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Full Length Research Paper

The role of hepatoprotective effect of a flavonoid-rich extract of *Salvia plebeia* R.Br. on carbon tetrachloride-induced acute hepatic injury in mice

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***Salvia plebeia* R.Br. is a widely distributed grass, which has been used as a folk medicine for the treatment of hepatitis. In this study, a simple method for preparative separation of the flavonoid-rich extract of *S. plebeia* was established with macroporous resins, the method can be referenced for large-scale enrichment of flavonoid-rich extract from herbal raw materials. The major constituents of FESP, including luteolin-7-glucoside, nepetin-7-glucoside, homoplantaginin, luteolin, nepetin, and hispidulin were determined by HPLC-DAD analysis. The hepatoprotective effect of FESP was evaluated in carbon tetrachloride (CCl_4)-induced liver injury in mice. The biochemical results were supplemented by histopathological examination.**

Key words: Hepatoprotective activity, *Salvia plebeia* R. Brown (Lamiaceae), flavonoids, histopathological.

INTRODUCTION

Liver intoxication has increased as a result of exposure to high levels of environmental toxins, for the liver has an important role in detoxification (Jin et al., 2005). Carbon tetrachloride (CCl_4), a well-known potent hepatotoxic agent, is being used extensively to investigate hepatoprotective activity on various experimental animals. Hepatic damage induced by CCl_4 resulted in an increase in serum aspartate transaminase (AST) and serum alanine transaminase (ALT) concentrations (Berry et al., 1992). The elevation of concentrations of these two serum enzymes is generally regarded as one of the sensitive markers of hepatic damage (Venkateswaran et al., 1998).

Salvia plebeia R.Br. is a widely distributed grass in China and India. It has been used as a folk medicine for the treatment of hepatitis, cough, inflammation and haemorrhoids. *S. plebeia*, as a formulation in a Taiwan herbal remedy named "Chhit-Chan-Than", has been used in the treatment of hepatitis (Lin et al., 1995). The previous phytochemical studies on *S. plebeia* herbs from different provinces in China revealed that it mainly contained flavonoids like hispidulin, homoplantaginin, nepetin, nepetin-7-glucoside, luteolin-7-glucoside and luteolin (Jin et al., 2008). Qu et al. (2009) demonstrated the protective effects of homoplantaginin isolated from *S. plebeia* on hepatocyte injury *in vitro* and *in vivo*. The flavonoid hispidulin (6-methoxy-5, 7, 4'-trihydroxyflavone) has been observed to prevent bromobenzene-induced liver injury in mice (Ferrandiz et al., 1994). Weng et al. (2000) screened over 700 species of the most commonly used herbs using the oxidative stability instrument (OSI), *S. plebeia* was identified to be a potent antioxidant plant among them. These results supported the use of FESP for the treatment of hepatitis. Many flavonoid-rich extracts revealed hepatoprotective effect (Yuan et al., 2008; Wang et al., 2008). However, no reports were available on the protective potential of flavonoid-rich extract of *S. plebeia* (FESP) against experimentally induced hepatitis in mice.

Macroporous resins, a nontype ion-exchange groups

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with large pore structure of the polymer adsorbent, is one of the most efficient separation materials. The adsorption properties of macroporous resin were correlated with their surface adsorption, sieve classification, surface electrical property and hydrogen bonding interactions, and so forth (Zhang et al., 2009). In this study, a flavonoid-rich extract of *S. plebeia* R.Br. (FESP) was prepared by adsorption on macroporous resin and desorption by ethanol, the total flavonoid content and major constituents of FESP were determined by a colorimetric method and HPLC-DAD analysis, respectively. The hepatoprotective effect of FESP was evaluated in carbon tetrachloride (CCl_4)-induced liver injury in mice by observing biochemical results and histopathological examination of liver sections.

