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Studies on Protective Effect of DA-9601, *Artemisia asiatica* Extract, on Acetaminophen- and CCl_4-induced Liver Damage in Rats

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The hepatoprotective effect of DA-9601, a quality-controlled extract of *Artemisia asiatica*, on liver damage induced by acetaminophen (APAP) and carbon tetrachloride (CCl_4) was investigated by means of serum-biochemical, hepatic-biochemical, and histopathological examinations. Doses of DA-9601 (10, 30, or 100 mg/kg) were administered intragastrically to each rat on three consecutive days i.e. 48 h, 24 h and 2 h before a single administration of APAP (640 mg/kg, i.p.) or CCl_4 (2 ml/kg, p.o.). Four h and 24 h after hepatotoxin treatment, the animals were sacrificed for evaluation of liver damage. Pretreatment of DA-9601 reduced the elevation of serum ALT, AST, LDH and histopathological changes such as centrilobular necrosis, vacuolar degeneration and inflammatory cell infiltration dose-dependently. DA-9601 also prevented APAP- and CCl_4-induced hepatic glutathione (GSH) depletion and CCl_4-induced increase of hepatic malondialdehyde (MDA), a parameter of lipid peroxidation, in a dose-dependent manner. These findings suggest that pretreatment with DA-9601 may reduce chemically induced liver injury by complex mechanisms which involve prevention of lipid peroxidation and preservation of hepatic GSH.

Key words : DA-9601, *Artemisia asiatica*, Hepatoprotection, Acetaminophen, Carbon tetrachloride, Rat

INTRODUCTION

Artemisia asiatica Nakai (*Compositae*) locally known as "ssuk", is an edible herb with bitter taste, growing abundantly on mountains and sea-side region of Korea. The plant is known to possess hemostatic and anti-anorexic effect as well as controlling action against various gynecological disorders. We have recently shown that ethanol extract of *A. asiatica* has potent antigastritic and cytoprotective effects on experimental gastritis induced by alcohol (Oh *et al.*, 1997a) and non-steroidal antiinflammatory drug (Oh *et al.*, 1997b), gastric ulcer induced by acetic acid instillation (Oh *et al.*, 1996) and animal models of inflammatory bowel diseases (Ahn *et al.*, 1997). It is suggested that anti-oxidative effect and modulation of proinflammatory cytokines by DA-9601 play an important role in its mucoprotective mechanism (Ahn *et al.*, 1997).

Although several herbs from *Artemisia* spp. including *A. scoparia* (Gilani *et al.*, 1994), *A. absinthium* (Gilani and Janbez, 1995) and *A. maritima* (Janbaz and Gilani, 1995) were reported to show hepatoprotective action, the scientific studies on *A. asiatica* on chemically induced liver damage are lacking. In the present investigation, ethanol extract of the plant, DA-9601, was tested against acetaminophen (paracetamol, 4-hydroxyacetanilide)- and carbon tetrachloride (CCl_4)-induced liver damage in rats.

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MATERIALS AND METHODS

Preparation of extract, DA-9601

Dried aerial parts of *A. asiatica* (herbal name "Aeyop") were purchased from a local herbal store and authenticated with the help of a botanist at Seoul National University. The detailed method of extraction and preparation of test material was previously reported (Yang, 1995). Briefly, the herb was macerated in 95% ethanol for 20 h, then the extract was filtered and concentrated to one-twentieth (w/w) of initial herbal weight. The concentrated extract was mixed with adequate amounts of lactose and corn starch and dried to obtain powdered extract. The final extract

(code name DA-9601, lot. 97-L01) was then tested according to an indoor quality assurance procedure for physicochemical properties and contamination of microorganisms and heavy metals. The content of eupatilin, a flavone which is responsible for its antiulcer effect, was 0.45% (w/w). For administration, DA-9601 was suspended in 5% hydroxypropylmethylcellulose (HPMC).

Animals and housing

All experimental procedures were performed in conformity with the institutional *standard procedure for animal care and experiment* (SOP-ANC) of Dong-A Pharmaceutical Company. Male Sprague-Dawley rats weighing 230~250 g were obtained from Charles River Japan (Kanagawa, Japan). All animals were maintained on standard rodent chow (Cheil, Korea) and UV-sterilized tap water ad libitum in standard wire cages with a constant 12 h light/dark cycle. After a 7-day acclimation period, rats were treated according to experimental protocol.

