응용약물학회지, The Journal of Applied Pharmacology, 9, 112-118(2001)

Effects of *Malloti Cortex* Water Extract, Bergenin, and Acetylbergenin on Liver Fibrosis Induced by Bile Duct Ligation in Rats

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(Received March 21, 2001; accepted June 15, 2001)

Abstract – The effects of *Malloti Cortex* Water Extract (MCWE), bergenin (isolated as an active component from MCWE), and acetylbergenin (synthesized from acetylation of bergenin) on the liver fibrosis induced by bile duct ligation (BDL) in rats. We studied hydroxyproline (HYP) as a marker of collagen accumulation in the liver, alanine aminotransferase (s-ALT), aspartate aminotransferase (s-AST), and alkaline phosphatase (s-ALP) as serum markers of liver cell damage induced by BDL. MCWE, bergenin, and acetylbergenin decreased towards normal the accumulated levels of HYP in the liver and the elevated serum levels of s-ALT, s-AST and s-ALP. The results indicate that MCWE, bergenin, and acetylbergenin ameliorated the liver damage induced by BDL in rats.

Key words □ bile duct ligation, liver fibrosis, hydroxyproline, *Malloti Cortex* water extract

Ligation of the bile duct in rats is associated with liver fibrosis, cirrhosis and consequential portal hypertension (Koyama *et al.*, 1975; Kountouras *et al.*, 1984; Gross *et al.*, 1987; Zimmermann *et al.*, 1992). The liver of these BDL rats has both morphological changes and metabolic abnormalities (Yuro, 1990; Zimmermann *et al.*, 1994). Because this model is similar to human biliary liver fibrosis, it has been used as a satisfactory model for the evaluation of drugs which are beneficial for the liver fibrosis.

MCWE containing about 11~18% bergenin has been used as a folk oriental Med. for the treatment and therapy of gastrointestinal diseases such as gastritis, gastric ulcer, diarrhea and constipation (Okada *et al.*, 1973; Abe *et al.*, 1980). Bergenin, a major component of MCWE, is a *C*-glucoside of 4-*O*-methylgallic acid, which has been found to have antiulcer activity (Goel *et al.*, 1997).

We isolated bergenin as an active component from MCWE, and found it to possess hepatoprotective effects on carbon tetrachloride (CCl_4)- and galactosamine(GalN)-induced hepatotoxicity in rats (Lim *et al.*, 1999). We also synthesized acetylbergenin from bergenin by an acetylation process to modify its physiological activities. The hepatoprotective effect of bergenin has been reported, but using only one glutamic pyrubic transaminase (GPT) index in cultured rats hepatocytes (Hikino *et al.*, 1985). The antifibrotic effects of MCWE, bergenin, and acetylbergenin have not been reported yet.

The aim of present work was to study the effects of MCWE, bergenin, and acetylbergenin on the model of rat liver fibrosis induced by biliary obstruction. We studied HYP as a marker of collagen accumulation in the liver, ALT, AST and ALP as serum makers of liver cells damage, and histopathology of BDL rats livers in order to elucidate the potential protective effects of these compounds

MATERIALS AND METHODS

Preparation of MCWE, bergenin, and acetylbergenin

The cortex of *Mallotus japonicus* was collected from the Chungbuk Province of Korea and identified by Dr. K. S. Lee, College of Pharmacy, Chungbuk National University. A voucher specimen (CBNU415) was submitted to the herbarium of the university. Dried plant material (1.0 kg) was sliced, extracted three times with 10 l of distilled water for 2 h. The filtrate was evaporated under reduced pressure to obtain 130 g of MCWE, which was then dissolved in methanol. The methanol-soluble fraction was again concentrated under reduced pressure. Final separation was carried out using a silica gel column with a chloroform:methanol (4:1) solvent to give bergenin. Bergenin was further purified by several recrystallizations from methanol (Hay and Haynes, 1958). It was

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identified by TLC using an ethyl acetate:ethanol:water (100:17:13) mobile phase (R_f =0.5). Spots were detected under UV irradiation. The yield of bergenin was 15.9% (15.9 g). Acetylbergenin was synthesized by the acetylation method of Ramaiha *et al.* (1979) from bergenin isolated from MCWE as described previously (Lim *et al.*, 1999). Bergenin (10 g) was dissolved in acetic acid anhydride (1 l), with dry pyridine (200 ml), and sodium acetate anhydrous (1 g) added in, heated in a water bath for 6 h and worked up in the usual manner.

