Potential antioxidant properties and hepatoprotective effects of an aqueous extract formula derived from three Chinese medicinal herbs against CCl₄-induced liver injury in rats

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Abstract

The hepatoprotective effects of an aqueous extract formula (AEF) derived from Artemisia capillaris, Lonicera japonica and Silybum marianum (ratio 1:1:1) were evaluated by its antioxidant properties and its attenuation of carbon tetrachloride (CCl₄)-induced liver damage in rats. The antioxidant analyses revealed that the AEF showed higher 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical and superoxide anion radical scavenging activities as well as ferric reducing antioxidant potential (FRAP) and Trolox equivalent antioxidant capacity (TEAC) compared with the individual herbs, suggesting a synergism in antioxidation between the three herbs. The animal experiments showed that the CCl₄ treatment increased serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities, but decreased triglyceride (TG) and glutathione (GSH) levels as well as glutathione peroxidase (GPx), superoxide dismutase (SOD) and catalase (CAT) activities. However, AEF administration can successfully lower serum ALT and AST activities, restore the GSH level, ameliorate or restore GPx and CAT activities as well as improve SOD action depending on AEF dosage. Histological examination of liver showed that CCl₄ increased the extent of bile duct proliferation, necrosis, fibrosis and fatty vacuolation throughout the liver, but AEF can improve bile duct proliferation, vacuolation and fibrosis, and restore necrosis. The present study demonstrated the hepatoprotective potential of AEF as an alternative to the traditional silymarin.

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1. Introduction

Chronic liver diseases are common worldwide and are caused as a result of long-term exposure to toxic chemicals, drugs, alcohol and/or viral infection. Liver diseases are characterized by a progressive evolution from steatosis to chronic hepatitis, fibrosis, cirrhosis, and hepatocellular carcinoma [1,2], and are associated with high morbidity and mortality.

The carbon tetrachloride (CCl₄) is a colorless, odorless, volatile and highly toxic chemical which enters human body via respiratory tract, digestive tract and skin [3]. In the liver, CCl₄ forms the trichloromethyl radical (CCl₃) through the action of cytochrome P450 enzymes and later reacts with oxygen to form the highly reactive derivative trichloromethylperoxy radical (CCl₃O₂). Both radicals can bind to cellular molecules (i.e. nucleic acids, proteins, lipids and carbohydrates) [4], and induce lipid peroxidation, damage the membranes of liver cells and organelles, cause the swelling and necrosis of hepatocytes and result in the release of cytosolic enzymes such as aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP) into the circulating blood [5,6]. The most remarkable pathological characteristics in CCl₄-induced hepatotoxicity are fatty liver, cirrhosis and necrosis [7]. Because symptomatically induced cirrhotic response in animals by CCl₃ has been shown to be superficially similar to human cirrhosis of the liver, CCl₄ has been widely used in animal models to investigate chemical toxin-induced liver damage [8,9].

Because free radicals and reactive oxygen species play a central role in liver disease pathology and progression, dietary antioxidants have been proposed as therapeutic agents to prevent oxidative stress-related liver pathologies [1,2,10]. Natural antioxidants may act not only as conventional hydrogen-donating compounds but, more importantly, may exert modulatory effects in cells through actions in antioxidant, drug-metabolizing and repairing enzymes as well as working as signaling molecules in important cascades for cell survival [11]. Additionally, recent studies have suggested that natural antioxidants in complex mixtures are more efficacious than...
pure compounds in preventing oxidative stress-related pathologies due to particular interactions and synergisms [2].

Chinese medicinal herbs have frequently been used to treat liver disorders in traditional Chinese medicine. For instance, Artemisia capillaris (Compositae) has been used to treat diseases of liver and gallbladder [12]; a number of terpenoid acids, polyenes, phenols, coumarins, chromones and flavonoids have been identified to be potent antipathotocytic constituents [13]. Lonicer a japonica (Caprifoliaceae) has been used as one of the principal ingredients in herbal formulations for the treatment of hepatitis [14], and a methanol extract of L. japonica was found to alleviate CCl₄-induced hepatic damage in rats [15]. Sil y bum marianum (Asteraceae), also called milk thistle, is a popular plant and its seed extract, the silymarin, exhibits antioxidant, anticarcinogenic, anti-inflammatory, hepatoprotective and growth modulatory effects [16]. In animals, silymarin reduces liver injury caused by acetaminophen, CCl₄, radiation, iron overload, phenylhydrazine, alcohol, cold ischemia and Amanita phalloides [17].