Experimental design

DA-9601 was administered daily to rats at doses of 0, 10, 30 or 100 mg/kg p.o. for 3 consecutive days. Two h following the last dose of DA-9601, acetaminophen (APAP; 640 mg/kg, i.p.) or carbon tetrachloride (CCl_4; 2 ml/kg, p.o.) was administered. At least eight rats per test condition were used. Animals were killed by CO_2 inhalation 4 h or 24 h following hepatotoxin treatment. Blood was subsequently collected from the inferior vena cava, and serum was separated by centrifugation (3,000 rpm for 15 min) and estimated for serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), and lactate dehydrogenase (LDH) using automatic analysis system (Spectrum, Abbott). Liver sections were removed and either fixed in 10% neutral buffered formalin for histological evaluation or frozen (-70°C) for determination of tissue malondialdehyde (MDA) and glutathione (GSH) levels.

Histologic observation

Samples of gross lesion were excised and fixed in 10% neutral buffered formalin solution, then processed by routine techniques for paraffin embedding. After sectioning, each specimen was stained with hematoxylin and eosin and examined with light microscope (BH-2, Olympus) by a pathologist blinded to the study.

Determination of tissue MDA and GSH

The content of hepatic malondialdehyde (MDA) was determined using the method of Ohkawa *et al.* (1979). In brief, after mincing and trimming, the pieces of liver were homogenized with 4 volume of ice-cold 0.1 M potassium phosphate buffer (pH 7.4) solution. Then reaction mixture containing 0.2 ml of the homogenate, 8.1% sodium dodecyl sulfate, 20% acetate buffer (pH 3.5), and 0.8% thiobarbituric acid (TBA) solution were mixed well for 3 min and incubated at 95°C for 60 min. TBA reactive substance, MDA, was extracted with a butanol-pyrimidine mixture solution. The absorbance measured at 532 nm was expressed as nmol/mg protein. The content of hepatic glutathione (GSH) was determined by a spectrophotometric method of Griffith (1980) and expressed as nmol/mg protein. Protein content was determined by the method of Lowry *et al.* (1951) using BSA as the standard.

Statistical analyses

All data are expressed as mean±standard error of the mean. Data were analyzed statistically using an analysis of variance (ANOVA) procedure. Between group variance was determined using Scheffe's t-test. P values of ≤ 0.05 were considered statistically significant.

RESULTS

Effect of DA-9601 on APAP-induced hepatotoxicity

Table I summarizes the change in serum concen-

Table I. Hepatoprotective effect of DA-9601 on acetaminophen (APAP) -induced biochemical change in rat serum

Treatment	ALT (IU/L)		AST (IU/L)		LDH (IU/L)	
	4 h[a]	24 h	4 h	24 h	4 h	24 h
Normal	48.8±2.9		100.2±3.9		809.0±52.2	
APAP	67.8±1.1[b]	148.0±26.1[b]	210.1±10.8[b]	542.1±91.7[b]	1226.1±112.0[b]	2567.2±258.3[b]
APAP+DA-9601 10 mg/kg	59.2±3.6	77.2±11.6[c]	196.8±25.6	318.7±43.0[c]	1062.7±86.8	2431.8±240.8
APAP+DA-9601 30 mg/kg	50.2±4.1[c]	64.8±6.9[c]	124.7±20.9[c]	239.7±31.8[c]	895.0±90.3	2037.0±177.1
APAP+DA-9601 100 mg/kg	49.1±3.1[c]	61.6±8.8[c]	136.5±13.2[c]	217.1±30.1[c]	966.1±75.1	1663.6±241.7[c]

[a]: Four h after single intraperitoneal administration with 640 mg/kg of APAP. Each value represents mean±S.E.M. from 8 rats. Statistical analysis was evaluated by the Scheffe's t-test.
[b]: $p<0.05$, significantly different from the normal group.
[c]: $p<0.05$, significantly different from the APAP group.

tration of ALT, AST, and LDH in rats following administration of APAP in the presence or absence of DA-9601. Serum levels of ALT, AST, and LDH increased significantly 4 h following APAP treatment ($p<0.05$), which became more evident 24 h following APAP. Pretreatment of animals with DA-9601 alleviated the increase of these enzymes both 4 h and 24 h after APAP in a dose-related manner, which reached statistical significance when compared to APAP control ($p<0.05$). There was no inter-group difference in hepatic content of MDA (Table II). The estimated value of liver GSH in normal rats were 4.09±0.14 nmol/mg protein, which were decreased significantly ($p<0.05$) to 3.05±0.21 and 2.65±0.18 nmol/mg protein 4 h and 24 h after APAP, respectively (Fig. 1). However, pretreatment of rats with DA-9601 totally protected APAP-induced decrease of GSH in a dose-dependent fashion. Hepatic GSH level was even higher than normal in high dose group of DA-9601 (100 mg/kg) both 4 h and 24 h following APAP ($p<0.05$, respectively).