Animal experiment

Specific-pathogen-free adult female Sprague-Dawley rats (200~250 g), supplied from the Animal laboratory of Korea Food and Drug Administration, were housed individually in wire bottom cages with food and water *ad libitum* throughout these experiments.

Rats were anesthetized with ether and subjected to a ligation of the common bile duct according to the Kountouras method (Kountouras *et al.*, 1984), with a minor modification. Briefly, a midline incision in the abdomen was made. The distal and proximal complete ligation in the common bile duct was performed using 5-0 silk (Ethicon, germany). After double ligation, the midpoint of the common bile duct was cut. BDL rats (143) were randomly divided into 11-treatment groups. A sham operation of 12 rats was performed in the same way without BDL following which the abdomen was closed and the animal allowed to recover. From the second day after operation, each dose of MCWE (150, 300 and 600 mg/kg, P.O.), bergenin (50, 100 and 200 mg/kg, P.O.), acetylbergenin (50, 100 and 200 mg/kg) and 40 mg/kg of silymarin (SIL) as a positive control were administered to the BDL rats for 4 weeks (Boigk *et al.*, 1997). Twenty four hour after the last administration, the abdomen was opened to collect blood from the abdominal aorta and to remove the liver under light ether anesthesia.

Assessment of hepatic injury

Blood obtained from the abdominal aorta was allowed to clot at room temperature for 30 minutes and centrifuged 900 × g for 15 min at 4°C (Joan 3.22, France) to collect serum. All serums were stored at -70°C for later analysis. Serum levels of s-ALT, s-AST and s-ALP were measured by a spectrophotometric method using a photometer 5010 (Boehringer Mannheim, Germany), using each test reagent (Boehringer Mannheim, Germany).

Determination of hydroxyproline

Liver HYP contents were examined as an index of collagen accumulation (Murawaki *et al.*, 1991; Okuno *et al.*, 1991; Schaff *et al.*, 1991; Fort *et al.*, 1998). The HYP was measured by a modified Jamall method (1981). Briefly, 180-220 mg of liver was homogenized in 5 ml of 6 N HCl and was then hydrolyzed at 120°C for 18 h in a Teflon-capped glass vial. After cooling, the hydrolysates were filtrated through a 0.45 μm Millipore filter. A 50 μl of aliquot was allowed dried under a vacuum over a sodium hydroxide/calcium sulfate desiccant. For removing the residual HCl, methanol 50 μl was added to the residue and then evaporated to dryness. The final residue was dissolved in 1.2 ml of 50% isopropanol/H_2O and incubated with 200 μl of 1.67% Chloramine-T in acetate citrate buffer (pH 6.0) at room temperature for 10 min. 410 mmol/l of *p*-dimethylaminobenzaldehyde was added and incubated for 90 min at 50°C. After cooling to room temperature, absorbance was read at 558 nm. The HYP concentration of each sample was determined from a standard calibration curve using a HYP standard (Merck, USA).

Histopatholoical study

For histological observation, the median lobe of the liver was fixed in 10% phosphate-buffered formalin (Sigma, USA) and embedded in paraffin. 5-3 μm thick sections stained with hematoxylin and eosin were observed under a light microscope (Olympus BH-1, Japan).

Statistical analysis

The data are expressed as mean ± SE. The evaluation of statistical significance was determined by a one-way analysis of variance with Student t-test for post-hoc comparisons. Values of $P<0.05$ were considered to indicate a significant difference.