MATERIALS AND METHODS

Chemicals

CCl_4 was purchased from Changjiang Chemical Co., Ltd (Shanghai, China). Assay kits for ALT and AST were obtained from the Jiancheng Institute of Biotechnology (Nanjing, China). Carboxy methyl cellulose (CMC) was purchased from Shanghai Shanpu Chemical Co., Ltd (Shanghai, China). Bifendate (DDB, 99.0% purity) was purchased from Zhejiang Wepon Pharmaceutical Company (Zhejiang, China). Rutin was purchased from Sigma Aldrich Co. (St. Louis, MO, USA), and the purity is above 98%. All chemicals were analytical grade.

Plant materials

S. plebeia was purchased from a crude drug market in Bozhou, Anhui Province, China and identified by Professor Yan-hua Lu. The voucher specimen (NO.20061021) was deposited at State Key Laboratory of Bioreactor Engineering, East China University of Science and Technology, Shanghai, 200237, China.

Preparation of flavonoid-rich extract of *S. plebeia* (FESP)

The aerial parts of *S. plebeia* were milled into powder and oven-dried at 50°C until constant weight was reached (Zou et al., 2004). A 250 g of milled material was suspended 1:20 (v/v) in 50% ethanol at 70°C for three times. Then, the crude extracts were concentrated by rotary evaporator at 70°C. The concentrated extracts were dissolved in water by ultrasonication, and then the solution was centrifuged at 3000 rpm for 10 min to obtain a clarified solution. The solution was poured in a column previously packed with HZ820 macroporous resin (Shanghai Huazhen Science and Tech. Co., Ltd, column of 40 × 3.4 cm, i.d.). The performance and adsorption characteristics of fourteen macroporous resins including HZ802, HZ801, HZ818, HZ820, LSA40, LSA20, DA201, NKA9, PA030060, PA080100, PA100120, AB8, PVA124 and 1799 had been evaluated. The results confirmed that HZ820 resin was preferred choice, which offered the best adsorption and desorption capacities for the FESP among the tested resins (dates not shown).

The solution was pumped down through the column at a speed of 2.5 bed volumes/h (BV/h). When the content of total flavonoid in the effluent achieved a value of about 10% of the loaded solution, as measured by the colorimetric method, the resin was thought to be saturated and the loading was then stopped. The resin was washed with 5 BV distilled water to remove the sugars and other water-soluble compounds, then 70% ethanol was used to elute the flavonoids at a speed of 4.0 BV/h. The effluent between 0.5 and 3 BV was collected, and the solvent was removed with a rotary evaporator at 70°C to afford a final extract (7.75 g).

Preparation of aqueous extract of *S. plebeia* (AESP)

A 100 g of dried and milled aerial parts of *S. plebeia* were subjected to hot water, decocted for 30 min at 100°C for three times with 1000 mL of distilled water, and then filtered. The filtrate was evaporated to dryness to prepare the total aqueous extract (AESP, 5.40 g).

Determination of total flavonoid content

The total flavonoid content of FESP was determined by use of a slightly modified colorimetric method described previously (Jia et al., 1999). A 0.5 mL aliquot of appropriately diluted sample solution was mixed with 2 mL of distilled water and subsequently with 0.15 mL of a 5% $NaNO_2$ solution. After 6 min, 0.15 mL of a 10% $AlCl_3$ solution was added and allowed to stand for 6 min, then 2 mL of 4% NaOH solution was added to the mixture. Immediately, water was added to bring the final volume to 5 mL, and then the mixture was thoroughly mixed and allowed to stand for another 15 min. Absorbance of the mixture was determined at 510 nm versus prepared water blank. Rutin was used as standard compound for the quantification of total flavonoid. All values were expressed as milligrams of rutin equiv per 1 gram of FESP. Data were reported as means ±SD for three replications.

