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Protective effects of *Flos lonicera* extract on acute liver injury by dimethylnitrosamine-induced in rats

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Abstract The aim of this study is to investigate effects of *Flos lonicera* extract (FLE) on acute liver injury model rats which induced by 35 mg/kg dimethylnitrosamine (DMN). Model rats were divided into hepatic injury control group (administrated with water), FLE group (administrated with FLE) and silymarin group (administrated with silymarin which is hepatotherapeutic drug) as positive control. They were examined including ALT, AST, ALP, γ-GT, ALB and TP levels in serum, and MDA, GPx levels in liver tissue. In addition, pathologic changes, particularly fibrosis, were examined by Azan staining. The results revealed that the ALT, AST, ALP, γ-GT, MDA GPx and liver fibrosis degree in the LJE group were lower than the silymarin group and control group, ALB and TP were higher than the silymarin group and control group. These results suggested that LJE may help in inhibiting of acute liver injury greater than silymarin.

Keywords *Lonicera japonica* · Acute liver injury · Protective effect · Dimethylnitrosamine

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Introduction

Recently, many chemicals that were inhaled or swallowed can damage the liver. Drug-induced liver injury is the most frequent cause of acute liver failure [1]. Dimethylnitrosamine (DMN) is a potent hepatotoxin, carcinogen and mutagen. A high dose of DMN is administered by one injection to the experimental animal, which causes submissive necrosis that resembles human fulminate hepatitis [2]. Therefore, DMN has been widely used as a model agent to study the mechanisms of liver injury.

According to "Chinese Pharmacopoeia" of 2005 version, *Lonicera japonica* belong to *caprifoliaceae* and *lonicera*. *Flos lonicera* is the flower buds of several medicinal *Lonicera* species, commonly used traditional chinese medicine for the treatment of sores, carbuncles, furuncles, swelling and affections caused by exopathic wind-heat or epidemic febrile diseases at the early stage [3] and has been proven to be responsible for the various biological activities such as hepatoprotective, cytoprotective, antimicrobial, antioxidative, antiviral and anti-inflammatory effects of the herbal remedy [4, 5]. A number of compounds including organic acids, flavonoids, iridoid glycosides and saponins have been isolated from *Lonicera* species [6, 7], that have different kinds of activities [8–10]. In China, the clinical use of *Flos lonicera* keep increasing for the inflammatory disease such as hepatitis and for the microbial disease such as influenza. However, the scientific basis to these effects is not clear. So, the present study was designed to evaluate the protective effect of *Flos lonicera* extract (FLE) on functional and morphological prevention activity for acute liver injury rat model induced by DMN, compared with silymarin which is famous hepatotherapeutic drug in Europe.

Materials and methods

Materials and reagents

Flos lonicera was purchased from Golden days Pharmaceutical Co., Ltd. (Jiamusi, China) and extracted with 75% ethanol. DMN was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan); SOD Assay Kit-WST was purchased from Dojindo Laboratories, Inc. (Kumamoto, Japan); Glutathione Peroxidase Assay Kit was purchased from Cayman Chemical Company (Tokyo, Japan). 1,1-diphenyl-2-picrylhydrazyl (DPPH) and chlorogenic acid were purchased from Nacalai Tesqque Inc. (Kyoto, Japan); oleanolic acid and quercetin were obtained from the National Institute for Control of Pharmaceutical and Biological Products. (Beijing, China). Silymarin was purchased from Madus AG (Cologne, Germany) and all the other reagents used were of analytical grade.

Preparation of FLE

Flos lonicera was weighed accurately 5 g and refluxed with water, 25% ethanol, 50% ethanol, 75% ethanol and 95% ethanol (100 ml) for 3 h and three times in total, respectively. Every time the extracts were rapidly vacuum-filtered through a sintered funnel put filtrate together. The extract was dried under reduced pressure using rotary flash evaporator until dry. Afterwards, the extract was removed with corresponding solvent for use.

Determination of superoxide dismutase (SOD) activity

SOD Assay Kit-WST was used to determine SOD activity. WST can produce a water-soluble form azan dye upon reduction with a superoxide action. The rate of reduction with O_2^- is linearly related to the xanthine oxidase activity and is inhibited by SOD. Therefore SOD activity as an inhibition activity can be quantified by measuring the decrease in the color development at 450 nm and 37°C after 20 min incubation. To evaluate the SOD activity the concentration curve was prepared for each extract using the measurement the concentration of 50% scavenging activity (IC50).