Although the pharmacological effects of A. capillaris, L. japonica and S. marianum have been reported, there is no scientific evidence yet regarding the safety and efficacy of these herbal products in combination. The present research therefore has the objective to explore the antioxidant properties and hepatoprotective effects of an aqueous extract formula (AEF) derived from a combination of A. capillaris, L. japonica and S. marianum against CCl₄-induced liver injury in rats.

2. Materials and methods

2.1. Plant material and AEF preparation

The three medicinal herbs used in the present study, i.e. A. capillaris, L. japonica and S. marianum, were collected in the mountainous areas of Taipei. The herbs were boiled in ten volumes the time of water for 4–5 h, then reduced to 1/10 in a 37 °C rotary evaporator after filtration under vacuum. They were freeze-dried, ground to fine powder and stored at 4 °C until use. The AEF was made by mixing the extracts at 1:1:1 ratio.

2.2. Antioxidant property analyses of the herbs and AEF

The antioxidant properties of each herb and the AEF were evaluated following the total phenolic and flavonoid content analyses, 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay, superoxide anion radical scavenging assay, ferric reducing antioxidant potential (FRAP) assay, and Trolox equivalent antioxidant capacity (TEAC) assay. The total phenolic content of the samples was evaluated following the Folin–Ciocalteu colorimetric method described by Kujala et al. [18] and expressed as mg of gallic acid per g of dry plant material. The total flavonoid content was estimated using the aluminum chloride colorimetric method described by Chang et al. [19] and expressed as mg of quercetin per g of dry plant material. The DPPH assay was conducted according to the colorimetric method described by Hsu et al. [20] and the DPPH scavenging activity (%) was calculated as $1 - \frac{A_{Sample}}{A_{control}} \times 100$. The superoxide anion radical scavenging assay was performed based on the method of Lin et al. [21] and the superoxide anion radical scavenging activity (%) was calculated as $1 - \frac{A_{Sample}}{A_{control}} \times 100$. The FRAP assay was modified from a method of Benzie and Strain [22] and the results were expressed as mg of Vitamin C equivalent per g of dry plant material. The TEAC assay was performed following the procedure of Re et al. [23] and the results were expressed as Trolox equivalent per g of dry plant material.

2.3. Animal treatments

Fifty male Sprague–Dawley rats, 6 weeks old and weighing about 200–250 g, were purchased from the BioLASCO Company. They were housed in an environmental controlled room maintained at 22 ± 2 °C, 65 ± 5% relative humidity and 12 h light and dark photoperiod. Animals were given standard pellet diet (LabDiet® 5001, Rodent Diet, USA) and tap water ad libitum. The rats were assimilated to laboratory conditions for 7 days before being subjected to hepatotoxicity studies.

Rats were randomly divided into five groups of ten each: normal control group, CCl₄ negative control group, silymarin positive control group, low dose and high dose AEF groups. Twice a week, the normal control group received 0.1 ml 100 g⁻¹ body weight of olive oil whereas the remainder groups received 20% of CCl₄ dissolved in olive oil. Four times a week, rats of the normal control and the CCl₄ negative control groups were fed with distilled water, those of the silymarin positive control group were administered with silymarin at 200 mg kg⁻¹ of rats, those of the low dose AEF group were fed with AEF at 0.28 g kg⁻¹ of rats, and those of the high dose AEF group were administered with AEF at 1.4 g kg⁻¹ of rats. By the end of the eighth week, the rats were sacrificed under ether anesthesia. Blood and liver samples were collected for biochemical and histopathological analyses. All experimental protocols were approved by the Institutional Animal Care and Use Committee for National Pingtung University of Science and Technology.