High-performance liquid chromatography (HPLC) analysis

In the previous study on *S. plebeia*, six reference compounds (hispidulin, homoplantaginin, nepetin, nepetin-7-glucoside, luteolin and luteolin-7-glucoside) were isolated and confirmed by UV, IR, ESI-MS, ^{1}H NMR and ^{13}C NMR (Dates were consistent with Weng et al. (2000); Gu et al. (2001)). The HPLC method was validated and standardized (Jin et al., 2008). Briefly, 20.6 mg FESP were dissolved by 50 mL methanol and then filtered through a 0.45 μm membrane filter. Analysis was carried out at 30°C on a Zorbax Eclipse XDB-C18 column (250 × 4.6 mm, 5 μm). A linear gradient elution of eluents A (0.5% (v/v) aqueous glacial acetic acid) and B (methanol). The elution programme was conducted as follows: a linear gradient of 38 ~ 42% B with the range of 0.0 ~ 14.0 min, a linear gradient of 42 ~ 45% B with the range of 14.0 ~ 17.0 min, a linear gradient of 45 ~ 48% B with the range of 17.0 ~ 17.1 min, a linear gradient of 48 ~ 50% B with the range of 17.1 ~ 32.0 min and a linear gradient of 50 ~ 85% B with the range of 32.0 ~ 40.0 min. The detection wavelength was at 342 nm and the solvent flow rate was 1.0 mL/min. The injection volume was 5 μL.

Acute toxicity studies

The acute toxicity study for FESP was performed using Kunming mice (18 - 20 g) of either sex. The animals were fasted overnight prior to the experiment and maintained under standard conditions. FESP dissolved in 0.8% CMC, was administered orally to three groups of mice (six animals each) at doses increasing (1000, 1500, 2250 mg/kg b.w.) and found safe up to dose of 2250 mg/kg.

Animals and design

Kunming mice (18 ~ 20 g) of either sex were purchased from Shanghai Slac Laboratory Animal Co., Ltd (Shanghai, China) and

maintained under standard environmental conditions and had free access to feed and water. Experiments on animals were performed based on animal ethics guidelines of Institutional Animal Ethics Committee. After one week of acclimation, 70 mice were randomly divided into 7 groups with 10 mice per group: control group, CCl_4 group, FESP (50, 150 and 300 mg/kg b.w.) groups, AESP (300 mg/kg b.w.) group and bifendate (150 mg/kg b.w.) group. Control group and CCl_4 group animals were given the vehicle alone (0.8% CMC, 0.1 mL/10 g b.w., per day p.o.) for 7 days. FESP and AESP group animals were treated with plant extracts at dose level of 50, 150, 300 and 300 mg/kg b.w. (1%, w/v, 0.8% CMC, per day p.o.) for 7 days, respectively. Bifendate group animals were treated with standard drug, bifendate at dose level of 150 mg/kg b.w. (1%, w/v, 0.8% CMC, per day p.o.) for 7 days. Seven days after treatment, the mice (except the control group) were intraperitoneally injected with a mixture of carbon tetrachloride and olive oil (0.1%, v/v, 0.1 ml/10 g) to induce acute liver injury. All animals were then fasted for 16 h before they were sacrificed. Blood samples were collected from retroorbital plexus of mice, and allowed to clot for 45 min at room temperature. The serum was separated by centrifugation at 2500 rpm at 30°C for 15 min and used for the assay of marker enzymes, ALT and AST.

Biochemical determinations

The biochemical parameters like serum enzymes, AST and ALT were assayed using assay kits according to the manufacturer's protocols. Serum levels of ALT and AST were analyzed using a kinetic UV method defined by the International Federation of Clinical Chemistry (IFCC) that uses pyridoxal phosphate, and expressed as U/L.

Histopathological study

The liver tissue was dissected out and fixed in 10% formalin, dehydrated in gradual ethanol (50 ~ 100%), cleared in xylene, and embedded in paraffin. Sections of 5 µm were cut and stained with hematoxylin and eosin (H & E) for photomicroscopic observation (400 ×), including cell necrosis, fatty change, hyaline degeneration, ballooning degeneration, infiltration of kupfer cells and lymphocytes. Histological damages were scored as follows: 0: absent; +: mild; ++: moderate; and +++: severe.