Measurement of reactive oxygen-scavenging action using ESR

The ESR spectrum was measured using a JEOL JEX-RE3X ESR spectrophotometer (Nihon Densi Inc.). The measurement conditions are shown below. Power: 5 mW, Field: 336.0 ± 10 mT, Sweep time: 1 min, Modulation: 100 kHz 9.45 GHz, Time constant: 0.03 s, Receiver gain: 1 × 1000. To evaluate the scavenging activity of each radical, the concentration-radical scavenging rate curve was prepared for each extract using the measurement the concentration of 50% scavenging activity (IC50).

HPLC fingerprint of FLE

Twenty microlitre standards and sample were dissolved in 0.4% phosphoric acid and acetonitrile. The solution was filtered through 0.45 μm microporous membrane then analyzed in HPLC (Angilent 1100, USA). The gradient separation of FLE extracts was performed on a Diamonsil C18 column (250 × 4.6 mm, 5 μm) at 30°C. The mobile phase was 0.4% phosphoric acid (A) and acetonitrile (B) and the linear gradient was 95, 87, 70, 60 and 95% A at 0, 8, 20, 35 and 45 min with a flow rate of 1.0 ml/min, respectively. The wavelength of detection was 238 nm [11].

Measurement of chlorogenic acid content by LC–MS

Sample (5 μL) was analyzed with high performance liquid chromatograph with a mass selective detector (LC–MS 2010, SHIMADZU, Kyoto, Japan), using Shim-pack VP ODS column (150 × 2.0 mm i.d., SHIMAZU). The oven temperature was 25°C and the column was eluted with a gradient of 0.05% HCOOH (v/v) in water as A and CH_3CN (contain 0.05% HCOOH, v/v) as B at a flow rate of 0.25 ml/min (from 0 to 5 min, 15% B; from 6 to 20 min, 20–80% B).

The authentic chlorogenic acid was weighted accurately then dissolved in methanol and diluted to 20, 10, 5, 2.5, 0 μg/ml. The extracts were dissolved in methanol and diluted to 10 μg/ml. The solution was filtered through a 0.45 μm microporous membrane then directly injected into LC–MS. Three replicate injections were analyzed to determine the contents of chlorogenic acid in the herb with the average peak.

Treatment of rats

Male Wistar rats aged 7 weeks (150–180 g) were purchased from JAPAN SLC, Inc.(Hamamatsu Japan) and acclimated at 12–12 h light–dark cycle and 23 ± 2°C room temperature for 7 days. The rats were divided into four groups of eight rats each. The normal group was not treated. The control group was treated with an intraperitoneal injection of DMN at a dose of 35 mg/kg. [12, 13] The FLE group was intraperitoneally injected DMN at a dose of 35 mg/kg and intragastric administrated with FLE (according to the result of preparation of FLE, the extract of 75% ethanol was used and dissolved in distilled water) at a dose of 2 g/kg body weight for 2 weeks from the 1st day to 14th. [14] The silymarin group was treated with an

intraperitoneal injection of DMN at a dose of 35 mg/kg, followed by daily treatment with silymarin (suspended in distilled water) at a dose of 100 mg/kg for 2 weeks from the 1st day to 14th. [15–18] On the 14th day, all rats in each group were sacrificed under anesthesia with ether. Blood samples for biochemical analyses were obtained from the vein of abdominal cavity.

Serum indicator analysis

The rats were bled via abdominal vein after ether anesthesia on the final day of full experimentation. Once serum was prepared, the levels of TP, ALB, AST, ALT, γ-GT and ALP were determined by DRI-CHEM 5500 (Fuji Film, Tokyo, Japan) [19, 20].

Measurement of lipid peroxidation in liver tissue

Malondiadehyde in liver tissue was examined using the method of thiobarbitric acid reactive substance (TBARS) [21]. The concentration of TBARS was expressed as n moles of MDA per 100 mg protein using 1,1,3,3-tetraethoxypropane (TEP) as a standard. Briefly, 0.2 g of liver tissue was homogenized in 2 mL of ice-cold 11.5 g/L KCl; then 0.2 ml of homogenate was mixed with 1.5 mL of 0.2 M acetate buffer, 0.2 mL of 0.81% sodium dodecyl sulfate and 1.5 ml of 0.8% thiobarbituric acid then added distilled water up to 4 mL. After heating the mixture for 1 h at 100°C, 5 ml of mixed liquor (*n*-butanol: pyridine = 15:1 v/v) was added into the mixture, followed by vigorous mixing and centrifugation at 3000 rpm/min for 15 min. The intensity of fluorescence (Ex 515 nm, Em 553 nm) of the *n*-butanol layer was determined, and compared to the value from freshly prepared TEP as a standard.