Histopathological evaluation mirrored the biochemical parameters. Following APAP administration, sinusoidal congestion, centro-midzonal necrosis with mild inflammation, and many pyknotic cells around the lesions were present and increased in severity over time (Fig. 2). In contrast, pretreatment of rats with DA-9601 reduced these alterations in severity and limited the lesion to areas immediately adjacent to the central veins with a tendency of dose-dependency. Most of rats treated with high dose (100 mg/kg) of DA-9601 showed normal hepatic cords. Hepatocytes undergoing steatosis were rarely found in all APAP- treated rats.

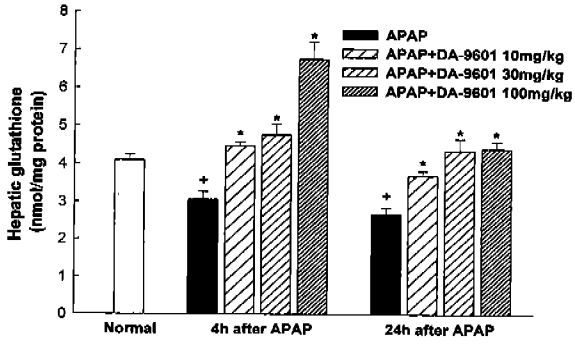

Fig. 1. Dose-response of DA-9601, an extract of *A. asiatica*, on hepatic glutathione content to intraperitoneal injection of 640 mg/kg APAP in rats. Each value represents mean±S.E.M. (n=8). +: $p<0.05$ significantly different from normal control. *: $p<0.05$ significantly different from APAP group.

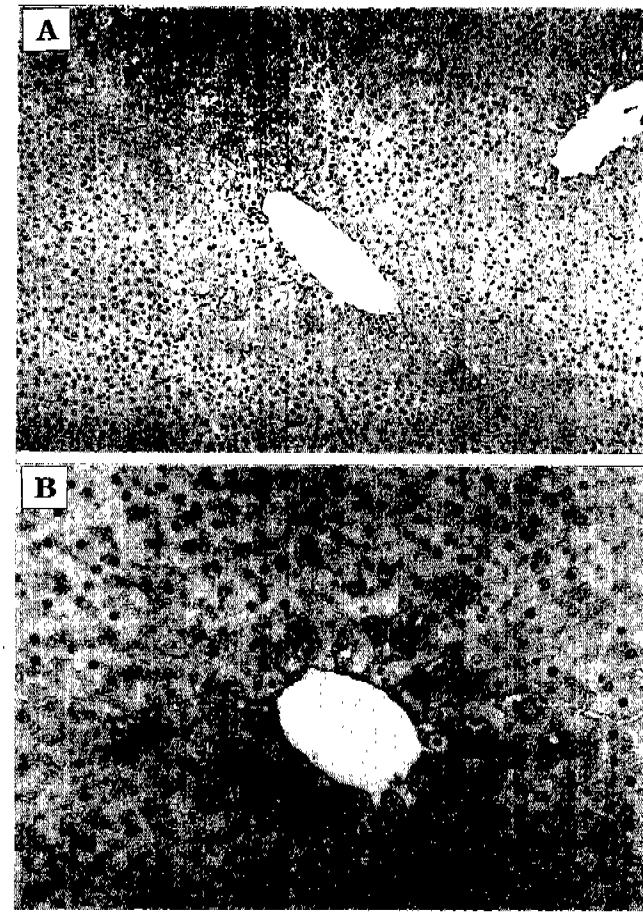

Fig. 2. The hepatic tissue of the rats sacrificed 24 h after APAP (640 mg/kg, i.p.). (a) APAP control, (b) DA-9601 (100 mg/kg, p.o.) at 48, 24 and 2 h before APAP. Central and middle zones showed degeneration, pyknosis and necrosis of hepatocytes, sinusoidal congestion and mild infiltration of inflammatory cells. Note clear demarkation of the lesion (a). H&E stain, ×40; Central zones showed minimal microvesicular degeneration of hepatocytes. Sinusoidal congestion was rarely observed (b). H&E stain, ×100.