Statistical analysis

Results were expressed as mean ± S.D and significant difference between groups was detected by means of a one-way ANOVA test followed by Dunett's *t*-test. Student's *t*-test was used for comparison between two groups.

RESULTS

The yield, total flavonoid content, and composition of FESP

The total flavonoid contents of AESP and FESP were different, which were 156.0 ± 2.15 and 622.3 ± 2.41 mg/g, respectively. The yield of AESP and FEHP were 5.4 and 3.1% (based on crude herb), respectively. Compared with reference standards, six flavonoids of FESP were identified as peak (2) luteolin-7-glucoside; (3) nepetin-7-glucoside; (6) homoplantaginin; (7) luteolin; (8) nepetin; (9) hispidulin (chemical structures shown in Figure 1). The chromatogram was shown in Figure 2. The results of the content (mg/g) of six flavonoids of FESP were shown in Table 1.

Acute toxicity studies

FESP did not show any sign and symptoms of toxicity and mortality up to 2250 mg/kg dose.

Protective effects of FESP on CCl_4-induced acute liver injury in mice

The results of hepatoprotective effect of extracts on CCl_4-intoxicated mice are shown in Table 2. In the CCl_4 group, serum AST and ALT were increased to 70.4 and 244.2 U/L, respectively, whereas these values were showed 28.5 and 30.0 U/L in control group. Pretreatment with FESP (50, 150 and 300 mg/kg b.w.) prevented the CCl_4-induced elevation of ALT and AST serum levels in a dose-dependent manner; the high dose FESP (300 mg/kg b.w.) showed extremely activity ($p < 0.001$). Serum AST and ALT of High dose FESP (300 mg/kg b.w.)-pretreated mice were close to bifendate group (150 mg/kg b.w.) and normal group.

Histopathological observation

Histology of the liver sections of control animals (Figure 3A) showed normal hepatic cells with well-preserved cytoplasm, prominent nucleus and nucleolus. The liver sections of CCl_4 group animals (Figure 3B) showed massive fatty changes, necrosis, ballooning degeneration, infiltration of lymphocytes and Kupffer cells and the loss of cellular boundaries. Treatment with FESP exhibited dose-dependent reversal of these changes induced by CCl_4 with few foci of necrosis of hepatocytes and fatty changes (Figure 3D-F). However, reversal at higher dose FESP (300 mg/kg b.w.) was similar to those of normal control group.

DISCUSSION

An evidence of hepatic injury is leakage of cellular enzymes into the plasma. Their estimation in the serum is a useful quantitative marker of the extent and type of hepatocellular damage. The rise in ALT activity is almost always due to hepatocellular damage and is usually accompanied by rise in AST (Ravikumar et al., 2005). Administration of FESP at different dose levels (50, 150 and 300 mg/kg) attenuated the increased levels of the serum enzymes, produced by CCl_4 and caused a