RESULTS

Mortality

Postoperatively, 21 rats died within 3 days due to the complications of a local infection (10 on the day of operation). The difference was not significant among the treatment groups (Table 1).

Body and relative liver weights

The gain of body weight in the BDL group was significantly increased over that of sham operated group ($P<0.01$).

Table I. Gain in body, and liver weight, and death rate of rat

Treatment		n	Gain in body weight (g)	Liver weight (g/100 g)	Death rate
sham		12	38.7 ± 5.1	2.58 ± 0.04	0/12
BDL		15	53.0 ± 7.0#	7.24 ± 0.11#	5/20
SIL	40 mg/kg	11	55.1 ± 3.7	6.51 ± 0.38***	4/15
MCWE	150 mg/kg	10	55.5 ± 4.4	6.22 ± 0.21***	2/12
	300 mg/kg	9	56.7 ± 3.5	6.24 ± 0.14***	3/12
	600 mg/kg	10	56.3 ± 8.4	6.07 ± 0.17***	2/12
Bergenin	50 mg/kg	10	55.6 ± 4.1	6.68 ± 0.19*	2/12
	100 mg/kg	10	56.7 ± 3.5	6.64 ± 0.16**	2/12
	200 mg/kg	9	57.6 ± 4.7	6.52 ± 0.17***	3/12
Acetylbergenin	50 mg/kg	10	56.0 ± 2.9	6.46 ± 0.16***	2/12
	100 mg/kg	9	56.9 ± 5.9	6.48 ± 0.19***	3/12
	200 mg/kg	9	55.7 ± 3.1	6.41 ± 0.14***	3/12

Silymarin (40 mg/kg/day), bergenin (50, 100 and 200 mg/kg/day) and acetylbergenin (50, 100 and 200 mg/kg/day) were given orally for 4 weeks. Values are means ± SE. n=number of rats. #P<0.01 for difference between sham-operated and BDL controls. *P<.05, **P<0.02 and *** P<0.01, for the difference the between BDL control and each treatment group.

No significant difference was observed between treatment groups. The relative liver weight of the BDL group was also dramatically increased during observation period compared to sham operation group (P<0.01). The relative liver weight was decreased by MCWE, bergenin, and acetylbergenin. SIL (40 mg/kg) also significantly decreased the gain of body weight (P<0.01) compared to BDL group (Table I).

Hepatic injury

The degrees of parenchymal cell damage and the loss of hepatic functions were analyzed by the examination of conventional serum hepatic enzymes, including s-ALT, s-AST, and s-ALP. The values in sham operated animals were 49.9± 4.0, 65.2± 4.4, and 56.2± 1.6 U/L, respectively (Figs. 1, 2 and 3). On the 28th day after BDL operation, the serum levels of s-ALT (P<0.01), s-AST (P<0.01) and s-ALP (P<0.01) were remarkably elevated relative to shams. These were; 119.9± 6.3 for s-ALT, 1157.7± 32.8 for s-AST, and 202.5± 6.1 for s-ALT, respectively. The groups treated with MCWE (300 and 600 mg/kg) had significantly decreased the elevation of these enzyme activities; 40-47% in s-ALT, 22-36% in s-AST, and 23-30% in s-ALP, respectively. Bergenin (100 and