Determination GPx activity in liver tissue

Determination GPx activity of liver tissue was carried out using glutathione peroxidase assay kit.

Histological examination

At the final of experiment, the liver and spleen were removed immediately and wet weighed. The liver specimens were fixed in 10% neutral buffered formalin and then dehydrated and paraffin-embedded for histochemical studies.

Statistical analysis

All of the values were expressed as the mean ± standard deviation (SD). Significant differences between the groups were statistically analyzed using Student's *t* test. The

differences between multiple groups were evaluated by a one-way analysis of variances (ANOVA), followed by Dunnett's test. *P* values less 0.05 were considered statistically significant.

Results

HPLC fingerprint of FLE

In all the extracts, the HPLC chromatogram pattern was almost similar. As the typical chromatogram, the FLE of 75% ethanol was shown in Fig. 1.

The HPLC fingerprint shows that FLE of 75% ethanol containing many types of compounds. Comparison with retention time and peak areas, the FLE of 75% ethanol contain mainly chlorogenic acid, oleanolic acid and quercetin [22, 23].

Effect on antioxidant activity

The inhibition activity of SOD, 95% extract was the highest but comparing with 75% extract was not significant. At the same time, the inhibition activity of DPPH, 75% extract was the highest and comparing with 95% extract was significant ($P < 0.05$). In addition, the content of chlorogenic acid in 75% extract was highest of all the extracts. The results were shown in Table 1.
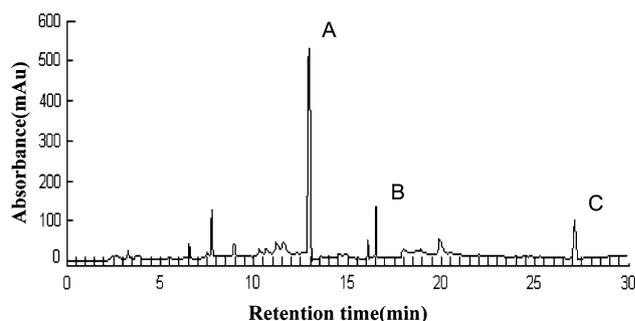

Fig. 1 HPLC profile of FLE. *A* was identified as chlorogenic acid, *B* was identified as oleanolic acid, and *C* was identified as quercetin

Table 1 Discovered yields, the IC$_{50}$ values of SOD activity and DPPH inhibition activity, chlorogenic acid contents on the different base FLE extracts

	Yield %	SOD activity IC$_{50}$ value (mg/mL)	DPPH activity IC$_{50}$ value (mg/mL)	Chlorogenic acid (mg/g dry weight)
Water	51.87 ± 0.90	0.17 ± 0.01	0.92 ± 0.08	3.40 ± 0.60
25% ethanol	43.07 ± 0.58	0.13 ± 0.02	0.31 ± 0.04	4.50 ± 0.60
50% ethanol	42.73 ± 0.92	0.13 ± 0.03	0.25 ± 0.01	4.70 ± 1.30
75% ethanol	40.87 ± 0.61	0.10 ± 0.02	0.20 ± 0.03	6.00 ± 0.10
95% ethanol	32.13 ± 0.70	0.09 ± 0.02	0.32 ± 0.03	3.60 ± 0.80