2.4. Serum biochemical analyses

Blood was drawn by cardiac puncture, allowed to clot for 1 h at room temperature and centrifuged at 12,000 rpm for 10 min to obtain the serum. The levels of AST and ALT, triglyceride (TG) and total cholesterol (TC) were estimated using commercial kits from Randox Laboratories Ltd. (U.K.).

2.5. Antioxidant property analyses of the liver cells

The glutathione (GSH) content was analyzed using a Glutathione Assay Kit (Cayman Chemical). The glutathione peroxidase (GPx) activity was determined following the method of Lawrence and Burk [24] and the results were expressed as U per mg of protein. Protein content of liver homogenates was determined according to Bradford [25]. The glutathione reductase (GRd) activity was determined according to the method of Bellomo et al. [26] and the results were expressed as U per mg of protein. The superoxide dismutase (SOD) analysis was performed according to Marklund and Marklund [27] and one unit of enzyme activity was expressed as 50% inhibition of NBT reduction per mg of protein. The catalase (CAT) analysis was modified from Aebi [28] and the results were expressed as U per mg of protein. The quantitative measurement of lipid peroxidation was conducted by measuring the concentration of thiobarbituric acid reactive substances (TBARS) using the method of Ohkawa et al. [29], and the TBARS content was expressed as μmol malondialdehyde (MDA) per mg of protein.

2.6. Histopathological examination of liver damage

Hepatic morphometry was assessed by light microscopy. Liver tissue (i.e. 1 cm × 1 cm × 0.5 cm) was taken from the largest right lobe of the liver and fixed in 10% of formalin solution for 1 week. The liver tissue was dehydrated using a sequence of ethanol solutions, embedded in paraffin and sectioned to 5 μm in thickness followed by staining with hematoxylin–eosin (HE) dye before subjecting to microscopic observation of bile duct proliferation, steatosis, lobular inflammation and necrosis. Masson’s trichrome stain was used for the observation of liver fibrosis. The histological indices of bile duct proliferation, steatosis, lobular inflammation and necrosis were evaluated based on the method of Jonker et al. [30] and Lawrence and Burk [24] as follows: grade 0: absent; grade 1: few; grade 2: mild; grade 3: moderate; grade 4: strong. Liver fibrosis was graded according to Ruwart et al. [31] and Boigk et al. [32] as follows: grade 0: absent, normal liver; grade 1: few, increase of collagen without formation of septa (small stellate expansions of the portal fields and/or increased pericellular collagen in the lobules);
grade 2: mild, formation of incomplete septa from portal tract to central vein (septa that do not interconnect with each other); grade 3: moderate, complete but thin septa interconnecting with each other so as to divide the parenchyma into separate fragments (incomplete cirrhosis); grade 4: same as grade 3 but with thick septa (complete cirrhosis). All the observations were made from stained sections extracted from the same location of the largest right lobe of the liver to avoid subjective interpretations.

2.7. Statistical analysis

All experimental data were analyzed by one way analysis of variance (ANOVA) using SAS (Cary, NC) software, and treatment means were compared by performing Duncan Multiple Range test (*P<0.05). All the experiments were conducted three times to confirm the reproducibility of the assays.

3. Results

3.1. Antioxidant properties of the herbs and AEF

The antioxidant properties of each herb and the AEF are summarized in Table 1. The highest phenolic content was found in L. japonica (i.e. 75.4 mg g⁻¹), followed by AEF (i.e. 52.6 mg g⁻¹), A. capillaris (i.e. 35.6 mg g⁻¹) and S. marianum (i.e. 26.4 mg g⁻¹). For the flavonoid content, both L. japonica and the AEF had the highest total flavonoid content (i.e. 9.6 mg g⁻¹), followed by A. capillaris (i.e. 6.1 mg g⁻¹) and S. marianum (i.e. 2.4 mg g⁻¹). In terms of the DPPH scavenging activity, it was observed that the AEF showed a higher scavenging activity (i.e. 141.8%) than L. japonica (i.e. 81.4%), A. capillaris (i.e. 66.0%) and S. marianum (i.e. 57.0%). However, they were all inferior to the control (i.e. 96%). Similar trend was observed for the superoxide anion scavenging activity, except that no difference in activity was found between A. capillaris and L. japonica (i.e. 71%).