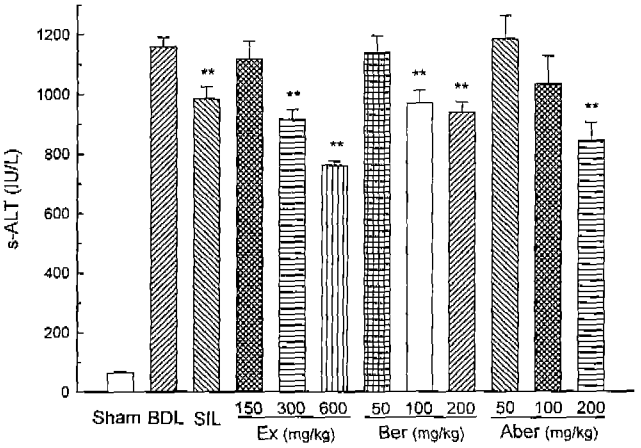

Fig. 1. Effects of MCWE, bergenin (Ber), and acetylbergenin (Aber) on serum levels of alanine aminotransferase (s-ALT). SIL (40 mg/kg/day), MCWE, Ber and Aber were orally administered for 4 weeks after operation. Data are expressed as means ± SE. The post hoc comparisons between groups are given as follows. #P<0.01 vs sham. **P<0.01 vs BDL alone.

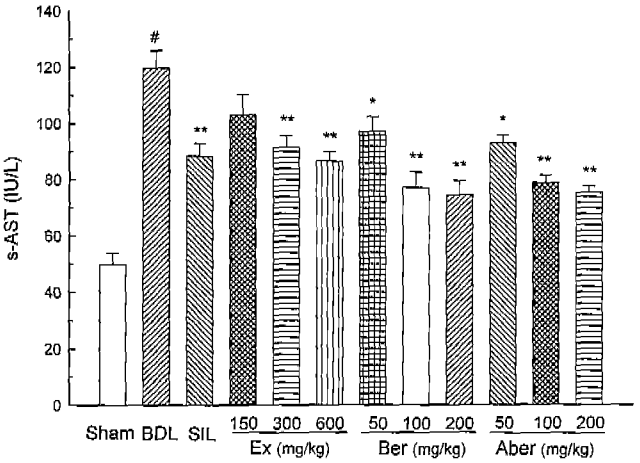

Fig. 2. Effects of MCWE, bergenin (Ber), and acetylbergenin (Aber) on serum levels of aspartate aminotransferase (s-AST). #P<0.01 vs sham. *P<0.05 and **P<0.01 and vs BDL alone.

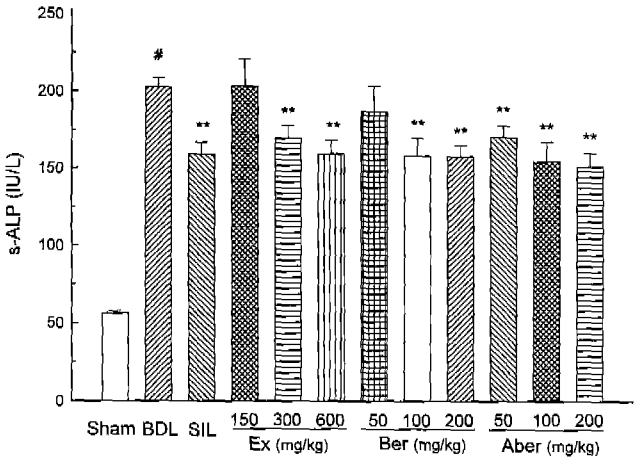

Fig. 3. Effects of MCWE, bergenin (Ber), and acetylbergenin (Aber) on serum levels of alkaline phosphatase (s-ALP). #P<0.01 vs sham. **P<0.01 vs BDL alone.

