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RESEARCH ARTICLE

# A comparison of the biological activities of ethyl acetate fractions from the stems and leaves of *Penthorum chinense* Pursh

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**Abstract** *Penthorum chinense* Pursh (PCP) is a popular traditional medicinal plant in China, widely used for the treatment of a variety of liver diseases. Although it has been long recognized that the main active elements of PCP are contained in ethyl acetate fraction (EAF), little is known so far in terms of the relative effectiveness of EAF derived from the stems versus leaves of this plant. In the current study, we prepared EAF by reflux extraction and sequential extraction from the stems (SEAF) and leaves (LEAF) of PCP and tested their hepatoprotective efficacies. The extract rates and flavonoid contents of LEAF were higher than those of SEAF. EAFs ($>50$ μg·mL$^{-1}$) prevented lipid accumulation in cells and protected against lipotoxicity injury when the concentration exceeded 25 μg·mL$^{-1}$. More than 95% free radicals released by 2,2-diphenyl-1-picrylhydrazyl (DPPH) were eliminated by 25 μg·mL$^{-1}$ SEAF and 50 μg·mL$^{-1}$ LEAF, respectively. Further, EAFs (25 μg·mL$^{-1}$) also showed protective antioxidant effects, with the activity of LEAF being significantly higher than that of SEAF. EAFs (10 mg·mL$^{-1}$) also showed similar unspecific bacteriostatic activity. In comparison with SEAF, LEAF contained more flavonoids and had a higher anti-oxidation capability and for these reasons we suggest it should be better for clinical use.

**Keywords** antibacterial, anti-oxidation, lipid accumulation, lipotoxicity, *Penthorum chinense* Pursh

## 1 Introduction

Liver is the most vital metabolic organ in vertebrates. Nutritional imbalance or ingestion of toxicity elements causes dysfunction of hepatocytes, which influences energy balance, with the resulting energy surplus being stored as lipid droplets throughout the body, especially in the liver[1]. The hepatic capacity to oxidize, store and export free fatty acids (FFAs) as triglycerides (TGs) is overwhelmed by their flux from the periphery or hepatic *de novo* lipogenesis and the excess FFAs in liver may cause lipotoxicity[2,3]. This is a vicious cycle that, if left untreated, leads to nonalcoholic fatty liver disease (NAFLD), cirrhosis, and even hepatomas[4]. The mechanism which leads to lipid accumulation and nonalcoholic fatty liver disease (NAFLD) is largely unknown. Currently, oxidative stress (OS) is recognized to be one of the most important factors[5,6], as well as intestinal flora released lipopolysaccharides (LPS)[7,8].

To date, no specific drug treatment exists for nonalcoholic fatty liver disease. Herbal tea has become increasingly popular and is associated with enhanced liver health. Ganhuangcao (Saxifragaceae, *Penthorum chinense* Pursh (PCP)) is a major medicinal plant in the Gulin District of Sichuan Province, China. It is also known as 'immortal grass' by Miao people, who have consumed this plant since ancient times[9] and who believe that drinking PCP tea can protect against liver damage[10]. For decades, the curative effect of PCP has been explored on liver disease treatment in clinic, including hepatitis B-induced liver fibrosis[11–13], alcoholic fatty liver caused by long-term drinking[14], nonalcoholic steatohepatitis[15], and cirrhosis[16]. Fundamental research on the active ingredients of PCP and their biological activities is ongoing. Some studies demonstrated that the main active elements are contained in the ethyl acetate fraction (EAF) from PCP[17–19], such as anti-bacteria (bacteriostatic) activity[20,21] and anti-oxidation activity[22]. It is common to separate the leaves from the stems of PCP when making tea[9,23,24]; however, the reasons for this are unclear. Therefore, in the current study, we compared and contrasted the biological activities of EAF in 70% ethanol extracts of PCP stems and leaves.

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## 2 Materials and methods

### 2.1 Materials

The stems and leaves of PCP were provided by Sichuan Traditional Chinese Medicine Decoction Co., Ltd. and appraised by Prof. Chunlei Fan, Zhejiang Chinese Medical University (Hangzhou, China). *Escherichia coli* ATCC35218, *Staphylococcus aureus* ATCC29213, *S. aureus* ATCC43300, and *Enterococcus faecalis* ATCC29212 were purchased from American Type Culture Collection (ATCC) (Manassas, VA, USA) and *Bacillus subtilis* CMCC1.1470 from the China Strain Preservation Center (Beijing, China). The human hepatocellular carcinoma cell line HepG2 (ATCC, Manassas, VA, USA) was provided by Associate Prof. Li Xu, Zhejiang Chinese Medical University.