Effect of DA-9601 on CCl_4-induced hepatotoxicity

As depicted in Table III, CCl_4 produced significant increase in all of serum ALT, AST, and LDH 4 h after administration, which was aggravated more prominently at 24 h. However, pretreatment of DA-9601

Table II. Hepatic malondialdehyde (MDA) content in rats treated with 640 mg/kg of APAP (i.p.) and DA-9601 (p.o.)

Treatment	Normal	APAP		APAP+DA-9601 (10 mg/kg)		APAP+DA-9601 (30 mg/kg)		APAP+DA-9601 (100 mg/kg)	
		4 h	24 h	4 h	24 h	4 h	24 h	4 h	24 h
MDA content (nmol/mg protein)	1.85±0.04	2.03±0.11	1.94±0.09	1.87±0.05	1.94±0.05	1.95±0.06	1.97±0.07	1.98±0.09	1.81±0.09

Each value represents mean±S.E.M. from 8 rats.

Table III. Hepatoprotective effect of DA-9601 on carbon tetrachloride-induced serum biochemical change in rats

Treatment	ALT (IU/L)		AST (IU/L)		LDH (IU/L)	
	4 h[a]	24 h	4 h	24 h	4 h	24 h
Normal	48.8±2.9		100.2±3.9		809.0±52.2	
CCl_4	84.5±4.4[b]	922.6±107.6[b]	213.2±19.6[b]	358.0±32.6[b]	1924.8±160.6[b]	4024.6±257.2[b]
CCl_4+DA-9601 10 mg/kg	74.7±5.8	836.1±98.8	190.8±11.8	311.7±16.2[c]	1868.9±127.8	3930.5±157.8
CCl_4+DA-9601 30 mg/kg	67.4±7.3	687.4±52.3[c]	160.5±4.6[c]	282.3±21.4[c]	1635.9±97.1	3403.3±293.4
CCl_4+DA-9601 100 mg/kg	58.8±2.9[c]	448.0±108.1[c]	132.4±6.9[c]	204.8±16.3[c]	1230.4±148.7[c]	2790.1±182.6[c]

[a]Four h after single oral administration with 2 ml/kg of CCl_4. Each value represents mean±S.E.M. from 8 rats. Statistical analysis was evaluated by the Scheffe's t-test.
[b]$p<0.05$, significantly different from the normal group.
[c]$p<0.05$, significantly different from the CCl_4 group.

Table IV. Hepatic glutathione (GSH) content in rats treated orally with 2 ml/kg of CCl_4 and DA-9601

Treatment	Normal	CCl_4		CCl_4+DA-9601 (10 mg/kg)		CCl_4+DA-9601 (30 mg/kg)		CCl_4+DA-9601 (100 mg/kg)	
		4 h	24 h	4 h	24 h	4 h	24 h	4 h	24 h
GSH content (nmol/mg protein)	4.09±0.14	3.34±0.28[a]	2.89±0.25[a]	3.32±0.16	2.78±0.24	4.10±0.19[b]	3.16±0.18	5.37±0.51[b]	5.39±0.52[b]

Each value represents Mean±S.E. from 8 rats. Statistical analysis was evaluated by the Scheffe's t-test.
[a]$p<0.05$, significantly different from the normal group.
[b]$p<0.05$, significantly different from the CCl_4 group.

attenuated the elevation of the biochemical parameters dose-dependently. And the rats receiving high dose (100 mg/kg) of DA-9601 and CCl_4 showed a significant reduction in all parameters both 4 h and 24 h following CCl_4 ($p<0.05$). GSH level in liver tissue decreased significantly ($p<0.05$) 4 h and 24 h after CCl_4 (Table IV). Similarly to APAP-induced liver damage, DA-9601 protected CCl_4-induced GSH decrease in a dose-related manner. In high dose group, tissue GSH value was higher than normal rats ($p<0.05$). CCl_4 treatment induced a significant increase in liver MDA to 4.59± 0.47 and 3.17±0.24 nmol/mg protein 4 h and 24 h after administration ($p<0.05$), and DA-9601 attenuated the lipid peroxides in a dose-dependent fashion. In high dose group of DA-9601, MDA levels 4 h and 24 h after CCl_4 were 1.94±0.10 and 1.84±0.08 nmol/mg protein, respectively, which were significantly lower ($p<0.05$) than the values of CCl_4 control and close to the normal value (Fig. 3).