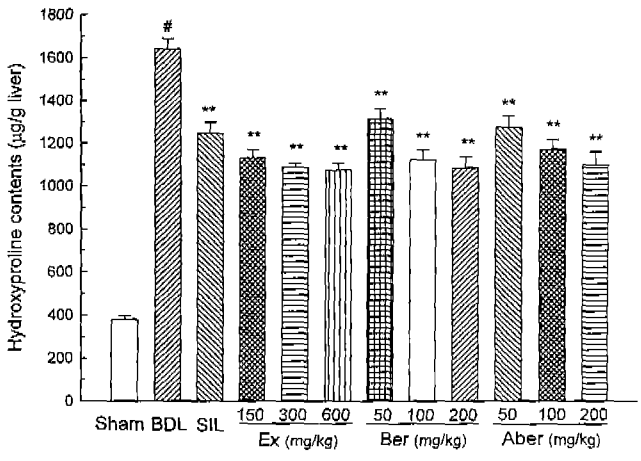

Fig. 4. Effects of MCWE, bergenin (Ber) and acetylbergenin (Aber) on hydroxyproline contents in the liver. MCWE, Ber, and Aber were orally administered for 4 weeks after the operation. HYP contents as a collagen accumulation index were determined in the liver as described in the Materials and Methods. Rats were killed on the 28th day after BDL operation. #P<0.01 vs sham. **P<0.01 vs BDL alone.

200 mg/kg, P<0.01) and acetylbergenin (200 mg/kg, P<0.01) also significantly inhibited elevation of activities of these enzymes, but still higher than normal activity. Bergenin (200 mg/kg) reduced elevation of s-ALT, s-AST and s-ALP by 64%, 20%, and 31%. Acetylbergenin (200 mg/kg) reduced the elevation of s-ALT, s-AST, and s-ALP by 63%, 29% and 35%, respectively. In the group treated with SIL, these levels were also significantly decreased compared to those of BDL, s-ALT (from 119.9-88.5 U/L, P<0.01) by 45%, s-AST (from 1157.7-984.2 U/L, P<0.01) by 16%, and s-ALP (from 202.5-158.8 U/L), P<0.01) by 30%, respectively. These results coincide with that of the SIL literature (Boigk *et al.*, 1997).

Hydroxyproline accumulation

HYP was used an index of the total collagen deposition in the liver during the observation period. BDL of rats led a 4.3-fold increase in the HYP content of the livers (378.0 vs 1641.0 μg/g liver, P<0.01), compared with sham (Fig. 4). A significant difference of HYP level was observed between SIL-treated rats and BDL-only rats (P<0.01). Administration of MCWE, bergenin, and acetylbergenin dose-dependently reduced HYP accumulation in the liver. MCWE (150, 300 and 600 mg/kg) significantly inhibited HYP accumulation in the liver by 24% (P<0.01), 40% (P<0.01), and 47% (P<0.01), respectively. Bergenin (50, 100 and 200 mg/kg) inhibited HYP accumulation in the liver by 26% (P<0.01), 41% (P<0.01), and 47% (P<0.01), respectively. Acetylbergenin (50, 100 and 200 mg/kg) inhibited HYP contents in the liver by 29% (P<0.01), 37% (P<0.01) and 43% (P<0.01), respectively. Treatment with SIL (40 mg/kg) inhibited HYP contents in the liver by 31.3% (P<0.01). No significant difference between bergenin and acetylbergenin treatments was observed.

Histopathology of liver

Hepatic lesions induced by BDL operation were characterized by a cellular proliferation in which bile duct epithelial cells and fibrocytes invaded the surroundings of the portal triad. Prolifering fibrocytes also invaded into the liver parenchyma (Fig. 5B). SIL (40 mg/kg) prevented proliferation of the fibrocytes (Fig. 5C). MCWE (600 mg/kg) reduced the hyperplasia of bile duct epithelial cells (Fig. 5D). Bergenin seemed to decrease the damaged area (Fig. 5E). Bergenin (200 mg/kg) and acetylbergenin (200 mg/kg) both seemed to decrease the area of damage. Acetylbergenin (200 mg/kg) markedly reduced the hyperplasia of bile duct epithelial cells and fibrocytes (Fig. 5F).