### 2.2 EAF preparation

The stems and leaves of PCP were powdered with a herb-grinding machine (Wuyi Haina Electric Appliance Co., Ltd., Jinhua, Zhejiang, China) and sifted through an 80-mesh screen. Powdered PCP stems and leaves were extracted twice with 70% ethanol for 1.5 h. The crude extract was concentrated by rotary evaporation and successively extracted with petroleum and ethyl acetate. The ethyl acetate extracts of stems (SEAF) and of leaves (LEAF) were dried with rotary evaporation and weighed.

### 2.3 Total flavonoids determination

SEAF, LEAF, and rutin standards were dissolved in 70% ethanol and mixed successively with 1 mL 5% sodium nitrite solution, 1 mL 10% aluminum nitrate solution, and 10 mL 4% sodium hydroxide solution. Finally, 70% ethanol was added to adjust the volume of each mixture to 25 mL. The absorbance ($A_{510}$) of the mixtures was measured at 510 nm over 15 min.

### 2.4 Cell culture

HepG2 cells were cultured in a 96-well plate containing 200 mL Dulbecco's Modified Eagle Medium (DMEM) with 10% (V/V) fetal bovine serum (containing 100 $U \cdot L^{-1}$ penicillin and 100 $mg \cdot L^{-1}$ streptomycin). The cell culture was grown in a 5% $CO_2$ incubator at 37°C with full humidity. To evaluate their cell protection activities, LEAF and SEAF solutions (12.5, 25, 50, and 100 $\mu g \cdot mL^{-1}$) were added to separate cell cultures. In addition, 0.4 $mmol \cdot L^{-1}$ $H_2O_2$, 0.5 $mmol \cdot L^{-1}$ palmitate (PA), and 0.5 $mmol \cdot L^{-1}$ oleic acid (OA) were added to cell cultures to assess their ability to protect against OS, lipotoxicity, and TG accumulation.

### 2.5 Apoptosis assessment

Morphological changes of apoptosis could be observed under the microscope, using Hoechst 33342 (Thermo Scientific, Middletown, VA, USA) staining. The HepG2 cells were fixed with 4% paraformaldehyde in phosphate buffered saline for 10 min. After staining with 5 $mg \cdot L^{-1}$ Hoechst 33342 for 10 min, the cells were visualized under a fluorescent microscope (Leica, Wetzlar, Germany).

### 2.6 Triglycerides content assessment

BODIPY (493/503) (Thermo Scientific, Middletown, VA, USA), 4,4-difluoro-1,3,5,7,8-pentamethyl-4-bora-3a,4a-diaza-s-indacene, is a kind of lipophilic fluorochrome, which can stain triglyceride droplet in cells. The HepG2 cells were fixed with 4% paraformaldehyde in phosphate buffered saline for 10 min. After staining with 1 $mg \cdot L^{-1}$ BODIPY 493/503 for 30 min, triglyceride droplet in cells were visualized under a fluorescent microscope (Leica, Wetzlar, Germany).

### 2.7 Cell viability evaluation

In this study, the cell viability was evaluated by measuring the amount of lactate dehydrogenase (LDH) released in the culture medium, using a LDH cytotoxicity assay kit (Thermo Scientific, Middletown, VA, USA) according to the manufacturer's instructions. The treated cell culture medium was transferred to a new plate and mixed with the reaction mixture from the kit. After 30 min, the stop solution was added to stop the chromogenic reaction. LDH activity was then determined spectrophotometrically at 490 nm.

### 2.8 Triglyceride determination

OA incubation induces TG accumulation in cells *in vitro*. In the current study, the concentration of TG in cells was determined by enzymatic colorimetry. The cultivated cells were lysed by lytic buffer at room temperature for 10 min followed by heating for 10 min at 70°C. The cell debris and denatured proteins in the pyrolytic solution were discharged with centrifugation at 2000 × *g* for 5 min at room temperature. TGs in this cell lytic solution were detected using a kit (Applygen Technologies Inc., Beijing, China) according to the manufacturer's instructions.