Table 2 shows the change in rat body weight and relative liver weight following 8 weeks of CCl₄, silymarin and AEF treatments. High dose AEF group (i.e. 229.6 g), and they were all inferior to the low dose AEF group (i.e. 246.9 g).

3.3. Serum biochemical analyses

The activities of ALT and AST and the levels of TG and TC in the serum 8 weeks following the treatments are summarized in Table 3. Results showed that the CCl₄ negative control group significantly elevated the level of ALT (i.e. 246.5 U l⁻¹) and AST (i.e. 346 U l⁻¹) compared with the normal control group (i.e. 34.4 and 106.3 U l⁻¹ respectively). The administration of low and high doses of AEF successfully lowered the ALT (i.e. 126.9–141.8 U l⁻¹) and AST (216–236 U l⁻¹) levels. The silymarin positive control group registered a significantly lower ALT level (i.e. 67.5 U l⁻¹) than the AEF groups. In terms of TG, the CCl₄ negative control group expressed a significantly lower TG level (i.e. 115.2 mg dl⁻¹) compared with the normal control group (i.e. 151.6 mg dl⁻¹), and none of the silymarin (i.e., 108.3 mg dl⁻¹) and low and high doses of AEF (i.e. 105 and 102.8 mg dl⁻¹) treatments were able to resume the TG levels. For the TC level, there was no significant difference among the different groups.

3.4. Antioxidant property analyses of the liver cells

The antioxidant properties of the liver cells 8 weeks following the treatments are presented in Table 4. It can be seen that the CCl₄ negative control group showed a significantly lower concentration of GSH (i.e. 38.8 µM) compared with the normal control group (i.e. 42.4 µM), whereas the silymarin positive control group (i.e. 42.4 µM), low dose AEF group (i.e. 42.3 µM) and high dose AEF group (i.e. 43.6 µM) all exhibited a similar GSH content than the normal control group. Regarding the GPx activity, the CCl₄ negative control group significantly decreased the enzyme activity down to 370.2 U mg⁻¹ protein after treatment. The low and high dose AEF groups were able to raise the enzyme activities to 673.4 and 531.7 U mg⁻¹ protein respectively. Only the silymarin positive control group could bring the enzyme activity (i.e. 788.8 U mg⁻¹ protein) back to a level similar to the normal control group (795.5 U mg⁻¹ protein). In terms of the GRd activity,
the silymarin positive control group showed a higher enzyme activity (i.e. 354.0 U mg⁻¹ protein) compared with all the other treatment groups which were not significantly different from each other. For the SOD activity, there was no significant difference between the CCl⁴ negative control group (i.e. 64.3 mg⁻¹ protein), the low dose AEF group (i.e. 72.5 U mg⁻¹ protein), and the normal control group (i.e. 96.5 U mg⁻¹ protein). By contrast, the silymarin positive control group (i.e. 151.1 U mg⁻¹ protein) and the high dose AEF group (i.e. 124.7 U mg⁻¹ protein) exhibited a comparatively higher enzyme activities. For the CAT activity, the lowest CAT activity was found with the CCl⁴ negative control group (i.e. 98.6 U mg⁻¹ protein). The CAT activity was raised in the low dose AEF group (i.e. 164.9 U mg⁻¹ protein) and was completely resumed to the level of the normal control group (i.e. 298.8 U mg⁻¹ protein) in the high dose AEF group (i.e. 261.8 U mg⁻¹ protein) and the silymarin positive control group (i.e. 247.5 U mg⁻¹ protein). Finally, there was no significant difference between the different treatment groups in terms of their MDA content.

### 3.5. Histopathological examination of liver damage

The morphology of the liver following 8 weeks of CCL₄ silymarin and AEF treatments is shown in Fig. 1. The liver of the normal control group was shiny and red, smooth on the surface and soft to the touch (Fig. 1A). Following the CCL₄ treatment, the color of the liver turned yellowish and the edge was obtuse and tough to the touch (Fig. 1B). Compared with the normal control group, the liver was swollen and rough on the surface with nodular protuberances. For the groups administered with silymarin (Fig. 1C) and AEF (Fig. D and E), although the liver could not restore the deep red color of the normal control group, the overall appearance was more reddish, smooth and shiny compared with the CCL₄-damaged liver.