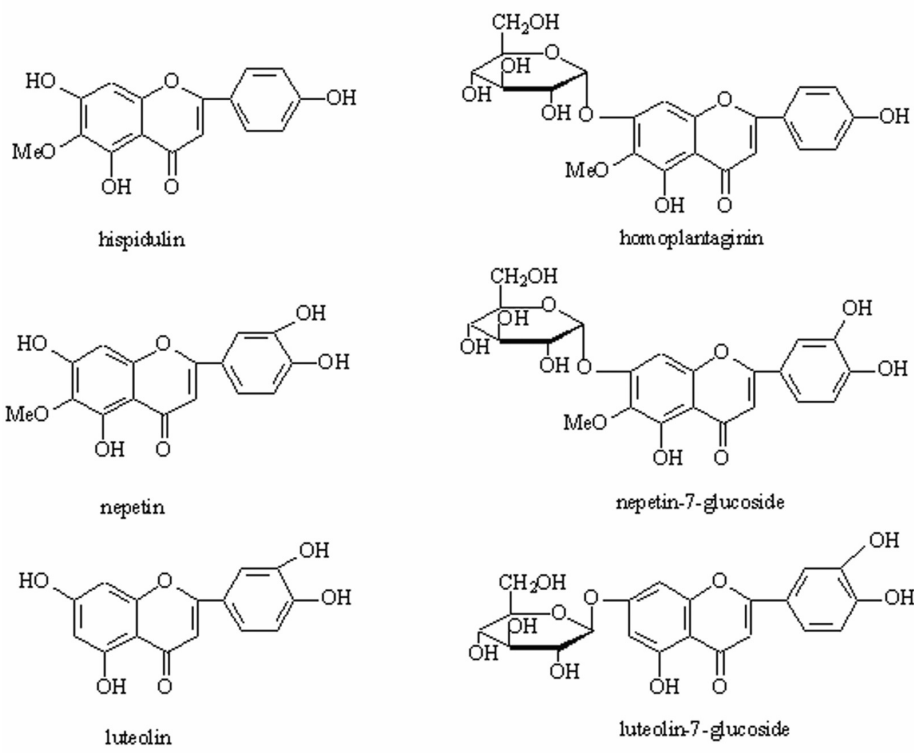

Figure 1. Chemical structures of hispidulin, homoplantaginin, nepetin, nepetin-7-glucoside, luteolin and luteolin-7-glucoside.