Fig. 2 Body weight **a**, *$P < 0.01$ compared with normal group, #$P < 0.01$ compared with normal group. Weighted of liver and spleen to total body weight **b** and **c**, *$P < 0.05$ compared with normal group (analysis of variance followed by Dunnett's test)
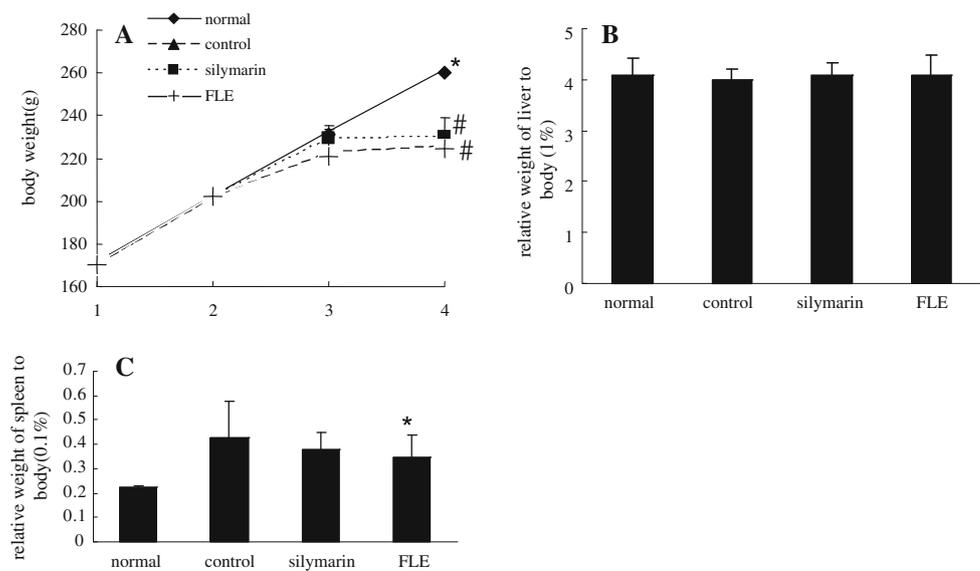
Animal experiments

Treatment with DMN caused a significant decrease in body weight, compared with the normal group as shown in Fig. 2a. The effects of the FLE on the wet liver weight and wet spleen weight of the rats were shown in Fig. 2b and c. Gross manifestation of body was decreased after injection DMN. In the control group and FLE group, body weight gain for 1 week was 1.7 and 8.9% of the normal group, respectively. On the whole, the final liver weight in the control group and FLE group were the same as those of normal group but liver colors of the control group were very dark brown and had too many voids. On the other hand, FLE group were light brown and had fewer voids than control group (data not shown). Spleen weights were increased in all groups compared to the normal group. However, in the FLE group the value tend to lower than in the control group and the silymarin group.

Serum indicator of liver function

The effects of FLE on the serum parameters in the liver injury model are shown in Fig. 3. AST, ALT, γ-GT and ALP were significantly suppressed by FLE treatment compared to control group as shown in Fig. 3a–c, whereas both TP and ALB were increased in the FLE group compared to control group and lower than normal group which was shown in Fig. 3d.

Antioxidant parameters in liver tissue

MDA and GPx levels were elevated after 1 week of DMN treatment and remained at a level significantly higher than

normal group. In agreement with this notion, DMN caused the increase both MDA and GPx levels in liver tissue but the effects of DMN on both parameters were inhibited by FLE administration. In control group MDA was increased 6.95 of FLE treatment group, simultaneously value of GPx in control was three times of FLE group as shown in Fig. 4a and b.

Histopathological findings

The amount of collagen fibers in the liver and liver sections collected from DMN-treated was evaluated by staining with Azan-Staining. There were almost no fibers in the liver of untreated normal group as shown in Fig. 5a. The DMN group showed increased collagen and displayed bundles of collagen fibers surrounding the lobules, forming large fibrous septa as shown in Fig. 5b. The thickening of these collagen fiber bundles was markedly reduced in the FLE group and silymarin group as shown in Fig. 5c and d. The histopathological fibrosis scores analysis confirmed that the liver fibrosis was significantly reduced by FLE treatment, as compared with the control group.

Discussion

Hepatic injury at the intermediate and crucial stage is characterized by reversibility. If treated properly at this stage, cirrhosis could be successfully prevented. However, it remains a problem to prevent cirrhosis or to control its progression. Great efforts have been made to find safe and effective drugs. Among the many drugs for liver injury, silymarin is the most clinically popular for patients and is