### 2.9 Free radical scavenging activity quantitation

DPPH (Sigma-Aldrich, Shanghai, China) was used as the free radical donor in this study. Each reaction mixture contained 50 μL of the sample solution at various concentrations (0, 3.125, 6.25, 12.5, 25, 50, and 100 $\mu g \cdot mL^{-1}$) and 1 mL of 1 $mmol \cdot L^{-1}$ DPPH solution in

ethanol. Each mixture was incubated for 30 min at 37°C and the absorbance was measured at 517 nm. The percentage radical scavenging activity of each mixture was calculated in comparison with the NC, using the following equation:

$$\text{Scavenging activity}\,(\%) = [(A_{\text{control}} - A_{\text{sample}})/A_{\text{control}}] \times 100$$

The $EC_{50}$ value of the compound was obtained with GraphPad prism.

### 2.10 Antibacterial activity identification

Antimicrobial activity was detected by using the Kirby Bauer agar diffusion method. Sterile 3MM Whatman filter paper discs (diameter 6 mm) were saturated with either 10 mg·mL$^{-1}$ SEAF or LEAF 70% ethanol solution. 70% ethanol was used as a negative control. An air-dried filter paper disc was placed on each LB agar plate containing different test strains of bacteria ($10^7$–$10^8$ cfu·mL$^{-1}$). Each test solution contained at least four repeats. The agar plates were placed in a 37°C incubator and the diameter of each inhibitory zone was measured after 20 h.

### 2.11 Statistical analysis

All experiments were repeated at least three times. The data from each group were expressed as mean±standard deviation. Differences between the groups were determined using the Student's *t*-test, analyzed with SPSS 22.0 software. Differences were significant at the $P < 0.05$ level.

## 3 Results

### 3.1 Preparation of EAF and flavonoid concentration

The extraction rates (g/g) of SEAF and LEAF were 3.55%±0.24% and 8.73%±2.31%, respectively. Flavonoid content was calculated according to the standard curve of rutin. The total flavonoid concentration (g/g) in SEAF and LEAF was 16.05%±1.60% and 39.27%±6.54%, respectively.

### 3.2 Demonstration of triglyceride accumulation and lipotoxicity attenuation inhibitory effects

OA, a monounsaturated fatty acid, promoted the formation of triglyceride-enriched lipid droplets in HepG2 cells which could be stained by fluorochrome BODIPY and observed under the microscope (Fig. 1(a)). The total TG accumulation in cells from the treatment groups was calculated and compared with that of the control group (C, untreated), as shown in Fig. 1(b). In this experiment,