DISCUSSION

In the present study, administration of MCWE, bergenin, and acetylbergenin decreased not only the elevated serum enzyme activities such as s-ALT, s-AST, and s-ALP, but also the accumulation of HYP in the liver induced by BDL. Our previous study (Lim *et al.*, 1999), the elevated serum enzymatic activities of ALT, AST, and sorbitol dehydroxylase, and the decreased activities of glutathione-S-transferase and glutathione reductase caused by CCl_4 and galactosamine-induced

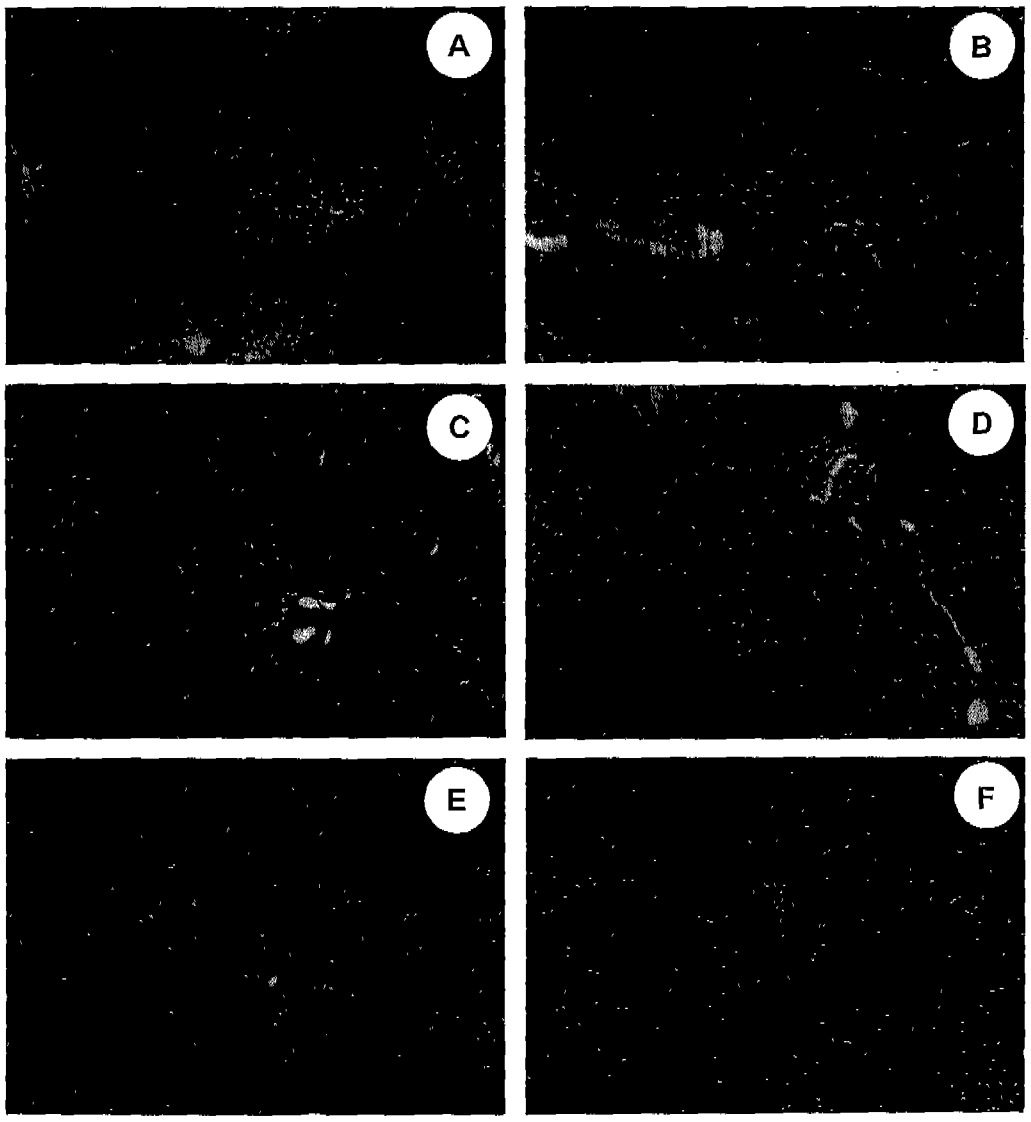

Fig. 5. Light micrographs of liver sections obtained from sham-operated and BDL rats. A: sham, B: BDL control, C: SIL (40 mg/kg) treated, D: MCWE (600 mg/kg), E: Ber (200 mg/kg) treated, and F: Aber (200 mg/kg) treated. Biliary hyperplasia with fibrosis surrounding the portal triad was induced by BDL by week 4 (A). Structure of liver parenchyma is preserved by administration of MCWE, Ber, and Aber, as well as by SIL. (H & E staining) (Original magnification×100).