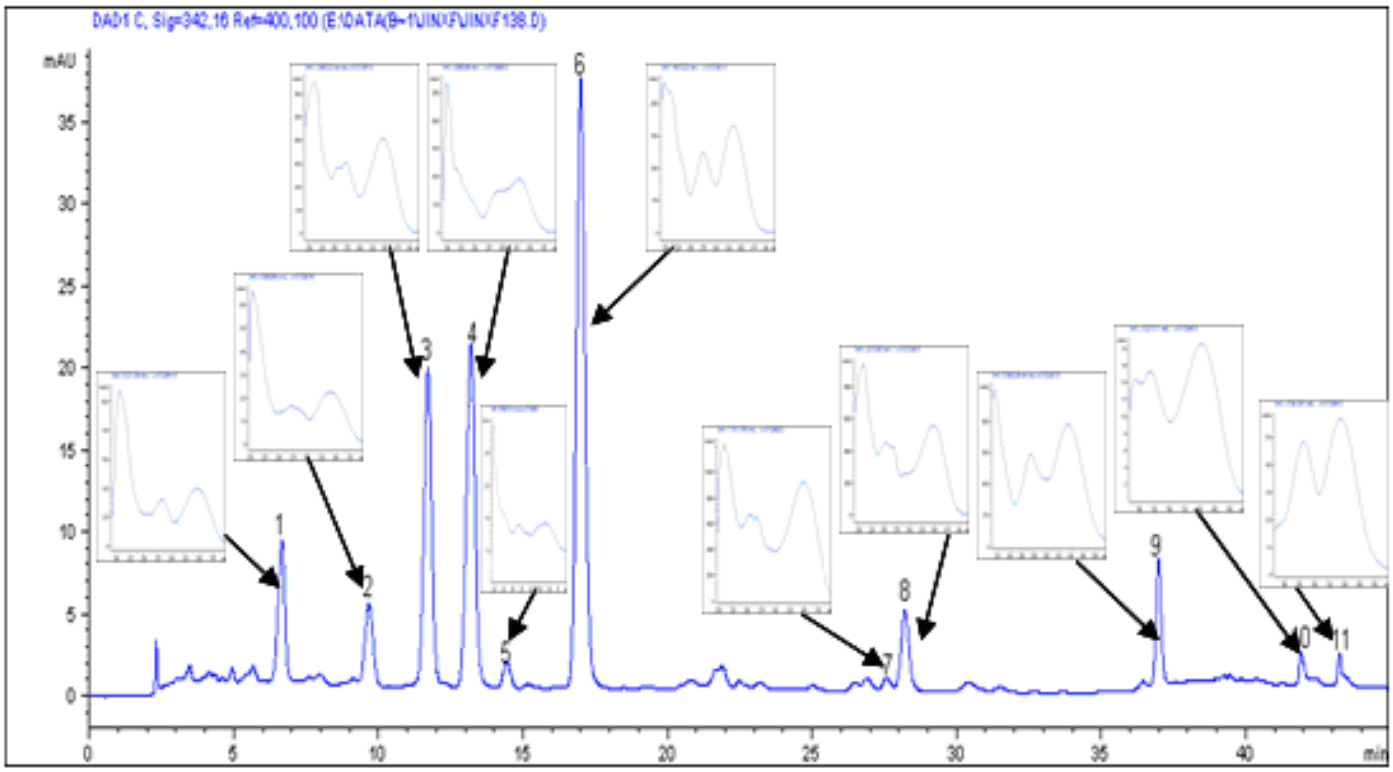

Figure 2. HPLC chromatogram of FESP at 342 nm wavelength and the DAD scans of main contents: peak (2) luteolin-7-glucoside; (3) nepetin-7-glucoside; (6) homoplantaginin; (7) luteolin; (8) nepetin; (9) hispidulin; (1), (4), (5), (10), (11) other unknown contents.

Table 1. Content of six flavonoids of FESP by HPLC (mg/g, n = 3).

Flavonoids	Content
Luteolin-7-glucoside	25.20±0.08
Nepetin-7-glucoside	50.70±0.21
Homoplantaginin	95.90±0.18
Luteolin	1.60±0.01
Nepetin	8.60±0.01
Hispidulin	7.40±0.02

Table 2. Hepatoprotective effect of FESP in CCl_4 intoxication mice

Group	Dose (mg/kg)	AST (U/L)	ALT (U/L)	Severity of liver injury
Control	—	28.5±7.8	30.0±13.5	0
CCl_4	—	70.4±34.9$^{\#\#}$	244.2±81.5$^{\#\#\#}$	+++
Bifendate	150	38.6±11.4***	36.5±17.2***	+
FESP	300	36.7±10.8***	35.9±18.4***	+
	150	40.5±13.2**	80.3±26.8***	++
	50	41.2±13.0**	81.8±32.9***	++
AESP	300	51.2±17.4*	176.4±124.2*	+++

[a] Values are mean ± S.D., n = 10, [b] 0: absent; +: mild; ++: moderate; +++: severe, Compared with the control group: $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, Compared with the CCl_4 group: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

