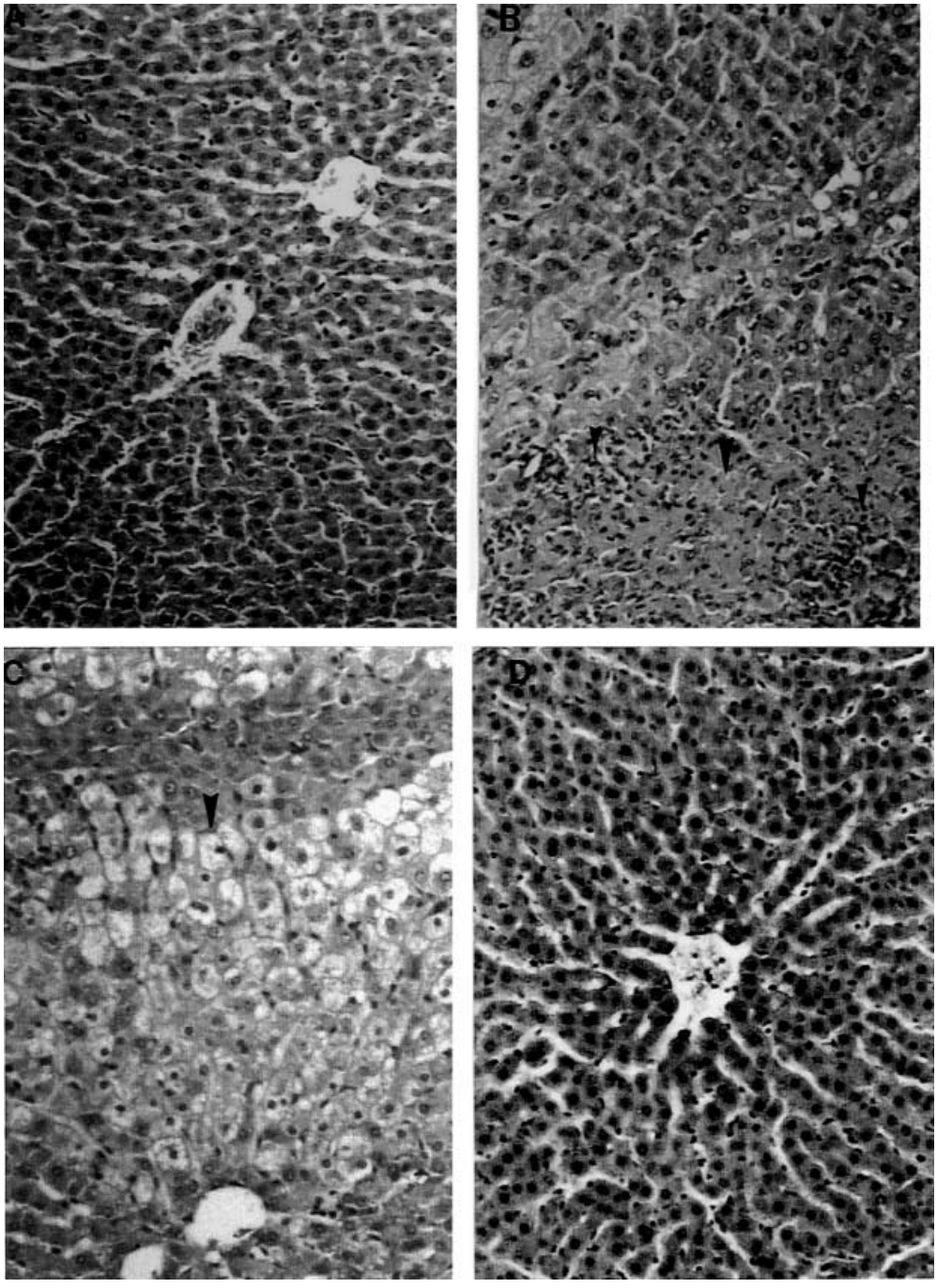

Fig. 4A–D Effects of esculetin on t-BHP-induced liver damage. **A** section of liver from a control animal treated with solvent; **B** section of liver from an animal treated with 0.1 mmol/kg t-BHP, showing severe neutrophil leukocyte infiltration (*small arrow*), degeneration and necrosis (*large arrowhead*); **C** section of liver from an animal pretreated with 0.5 mg/kg esculetin and then with t-BHP, showing partial hepatocyte degeneration and swelling (*large arrowhead*); **D** section of liver from an animal pretreated with 5 mg/kg esculetin and then with t-BHP, showing no pathological change. Hematoxylin/eosin staining; ×100

hepatic concentration of GSH was altered by toxin treatment. Although we did not determine the time of maximum GSH depletion induced by t-BHP in rat liver, our study showed that t-BHP had reduced the GSH level significantly after 18 h and that pretreatment with esculetin effectively attenuated this effect. Since it has been reported that mice treated with 25 mg/kg esculetin orally for 30 days show an increased glutathione reductase activity (Martin-Aragon et al. 1998), in addition to free radical scavenging, the effect of esculetin on the GSH system shown in this study needs further investigation.

The results of this study demonstrated that esculetin inhibits hepatotoxicity induced by t-BHP via its antioxidant potential. Many natural antioxidant products are capable of preventing or inhibiting the process of carcinogenesis (Rice-Evans 1999; Surh 1999). We therefore speculate that esculetin may show a chemopreventive effect in living systems.

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