Rats which received vehicle prior to CCl_4 showed centrilobular coagulative necrosis with balooning degeneration of hepatocytes 4 h after CCl_4, which became more extensive 24 h after CCl_4, involving centrilobular and midzonal hepatocytes. The necrotic areas were accompanied by inflammatory cellular infiltration and pyknotic hepatocytes (Fig. 4). However, rats given DA-9601 prior to CCl_4 showed minimal necrosis of centrilobular area surrounded by cells undergoing steatosis. Hepatocytes in midzonal and portal area were mostly spared in middle (30 mg/kg) and high (100 mg/kg) dose of DA-9601.

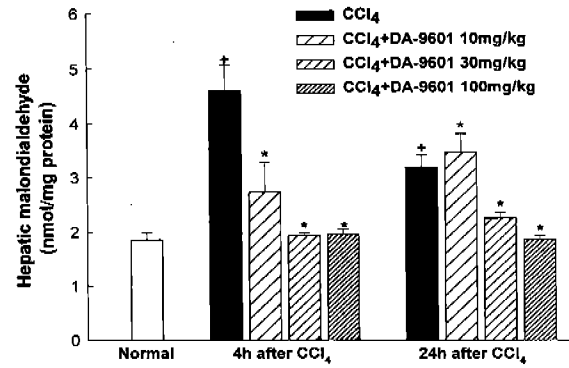

Fig. 3. Effect of DA-9601 on hepatic malondialdehyde content in the rat treated orally with 2 ml/kg of carbon tetrachloride (CCl_4). Each value represents mean±S.E.M. (n=8). +: $p<0.05$ significantly different from normal control. *: $p<0.05$ significantly different from CCl_4 group.

DISCUSSION

The objective of this study was to investigate the hepatoprotective effect of DA-9601, a herbal antiulcer agent derived from *Artemisia asiatica*, using animal models of liver damage. In the previous reports, the beneficial effect of DA-9601 on experimental gastritis (Oh *et al.*, 1997a, b), gastric ulcer (Oh *et al.*, 1996), and inflammatory bowel disease (Ahn *et al.*, 1997) was suggested. DA-9601 shows these cytoprotective and antiinflammatory effect through the complicated mechanisms including increase of mucus secretion, preservation of mucosal prostaglandins and GSH, anti-