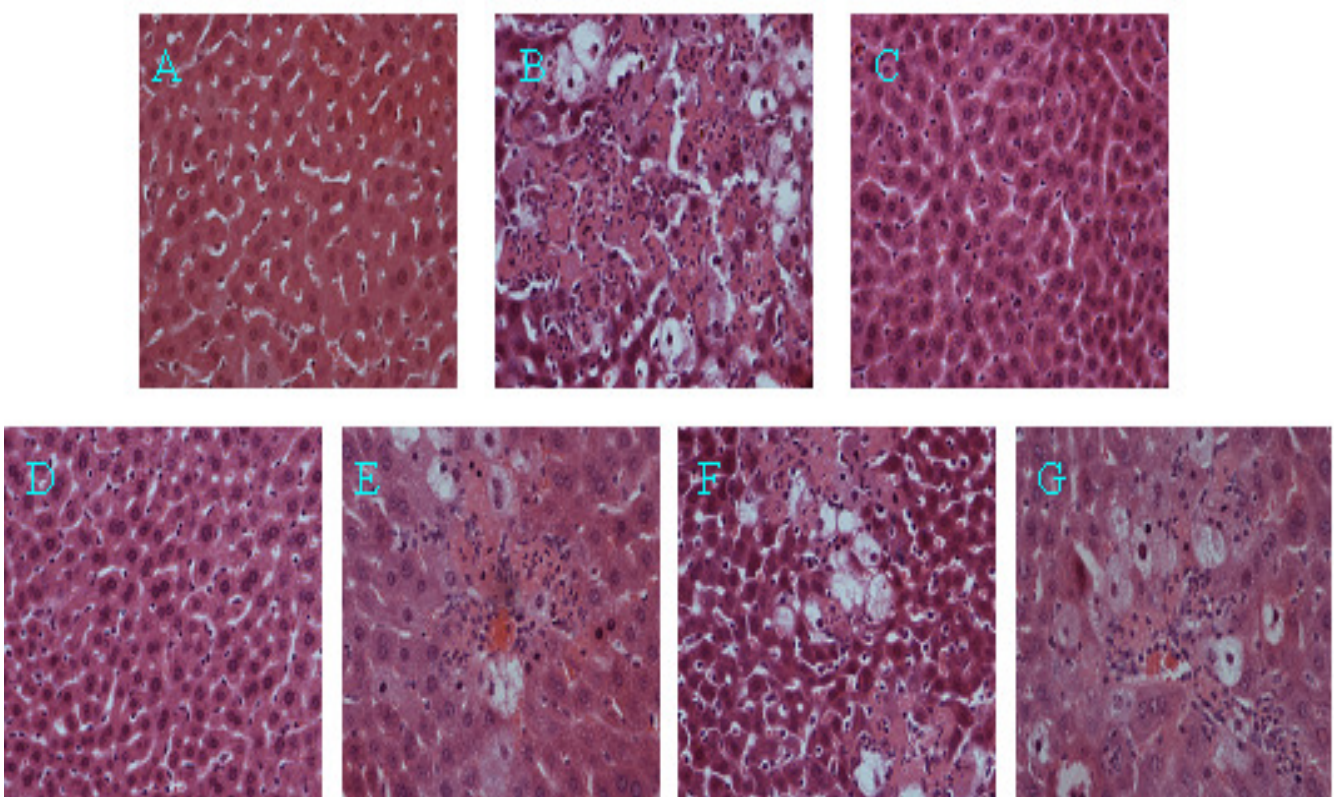

Figure 3. Histopathological changes in liver sections after CCl_4 intoxication and prevention by the treatment with FESP and AESP. Liver tissues were stained with H and E (400 ×). A: Normal control; B: CCl_4 control; C: CCl_4+ Bifendate (150 mg/kg); D: CCl4+FESP (300 mg/kg); E: CCl_4+FESP (150 mg/kg); F: CCl_4+FESP (50 mg/kg); G: CCl_4+AESP (300 mg/kg).

subsequent recovery towards normalization comparable to the control group animals. Although, pretreatment with AESP (300 mg/kg b.w.) showed decrease of the levels of serum AST and ALT ($p < 0.05$) compared to CCl_4 group, it seemed inferior to low dose FESP (50 mg/kg b.w.). The hepatoprotective effect of the FESP was further accomplished by the histopathological examinations. The histological observations basically supported the results obtained from biochemical index. The biochemical index and histopathological appearances were in a dose-dependent manner.

Many studies have reported the hepatoprotective effect of the extract of herb medicine. However, little literature indicated the main active component. Traditionally, the raw materials of *S. plebeia* were extracted by boiling water and administrated orally. In spite of its effect, the stupendous dosage, as well as unclear chemical composing, is the latent hazardous factor of administrating crude extract of the herbal medicine. In the present study, qualitative and quantitative analysis of six main content flavonoids of FESP was carried out by HPLC-DAD using the condition reported before by their laboratory. Five unknown contents were also conjectured to be the flavonoids by the DAD scans. All of these were in favour of illuminating the chemical composition of FESP. According to the study, the isolated flavonoids processed potent hepatoprotective activities and were conjectured as the main active contents. However, the convinced protective mechanism of flavonoids in liver injury must be ascertained in future study.

Conclusion

In this study, they firstly reported the extracting technology of flavonoid rich extract of *S. plebeia* (FESP), and its striking beneficial effects in preventing carbon tetrachloride induced cute hepatic injury in mice. The contents of FESP were mainly flavonoids, six of which have been identified as hispidulin, homoplantaginin, nepetin, luteolin luteolin-7-glucoside and nepetin-7-glucoside.

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