The appearance of HE-stained liver following 8 weeks of CCL₄ silymarin and AEF treatments is shown in Fig. 2. Normal tissue structure and intact liver cells without fatty vacuolation were observed in the normal control group (Fig. 2A). Following the CCL₄ treatment, the normal architecture of liver was lost with the appearance of vacuolated hepatocytes (Fig. 2B). The different degrees of fatty vacuolation scattered throughout the liver tissue evidenced that CCL₄ had successfully induced liver damage (Fig. 2B). In the silymarin positive control group, although some vacuolation was observed it was less pronounced than in the case of the CCL₄ negative control group B (Fig. 2C). In liver administered with AEF, the vacuolation was greatly reduced and the proportion of normal cells increased (Fig. 2D and E).

Representative histological micrographs of Masson’s trichrome stained-liver following 8 weeks of CCL₄ silymarin and AEF treatments are provided in Fig. 3. In the normal control group, liver sections showed normal hepatic cells without fibrosis (Fig. 3A). The livers of rats treated with CCL₄ showed numerous hepatic lobules surrounded by thick fibrotic tissue, resulting in the formation of continuous fibrotic septa (Fig. 3B). The collagen of these fibrotic tissues showed a blue color when stained by Masson’s trichrome. The lesions of silymarin-treated rats were observed to a much milder degree than in the CCL₄ negative control group (Fig. 3C). Similarly, the groups administered with AEF showed mild degrees of fibrosis and the formation of fragmented and incomplete septa (Fig. 3D and E).

The extent of liver bile duct proliferation, steatosis, lobular inflammation and liver necrosis scores of rats following 8 weeks of CCL₄, silymarin and AEF treatments are presented in Table 5. It was observed that the CCL₄ negative control group (i.e. 2.0) showed the highest degree of bile duct proliferation followed by the silymarin positive control group (i.e. 1.2), low dose AEF group (i.e. 0.8) and high dose AEF group (i.e. 0.8). The normal control group showed no bile duct proliferation. For steatosis, all the treatment groups showed comparable extent of steatosis, whereas no steatosis was observed in the normal control group. Similar results were recorded for lobular inflammation, where all the treatment groups showed similar degree of inflammation. No inflammation was observed for the normal control group. Regarding liver necrosis, the CCL₄ negative control group showed the highest level of necrosis (i.e. 1.8), while the remaining groups were all comparable with the normal control group. In the same way, the CCL₄ negative control group resulted in a higher degree of liver fibrosis (i.e. 1.8) than in the other treatment groups. No fibrosis was observed in the normal control group.

### 4. Discussion

The application of Chinese medicinal herbs in treating various health disorders has become more and more popular on a global scale in recent years. Medicinal herbs contain a wide array of chemical compounds that may act individually, additively or in synergy to improve health. The present study explores the antioxidant properties and protective effects of an aqueous extract formula derived from a combination of three Chinese medicinal herbs, i.e. A. capillaris, L. japonica and S. marianum, against CCL₄-induced liver injury in rats.

Tocopherols are widely used as safe natural antioxidants, but they have been reported to be not as effective as synthetic antioxidants and also the manufacturing cost is high [33]. The search for potent antioxidants from natural sources (i.e. plant materials) is suggested. Tocopherols are used as the control in the present study to compare