Fig. 3 Serum biochemical analyses. **a** Value of AST and ALT, AST, *$P < 0.01$ compared with normal group, #$P < 0.05$ compared with control group; ALT, *$P < 0.01$ compared with normal group, #$P < 0.01$ compared with control group. **b** Value of ALP *$P < 0.01$ compared with normal group, #$P < 0.01$ compared with control group; **c** value of γ-GT, *$P < 0.01$ compared with normal group, #$P < 0.05$ compared with normal group; **d** value of TP and ALB, ALB, *$P < 0.01$ compared with normal group, #$P < 0.05$ compared with control group (analysis of variance followed by Dunnett's test)
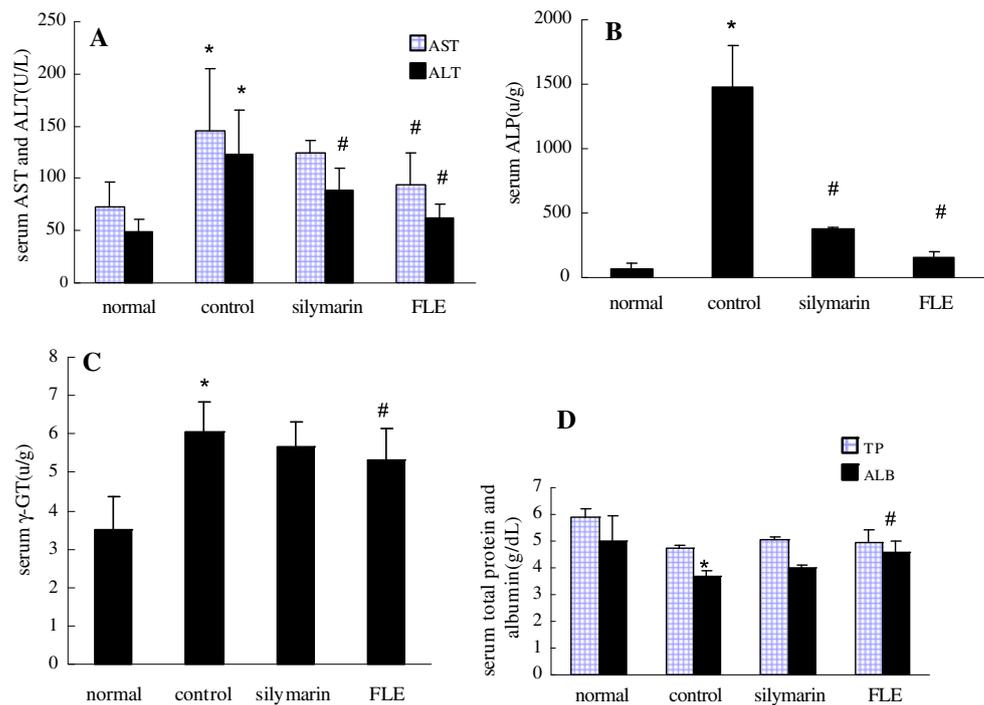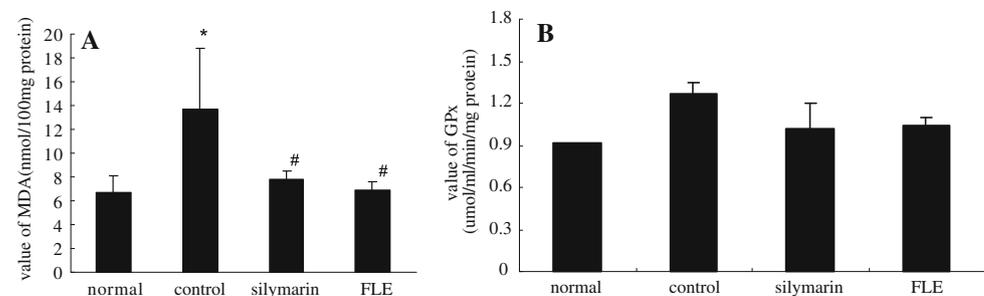
Fig. 4 MDA and GPx were measured in liver tissue. **a** Value of MDA, *$P < 0.05$ compared with normal group, #$P < 0.05$ compared with control group; **b** GPx, *$P < 0.01$ compared with normal group, #$P < 0.01$ compared with control group (analysis of variance followed by Dunnett's test)

known to have hepatotherapeutic and anti-fibrotic properties [24–27]. Therefore we used silymarin as the positive control in this experiment. But it was never found that the protective contribution and antifibrotics effect of *Flos lonicera* on acute liver injury. In present studies, the experiments were carried out to evaluate the effects obtained from the *Flos lonicera* on animal models of acute liver injury in rats by examining the levels modification of ALT, AST, ALP, γ-GT, MDA, GPx, ALB, TP and pathologic changes. We constructed an acute liver injury model using Wistar rats as in previous studies [27–31], showing marked liver dysfunctions, such as low serum albumin, high level of γ-GT, ALP, AST, and ALT, and increased MDA and GPx levels in liver tissues.