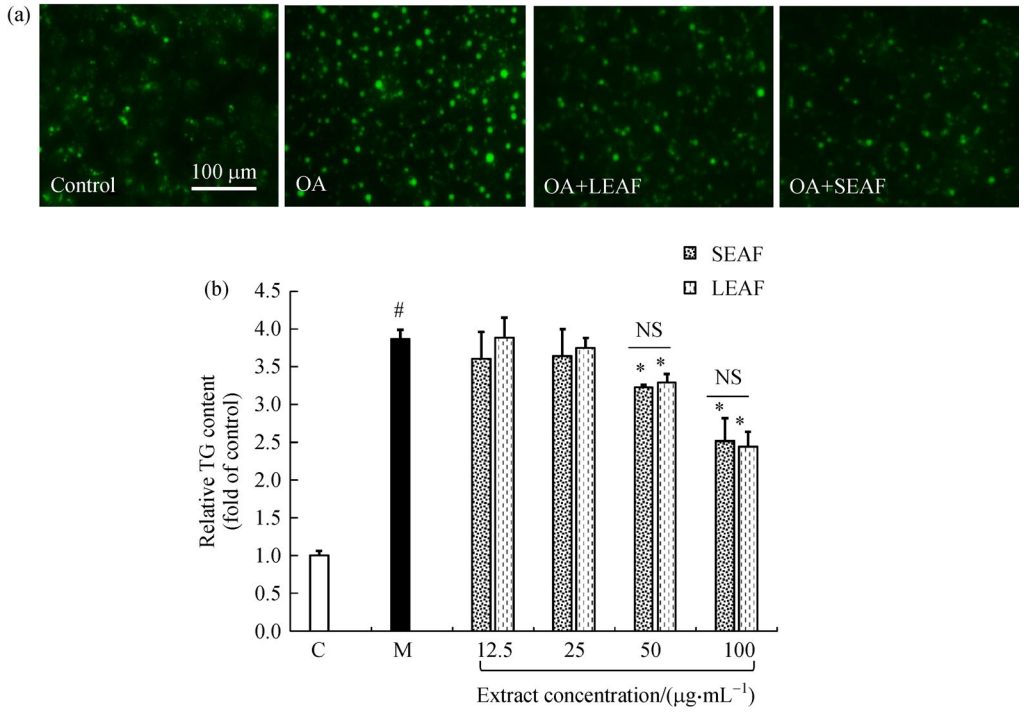


**Fig. 1** Cell protective efficiency of SEAF and LEAF against OA induced lipid accumulation. (a) The images of BODIPY stained HepG2 cells; (b) the relative TG content. Control (C) and model (M) groups are untreated and 0.5 mmol·L$^{-1}$ OA treated HepG2 cell groups, respectively. The others are sample groups that 12.5, 25, 50, 100 μg·mL$^{-1}$ SEAF and LEAF protected HepG2 cell groups, against 0.5 mmol·L$^{-1}$ OA treatment. # indicates significant difference between the C and M groups; * indicates significant difference between the sample and M groups; NS indicates nonsignificant difference between the same concentrations of SEAF and LEAF ($P < 0.05$).

the accumulated TG in the model group (M, 0.5 mmol·L$^{-1}$ OA treated) increased to 3.87±0.12. The inhibitory effects of LEAF and SEAF (> 50 μg·mL$^{-1}$) were similar. In detail, the concentration of TG in the 12.5, 25, 50, and 100 μg·mL$^{-1}$ LEAF groups was 3.61±0.35, 3.64±0.36, 3.22±0.04, and 2.52±0.30, respectively, whereas that in the 12.5, 25, 50, and 100 μg·mL$^{-1}$ SEAF groups was 3.89±0.26, 3.75±0.13, 3.29±0.114, and 2.44±0.19, respectively.

The excess free fatty acids, such as PA, have a toxicity effect and reduce cell viability. In the model group (M), which was treated by 0.5 mmol·L$^{-1}$ PA, the relative released LDH increased by 7.97±0.19 folds. The results showed pretreatment with SEAF and LEAF attenuated the lipotoxicity of PA in a dose dependent manner (Fig. 2; Table 1).

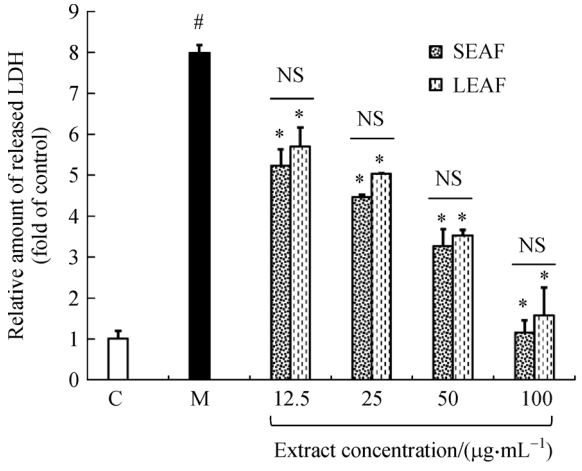


**Fig. 2** Lipotoxicity attenuation efficiency of SEAF and LEAF against PA induced cell injury, measured as the relative amounts of released LDH. Control (C) and model (M) groups are untreated and 0.5 mmol·L$^{-1}$ PA treated HepG2 cell groups, respectively. The others are sample groups that 12.5, 25, 50, 100 μg·mL$^{-1}$ SEAF and LEAF protected HepG2 cell groups, against 0.5 mmol·L$^{-1}$ PA treatment. # indicates significant difference between the C group and M group; * indicates significant difference between the sample group and M group; NS indicates nonsignificant difference between the same concentrations of SEAF and LEAF ($P < 0.05$).

Lipid accumulation is a regular feature of dysfunctional hepatic cell. This result explored the protective activities of LEAF and SEAF against lipid accumulation induced by OA and lipotoxicity induced by PA. There were no significant differences in the activities between SEAF and LEAF.