### Table 3

<table>
<thead>
<tr>
<th>Group</th>
<th>ALT (U/L)</th>
<th>AST (U/L)</th>
<th>TG (mg/dL)</th>
<th>TC (mg/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control</td>
<td>34.4±2.8a</td>
<td>106.3±14.3b</td>
<td>151.6±9.8c</td>
<td>147.1±10.6d</td>
</tr>
<tr>
<td>CCL₄ negative control</td>
<td>246.5±26.8b</td>
<td>346.0±84.2b</td>
<td>115.2±11.7b</td>
<td>137.4±16.8b</td>
</tr>
<tr>
<td>Silymarin positive control</td>
<td>67.5±20.11a</td>
<td>186.2±43.3a</td>
<td>108.3±47.4a</td>
<td>147.8±16.5a</td>
</tr>
<tr>
<td>Low dose AEF</td>
<td>141.8±23.3b</td>
<td>216.0±53.9b</td>
<td>105.0±30.7b</td>
<td>143.3±10.4b</td>
</tr>
<tr>
<td>High dose AEF</td>
<td>126.9±13.6b</td>
<td>236.8±30.1b</td>
<td>102.8±14.5b</td>
<td>139.0±10.8b</td>
</tr>
</tbody>
</table>

Data are presented as mean±SD (n=8).

### Table 4

<table>
<thead>
<tr>
<th>Group</th>
<th>Liver</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>GSH (μM)</td>
</tr>
<tr>
<td>Normal control</td>
<td>42.1±3.8a</td>
</tr>
<tr>
<td>CCL₄ negative control</td>
<td>38.8±3.7a</td>
</tr>
<tr>
<td>Silymarin positive control</td>
<td>42.4±1.4a</td>
</tr>
<tr>
<td>Low dose AEF</td>
<td>42.3±2.0a</td>
</tr>
<tr>
<td>High dose AEF</td>
<td>43.6±1.0a</td>
</tr>
</tbody>
</table>

Data are presented as mean±SD (n=8).

Numbers within a column not sharing the same letter are significantly different from each other (P<0.05).
with the antioxidant properties of *A. capillaris*, *L. japonica* and *S. marianum* and their combined formula.

Phenolics, such as flavonoids, phenolic acids, stilbenes, lignans, lignin and tannins, are common in leaves, flowering tissues, and woody parts such as stems and barks of diverse plant species [34]. The total phenolic compounds are often closely related with the antioxidative activity of the plant. The antioxidant activity of phenolics is principally due to their redox properties, which allow them to act as reducing agents, hydrogen donors and singlet oxygen quenchers [20]. The FRAP and TEAC assays are widely used in the evaluation of antioxidant components as the DPPH is an oxidizing radical often used to evaluate the antioxidative activity of drugs and plant extracts [35], and the superoxide anion \( \left( \text{O}_2^- \right) \) plays an important role in the initial and cycling reactions of lipid peroxidation [36]. Results from the present study showed that *L. japonica* alone had a higher total phenolic content than the AEF, but the total flavonoid content was comparable between *L. japonica* and the AEF. In addition, the DPPH and superoxide anion scavenging activities, as well as the FRAP and TEAC of the AEF were all superior to each of *A. capillaris*, *L. japonica* and *S. marianum*, suggesting the higher antioxidant properties of AEF compared with the herbs when used alone. Our results are in agreement with previously reported findings where it has been observed that when the Chinese herbs were used in combination, they can exert a synergetic effect and their effectiveness during treatment is superior to when the herbs were used alone. For instance, the combination therapy of *Schisandra chinensis* and *Astragalus polysaccharides* produced synergistic effects of antioxidation and thus hepatoprotection in CCl\(_4\)-treated rats [37]. Similarly, the mixture of *S. chinensis*, *Astragalus membranaceus*, *A. capillaris* and *Coriolus versicolor* extracts had a potent protective effect against oxidative stress induced by the administration of CCl\(_4\) in rats [38].