Liver dysfunction such as liver cirrhosis and portal hypertension cause hypersplenism (Splenomegaly) [32]. We examined wet spleen weight which is indicator of liver dysfunction. Spleen weights were increased in all groups compared to the normal group. However, in the FLE group

the value tend to lower than in the control group and the silymarin group. Therefore, liver dysfunction degree of FLE group is lower than the control group and the silymarin group. FLE administration showed major reducing effects on the increased levels ALP, AST, and ALT induced by DMN treatment, and slight restorative effects on organ weight, serum albumin and GPx in the tissue. In contrast to other biomarkers, the production of lipid peroxidation, measured as MDA, accelerated continuously up to the endpoint of this experiment. This was partially restored by the administration of FLE (Fig. 4). Enhancement of oxidative stress has been reported to be implicated in DMN-induced fibrosis and possibly hepatocarcinogenesis in rats [33, 34]. Intraperitoneal administration of DMN to rats is known to cause liver fibrosis, characterized by hepatocell necrosis, increased connective tissue and formation of regenerative nodules [32]. Because oxidative stress in DMN-treated animal models is known to be a major cause of hepatocellular damage and fibrosis [35], it is likely that

Fig. 5 Histopathologic examined of liver tissue by Azan staining. Light microscopy showing normal liver tissue as shown in **a**, control group as shown in **b**, silymarin group as shown in **c**, FLE treatment group as shown in **d**
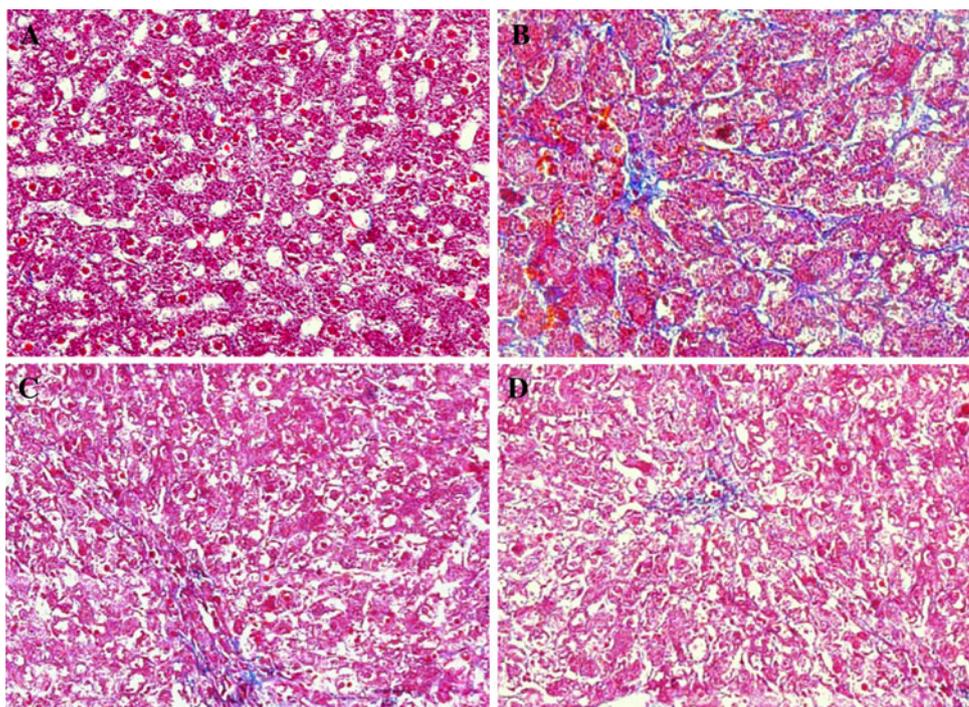
FLE has some hepatotherapeutic effects as a result of reducing DMN induced oxidative stress. Thus far, no effective anti-fibrotic or anticirrhotic treatments are available for clinical use [36]. This experiment demonstrated some anti-fibrotic effects of FLE administration and histological examination. In previous studies observed that FLE exhibited greater liver protective effects than silymarin.

Based on these results, we conclude that FLE possesses hepatotherapeutic properties against acute hepatocellular destruction. Which component in the extract hold the effects mentioned as above and the mechanism of these therapeutic effects of FLE on liver diseases, however, should be further clarified so that FLE can be used as effectively as possible as a means of treatment.

The content of chlorogenic acid was highest in FLE of 75% ethanol which has highest antioxidant activity suggesting that the antioxidation may be due to polyphenol compounds such as chlorogenic acid and its derivatives. The effort to identify other active component from FLE is ongoing in our laboratory.

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