### 3.3 Comparative evaluation of antioxidation efficiency

OS, which leads to lipid accumulation and NAFLD development, occurs as a result of excessive reactive oxygen species. The anti-oxidation efficiency of LEAF and SEAF was evaluated with DPPH free radicals scavenging (Fig. 3) and $H_2O_2$ treated HepG2 cell assay (Fig. 4), respectively. Scavenging of DPPH free radical is the basis of a common antioxidant assay. DPPH free radicals incubated with various dosages of LEAF or SEAF for 30 min at 37°C were eliminated in a dose-dependent manner (Fig. 3). The half maximum scavenging activity ($EC_{50}$) of EAF from stems and leaves was 7.30 and 12.72 μg·mL$^{-1}$, respectively.

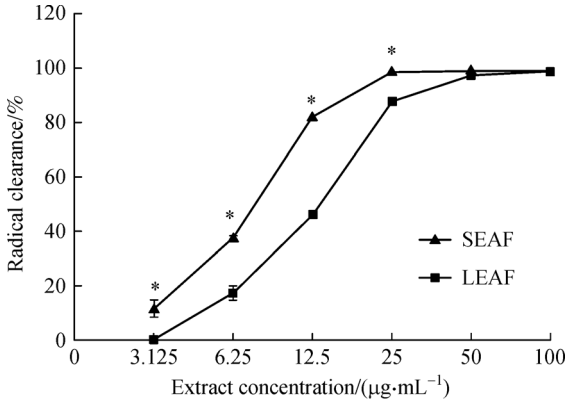


**Fig. 3** DPPH free radical clearance activities of SEAF and LEAF. * indicates significant difference between SEAF and LEAF ($P < 0.05$).

Based on the results of the free radical scavenging assessment, the protective effects of SEAF and LEAF against $H_2O_2$ treatment on HepG2 cells were evaluated by comparing the concentration of LDH in the different culture media. In a biological context, $H_2O_2$ can be partially reduced to hydroxyl radical which induces apoptosis. The concentration of LDH released by cells is proportional to the number of apoptotic cells. The apoptotic cells could be stained by Hoechst and observed under the microscope (Fig. 4(a)). The relative released LDH was calculated from the $A_{490}$ and compared with that of the control group (C). After $H_2O_2$ treatment, the released

**Table 1** Relative amount of released LDH in PA treated HepG2 assays protected by different concentrations of EAFs (μg·mL$^{-1}$)

| Sample | Relative amount of released LDH (fold of control) | | | | |
|---|---|---|---|---|---|
| | 0 | 12.5 | 25 | 50 | 100 |
| SEAF | 7.97±0.19 | 5.22±0.39 | 4.46±0.04 | 3.26±0.41 | 1.15±0.30 |
| LEAF | 7.97±0.19 | 5.69±0.47 | 5.02±0.02 | 3.51±0.15 | 1.56±0.68 |

LDH in the model group (M) increased significantly. In comparison with the M, more than 25 μg·mL$^{-1}$ SEAF and LEAF showed remarkable protective activities (Fig. 4(b); Table 2). At the same concentration, LEAF activity was significantly higher activity than that of SEAF, suggesting that the anti-oxidation properties of LEAF and SEAF were not identical.

### 3.4 Identification of bacteriostatic activity

The released of LPS by intestinal flora is one of major factors which lead to dysfunction of hepatic cells. Therefore inhibiting overgrowth of intestinal microorganisms could influence the LPS induced cell injury. In this work, the bacteriostatic activities of SEAF and LEAF were expressed as inhibition zone diameter (IZD) at a concentration of 10 mg·mL$^{-1}$ (Table 3). The results indicated that both LEAF and SEAF of PCP have anti-bacteria activities. Based on the IZD, the bacterial susceptibility, from strong to weak, was *E. coli* > *B. subtilis* > *S. aureus* ATCC43300 > *S. aureus* ATCC29213 > *E. faecalis* for SEAF, and *S. aureus* ATCC43300 > *B. subtilis* > *E. coli* > *S. aureus* ATCC29213 > *E. faecalis* for LEAF.