Enzymes such as ALT and AST are often used as biochemical markers of hepatic injury. In damaged liver cells, the ALT and AST leak into the blood stream leading to an increase in the plasma levels. Thus elevation of plasma AST and ALT activities in the serum could be regarded as an index of damage of the liver parenchyma cells [39,40]. Results from the present study showed that the CCl\(_4\) treatment significantly elevated the levels of ALT and AST by 86 and 69.2% respectively compared with the normal control group. These are in agreement with the findings of Mitra et al. [41] and Opoku et al. [42]. However, administration of low and high doses of AEF during CCl\(_4\) treatment significantly lowered the ALT activity by 42.5 and 48.5% and AST activity by 37.6 and 31.6% respectively as compared with the CCl\(_4\) negative control group, indicating potential hepatoprotective activity of the AEF. It was observed that the efficiency of AEF in lowering ALT activity was inferior to silymarin but was comparable to silymarin in terms of the AST activity. Generally, after healing of hepatic parenchyma and regeneration of hepatocytes, plasma levels of transaminases return to normal [43]. However, neither AEF nor silymarin were sufficient to restore ALT and AST activities to the level of the normal control group in the present study. Our results also showed that CCl\(_4\)
administration reduced the TG level by 24%. It has been suggested that changes in liver and plasma lipid concentrations may result from the reduction in the rate of formation in the liver of the protein moiety of the low-density lipoproteins of the plasma [44]. As for the AST and ALT activities, none of the silymarin and AEF treatments could restore the TG level back to the level of the normal control group. No significant difference in TC level was found between the different treatment groups. This implies that the TC level in rats was not influenced by CCl₄, silymarin or AEF administration.

The activity of the antioxidants and antioxidant enzymes and/or inhibition of free-radical generation are important means of protecting hepatic cells from CCl₄-induced damage [45]. GSH acts as an antioxidant both intracellularly and extracellularly in conjunction with various enzymatic processes that reduced hydrogen peroxide and hydroperoxide by oxidizing GSH to GSSG and other mixed disulfides [46]. GPx metabolizes H₂O₂ and hydroperoxides to non-toxic products and terminates the chain reaction of lipid peroxidation by removing lipid hydroperoxides from the cell membrane and thus stabilizes polyunsaturated membrane lipids [47]. GRd is involved in the detoxification of a range of xenobiotic compounds by their conjugation with GSH [48]. SOD converts the dismutation of superoxide anions into hydrogen peroxide (H₂O₂) [49] and CAT decomposes H₂O₂ to oxygen and water [35]. MDA is the major reactive aldehyde that appears during the peroxidation of biological membrane polyunsaturated fatty acids and is widely used as a marker of lipid peroxidation [50]. In the present study, the GSH content as well as the GPx, GRd, SOD and CAT activities were significantly decreased in the liver in response to CCl₄ treatment compared with the normal control group, suggesting that CCl₄ increased oxidative damage in the liver. However, feeding with low dose of AEF can elevate the GPx and CAT activities, and restore the GSH content to the normal control group level. Feeding with high dose of AEF can elevate GPx and CAT activities, restore the GSH content, and furthermore improve SOD activity compared with the normal control group. The availability of sufficient amount of GSH was found to increase the detoxification of active metabolites through the involvement of GPx [51]. No significant difference was found between the normal control and treatment groups in terms of their GRd activities, as well as their MDA level. This is in opposition to the results of Hung et al. [46] who showed that CCl₄ administration to rats induced significant production of MDA in the liver. Silymarin (a commercial hepatoprotective agent extracted from the thistle plant S. marianum) was used as a positive control in the present study and its administration to mice was found to restore the GSH content and the activities of GPx and CAT enzymes. It also elevated the activities of GRd and SOD to a higher level than those found in the

Fig. 2. Liver tissue sections of rats 8 weeks following CCl₄-induced chronic liver damage (HE stain, 10×, 200 μm). The liver damage was shown with fatty vacuolation (arrow). A: normal control group; B: CCl₄ negative control group (20% CCl₄); C: silymarin positive control group (20% CCl₄ + silymarin); D: low dose AEF group (20% CCl₄ + 0.28 g kg⁻¹ day⁻¹); E: high dose AEF group (20% CCl₄ + 1.4 g kg⁻¹ day⁻¹).
normal control group. Interestingly, the hepatoprotective effect of AEF at the high dose concentration was found to be as effective as the silymarin for GSH, SOD and CAT.