liver damage in rats were dose-dependently restored towards normal by administration of MCWE, bergenin, and acetylbergenin. Those resulted in the prevention of elevated hepatic malondialdehyde formation and depletion of reduced glutathione content in the liver of rats intoxicated with CCl_4 and galactosamine. Based on those results, it was reported that the possible mechanisms underlying the hepatoprotective effects of MCWE, bergenin, and acetylbergenin were related to glutathione-mediated detoxification, as well as their free radical suppressing activity.

Therefore, it was also presumed that the protective mechanisms of MCWE, bergenin, and acetylbergenin against the elevated or decreased serum enzymatic activities through glutathione-mediated detoxification, as well as free radical suppressing activity, could work in the present experiments.

The mechanisms underlying the inhibition by MCWE, bergenin, and acetylbergenin of HYP accumulation in the liver of BDL rats are not known. There are a few potential antifibrotic compounds, including prostaglandin E_1 and E_2, pentoxifylline, IFN-α and IFN-γ which have shown antifibrotic effects in animal studies (Peters *et al.*, 1989; Muriel *et al.*, 1991; Sakaida *et al.*, 1998; Desmouliere *et al.*, 1999). It has been reported that bergenin and norbergenin increase the synthesis of prostaglandin E_1 and E_2 in human colonic mucosa. Inter-

estingly, prostaglandin E_1 and E_2 inhibit the growth of hepatic stellate cells that are essential for the development of liver fibrosis (Ramadori *et al.*, 1987; Benyon *et al.*, 1998). However, antihepatofibrotic study has not been reported on bergenin with other potential compounds. On the basis of previous findings, it could tentatively presumed that MCWE containing 11-18% of bergenin, pure bergenin, and acetylbergenin may be deeply involved in producing prostaglandin E_1 and E_2, as well as other antihepatofibrotics. The antihepatofibrotic effects are confirmed by histopathological study, indicating that MCWE, bergenin, and acetylbergenin indeed ameliorated the liver damage induced by BDL in rats.

In the present study, no significant differences were observed between bergenin, and acetylbergenin treated groups on the 28th day of BDL in rats, despite the presumption that the activities of bergenin and acetylbergenin might be differentiated due to the different lipid solubility and metabolism of the two compounds. It has been reported that in *in vitro* primary cultured rat hepatocytes, the hepatoprotective effects of norbergenin as a hydrophilic polyphenol compound shows more active than that of acetylbergenin as a lipophilic compound (Kim *et al.*, in press). In addition, polyphenol compounds show hepatoprotective effects in primary cultured rat hepatocytes (Miyagawa *et al.*, 1997; Hikino *et al.*, 1985). But in *in vivo*, acetylbergenin showed more activity than bergenin against BDL induced rat hepatotoxicity. The result suggests that acetylbergenin, absorbed more easily due to its ability to cross the bilayer of intestinal cell membranes, results in increased protective activity, after being hydrolyzed into hydrophilic polyphenol compounds such as norbergenin and bergenin. Acetylbergenin, 100 mg has equivalent to about 65 mg of bergenin, calculated by molar concentration. We obtained similar hepatoprotective effect at the same concentration. In view of this, it could be said that acetylbergenin in this study also showed more activity than this of bergenin.

Further studies are needed to elucidate the effects of MCWE, bergenin, and acetylbergenin on antihepatofibrotic prostaglandins as well as parameters of fibrogenesis and fibrolysis.

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