## 4 Discussion

EAF have been reported to have the strongest activity of the fractionated extracts of PCP[18]. In this study we prepared the EAF from leaves and stems of PCP and the flavonoid concentration in LEAF and SEAF were

**Table 2** Relative amount of released LDH in $H_2O_2$ treated HepG2 assays protected by different concentrations of EAFs (μg·mL$^{-1}$)

| Sample | Relative amount of released LDH (fold of control) | | | | |
|---|---|---|---|---|---|
| | 0 | 12.5 | 25 | 50 | 100 |
| SEAF | 4.20 ± 0.08 | 4.12 ± 0.23 | 3.70 ± 0.06 | 2.68 ± 0.14 | 2.07 ± 0.04 |
| LEAF | 4.20 ± 0.08 | 4.20 ± 0.09 | 2.90 ± 0.03 | 1.90± 0.14 | 1.50 ± 0.15 |

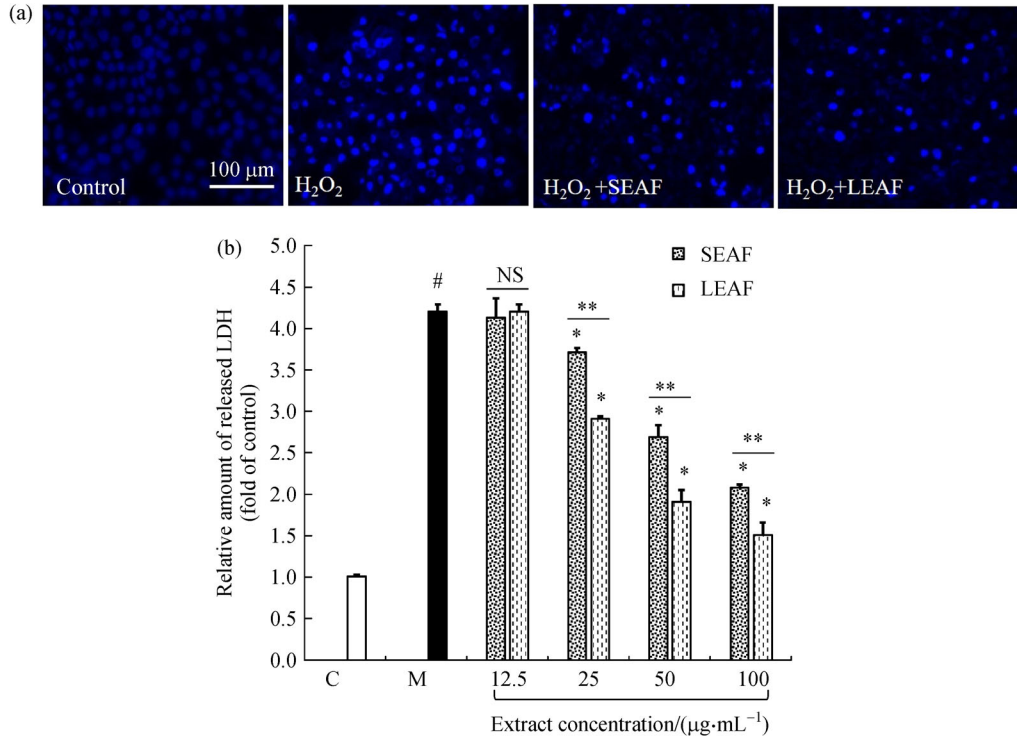


**Fig. 4** Cell protective efficiency of SEAF and LEAF against $H_2O_2$ oxidative damage. (a) The images of Hoechst stained HepG2 cells; (b) the relative amounts of released LDH. Control (C) and model (M) groups are untreated and 0.4 mmol·L$^{-1}$ $H_2O_2$ treated HepG2 cells, respectively. The others are sample groups that 12.5, 25, 50, 100 μg·mL$^{-1}$ SEAF and LEAF protected HepG2 cell groups, against 0.4 mmol·L$^{-1}$ $H_2O_2$ treatment. # indicates significant differences between the M and the C; * indicates significant differences between the EAF sample groups and M group; NS and ** indicate nonsignificant or significant differences, respectively, between the same concentrations of SEAF and LEAF ($P < 0.05$).

**Table 3** Inhibitory zone diameter (mean ± SD) of SEAF and LEAF against different bacterial strains

| Sample | Inhibitory zone diameter/mm | | | | |
|---|---|---|---|---|---|
| | *Escherichia coli* ATCC35218 | *Bacillus subtilis* CMCC1.1470 | *Enterococcus faecalis* ATCC29212 | *Staphylococcus aureus* ATCC29213 | *Staphylococcus aureus* ATCC43300 |
| SEAF | 13.50 ± 0.71 | 10.83 ± 0.58 | 9.33 ± 0.58 | 10.33 ± 0.58 | 10.75 ± 0.35 |
| LEAF | 10.50 ± 0.41 | 10.83 ± 0.68 | 9.32 ± 0.68 | 9.53 ± 0.58 | 11.25 ± 0.35 |

quantitated. The extraction ratio of EAF and the flavonoid content of leaves were more than twice that of those from the stems. Sun et al.[25] studied the total flavonoid content in the leaves of PCP at different growth stages. They found that the flavonoid content in the leaves was 4.3%–4.5% before flowering, which was higher than at other growth stages. Obviously, leaves gathered before flowering are a better source of PCP flavonoid preparations than stems.