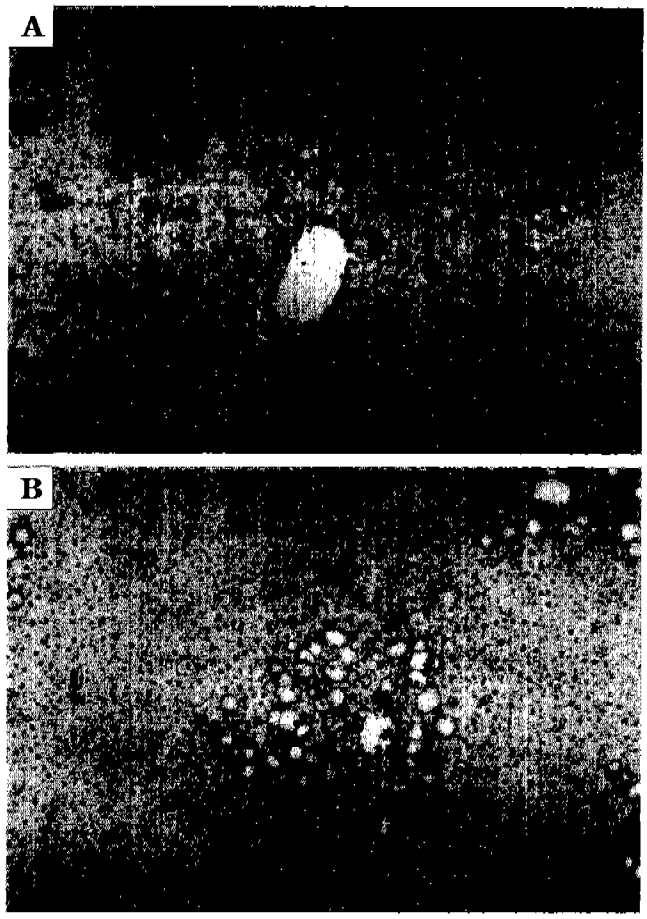

Fig. 4. The hepatic tissue of the rats sacrificed 24 h after CCl_4 (2 ml/kg, p.o.). (a) CCl_4 control, (b) DA-9601 (100 mg/kg, p. o.) at 48, 24 and 2 h before CCl_4. CCl_4-induced diffuse centro-midzonal necrosis surrounded by baloon cells and inflammatory cells was observed (a). H&E stain, ×100; Only central zones showed relatively mild necrosis and steatosis of hepatocytes (b). H&E stain, ×100.

oxidative effect and modulation of inflammatory cytokines (Ahn *et al.*, 1997; Oh *et al.*, 1996).

In the present study, to evaluate the hepatoprotective effect of DA-9601, we adopted liver injury models induced by carbon tetrachloride (CCl_4) and acetaminophen (APAP) which are commonly used for the screening of hepatoprotective drugs (Plaa and Hewitt, 1982). In the results, the sharp increase of serum transaminases (ALT, AST) and LDH by administration of these hepatotoxins was reduced by pretreatment with DA-9601 in a dose-related manner. The rise in serum levels of ALT, AST and LDH has been attributed to the damaged structural integrity of the liver (Chenoweth and Hake, 1962), because these are cytoplasmic in location and are released into circulation after cellular damage or death (Sallie *et al.*, 1991).

Both CCl_4- and APAP-induced liver damage models rely on the cytochrome P-450 system (CYP) to produce reactive metabolites. In CCl_4-induced liver injury, CCl_4 is first biotransformed by the CYP to produce the trichloromethyl free radical, which in turn covalently binds to cell membranes and organelles to elicit lipid peroxidation, disturb Ca^{2+} homeostasis, and finally result in cell death (Recknagel *et al.*, 1989). APAP is bioactivated by CYP to a toxic electrophile, N-acetyl-p-benzoquinoneimine (NAPQI) that produces liver damage unless it is conjugated with GSH (Nelson, 1990). Therefore, in addition to the acute increase of serum transaminases and morphologic alteration, increase of liver MDA, a typical parameter of lipid peroxidation, and depletion of hepatic GSH are important clinical findings in CCl_4- and APAP-induced liver damage, respectively. In the present study, pretreatment of DA-9601 showed hepatoprotective effect against both CCl_4 and APAP with dose-dependent reduction of the increase of transaminases activity and hepatic necrosis induced by hepatotoxins. In CCl_4 model, DA-9601 reduced liver MDA, a marker of lipid peroxidation. And in APAP model, DA-9601 also protected against depletion of hepatic GSH caused by APAP. Interestingly, these findings are consistent with the effect of DA-9601 on gastric mucosa. Recently we reported that DA-9601 increased GSH content in gastric mucosa and prevented the increase of mucosal MDA induced by noxious agents (Ahn *et al.*, 1997; Oh *et al.*, 1997a). Though polymorphonuclear cellular infiltration into liver tissue was often observed in hepatotoxin-treated animals, no inter-group difference in tissue MPO level was detected in this study (data not shown).

Because both CCl_4 and APAP must be activated by CYP to produce liver damage, it may be postulated that hepatoprotective action of DA-9601 is due to its inhibitory effect on the CYP. However, in the pharmacological study on DA-9601, it did not show any effect on barbiturate-induced sleeping time in mice, indicating no effect on CYP (Lee *et al.*, 1996). From these results, it was speculated that DA-9601 could ameliorate APAP- and CCl_4-induced liver damage not through its effect on CYP but through complex mechanisms including antioxidative effect and preservation of hepatic GSH. DA-9601 is safe as is obvious by the lack of any symptom of acute toxicity at an oral dose of as high as 5 g/kg (Yang, 1995), and has also been reported safe after subacute oral treatment (Kim *et al.*, 1996). These results suggest that DA-9601 could be used as a hepatoprotectant for prevention of toxic liver damage as well as a mucoprotectant. However, the hepatoprotective mechanisms and the active component(s) of DA-9601 require further investigation.

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