Microscopic examinations in the present study revealed that the severe liver damage induced by CCl₄ can be significantly reduced by the administration of AEF. This is in correlation with the results of the liver functional parameters of the serum and hepatic antioxidant enzyme activities. In the normal control group, liver sections showed normal hepatic cells. The livers of CCl₄-intoxicated rats revealed moderate to severe hepatocellular vacuolation, fibrosis, necrosis and swelling. Compared with the lesions observed in the CCl₄ negative control group, the lesions of the silymarin-treated rats were of a much milder degree. These animals showed slight to moderate diffuse necrosis of hepatocytes and slight to mild hepatocellular vacuolations. The same was observed in AEF-administered animals. In the semi-quantitative assessment, all scores in the CCl₄ control group were significantly higher than those of the normal control group, indicating that CCl₄ induced severe damage to the hepatic cells. All of the tested doses of AEF significantly decreased the scores of bile duct proliferation, liver necrosis and liver fibrosis to the same extent as the silymarin. Although the AEF and silymarin treatments did not lead to reduced steatosis and lobular inflammation on a statistical point of view, they may tend to reduce their extent.

5. Conclusions

The present study provides original evidence that the aqueous extract of *A. capillaris*, *L. japonica* and *S. marianum* used in combination can elicit a therapeutic effect on CCl₄-induced hepatic injury. The AEF possessed higher antioxidant properties compared with the herbs when used

Table 5
Liver bile duct proliferation, steatosis, lobular inflammation, necrosis and fibrosis scores 8 weeks following CCl₄, silymarin and AEF treatments.

<table>
<thead>
<tr>
<th>Group *</th>
<th>Bile duct proliferation</th>
<th>Steatosis</th>
<th>Lobular inflammation</th>
<th>Necrosis</th>
<th>Fibrosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control</td>
<td>0.0±0.0⁹</td>
<td>0.0±0.0⁹</td>
<td>0.0±0.0⁹</td>
<td>0.0±0.0⁹</td>
<td>0.0±0.0⁹</td>
</tr>
<tr>
<td>CCl₄ negative control</td>
<td>2.0±0.7⁸</td>
<td>1.8±0.5⁸</td>
<td>1.5±0.5⁸</td>
<td>1.8±0.5⁸</td>
<td>1.8±0.5⁸</td>
</tr>
<tr>
<td>Silymarin positive control</td>
<td>1.2±0.4⁹</td>
<td>1.5±0.5⁹</td>
<td>0.5±0.5⁹</td>
<td>0.5±0.5⁹</td>
<td>1.5±0.5⁹</td>
</tr>
<tr>
<td>Low dose AEF</td>
<td>0.8±0.3⁸</td>
<td>1.0±0.5⁸</td>
<td>0.7±0.4⁹</td>
<td>0.8±0.3⁸</td>
<td>1.0±0.5⁹</td>
</tr>
<tr>
<td>High dose AEF</td>
<td>0.8±0.6⁸</td>
<td>1.1±0.3⁸</td>
<td>0.7±0.4⁹</td>
<td>0.8±0.6⁸</td>
<td>1.1±0.3⁸</td>
</tr>
</tbody>
</table>

Data are presented as mean±SD (n=8).

Numbers within a column not sharing the same letter are significantly different from each other (P<0.05).

CCl₄ negative control (20% CCl₄); silymarin positive control (20% CCl₄ + silymarin); low dose AEF (20% CCl₄ + 0.28 g kg⁻¹ day⁻¹); high dose AEF (20% CCl₄ + 1.4 g kg⁻¹ day⁻¹).
alone. In animal testing, the administration of CCl4 resulted in significant hepatotoxicity in rats, as evidenced by the elevation of serum AST and ALT activities, increase of oxidative stress in hepatic tissues, and increased histopathological evidence of hepatic injury. However, the hepatotoxic effects induced by CCl4 can be suppressed with silymarin, a product of plant origin with hepatoprotective and antioxidative actions, and with AEF. Both the silymarin and AEF treatments showed analogous hepatoprotective effects in terms of the GSH content, AST and CAT activities, as well as the degree of liver bile duct proliferation, necrosis and fibrosis; silymarin appeared more effective than AEF only in the cases of Gpx and Grd activities, while AEF was superior to silymarin in ALT activity. Although it seemed difficult for the AEF to entirely heal the chronic hepatic damage induced by CCl4 through supplementation of AEF, the latter has promoted the regression of liver injury and hepatic regenerative capabilities.

References