To date, there have been few studies on the cell protection activities of PCP extracts against lipid accumulation and lipotoxicity. It was reported that Gansu granules (made from an extract of PCP) improved lipid metabolism, lowered blood sugar and reduced bodyweight[17]. In 2014, Xiao et al.[26] reported that PCP extract can reduce the concentration of TG in the serum and liver of a rat model of non-alcoholic fatty liver disease and alleviated fatty lesions in liver tissue. Those studies were carried out in clinic or with an animal model, *in vivo*. Our results validated these previous studies with cell assay, *in vitro*. ROS, a key factor, contributes to lipid metabolic disturbance in hepatocyte, and in a lipid-rich environment causes lipid accumulation[5,6]. So, we evaluated the antioxidant activities of both LEAF and SEAF with two kinds of assays. By DPPH assay, the free radical scavenging activity of LEAF ($EC_{50}$ = 12.72 $\mu g \cdot mL^{-1}$) was lower than that of SEAF ($EC_{50}$ = 7.3 $\mu g \cdot mL^{-1}$). By contrast, the $H_2O_2$ oxidant protection activity of LEAF (at concentrations > 25 $\mu g \cdot mL^{-1}$) was significantly higher than that of SEAF ($P < 0.05$). The differences in activity between LEAF and SEAF could result from variations in their chemical composition, as reported previously[27]. Tuo et al.[28] reported three more characteristic peaks in leaves than in stems, based on high performance liquid chromatography. Unfortunately, no studies have been conducted to identify these compounds. Whereas DPPH comprises stable free radical molecules, hydrogen peroxide ($H_2O_2$) is one of ROS which is generated as a short-lived product in biochemical processes, such as β-oxidation of long chain fatty acids[29]. And ROS appears to be a feature of liver diseases[30,31]. Therefore, leaves, not stems, of PCP are an ideal material for use as a liver diseases treatment.

Flavonoids are common active components in herbs, which show a variety of biological activities, including not only antioxidation but also bacteriostatic effects[32]. Previous studies reported that quercetin and pinocembrin-7-*O*-β-glucoside[33–35] are abundant in PCP. Both compounds showed antibacterial activity[36,37]. Previous studies also reported that the EAF of PCP has similar bacteriostatic activity against *Pseudomonas aeruginosa* ($G^-$) and *S. aureus* ($G^+$)[20,21]. The bacteriostatic activity of the two fractions were identified in the agar diffusion experiments with *E. coli*, *B. subtilis*, *E. faecalis* and two *S. aureus* strains, separately. There was no significant difference in the IZD obtained with the tested strains using SEAF and LEAF, apart from a higher IZD of SEAF on the *E. coli* plate. Although the flavonoid content of LEAF was more than twice that of SEAF, there was no significant difference in antibacterial activity between SEAF and LEAF. Thus, we hypothesize that SEAF and LEAF have nonspecific antibacterial activity and that the content of active compounds in leaves is not significantly different from that in stems. Our results suggest that this nonspecific antibacterial activity of EAF could reduce internal flora and prevent LPS induced liver damage by an indirect effect. This could be tested with current cell or animal models.

## 5 Conclusions

This study suggested that both LEAF and SEAF can prevent lipid accumulation in hepatic cells by their antioxidation activity. In addition, PCP may also influence the LPS induced liver injury by its bacteriostatic activities. Leaves of PCP were found to produce an EAF with a higher flavonoid content and present significant higher capability against hydroxyl radicals, than stems. Thus, we suggest that leaves extracts, rather than those from the stems, should be explored further for clinical use.

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**Compliance with ethics guidelines** Zhaolei Wang, Kai Jiang, Qinchao Ding, Shujun Liu, Xiaobing Dou, and Bin Ding declare that they have no conflicts of interest or financial conflicts to disclose.

This article does not contain any studies with human or animal subjects performed by any of the authors.

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