Available online at www.sciencedirect.com
ScienceDirect
Chinese Journal of Natural Medicines 2019, **17**(1): 0033–0042
Chinese Journal of Natural Medicines

•Special topic•

Secondary metabolites of petri-dish cultured *Antrodia camphorata* and their hepatoprotective activities against alcohol-induced liver injury in mice

WU Yu [1Δ], TIAN Wen-Jing [1Δ], GAO Shuo [1Δ], LIAO Zu-Jian [1], WANG Guang-Hui [1], LO Jir-Mehng [2], LIN Pei-Hsin [2], ZENG De-Quan [1], QIU Da-Ren [1], LIU Xiang-Zhong [1], ZHOU Mi [1], LIN Ting [1*], CHEN Hai-Feng [1*]

[1] *Fujian Provincial Key Laboratory of Innovative Drug Target, School of Pharmaceutical Sciences, Xiamen University, Xiamen 361005, China;*
[2] *Industrial Technology Research Institute, Taiwan 10001, China*

Available online 20 Jan., 2019

[ABSTRACT] *Antrodia camphorata*, a well-known and highly valued edible medicinal mushroom with intriguing activities like liver protection, has been traditionally used for the treatment of alcoholic liver disease. *A. camphorata* shows highly medicinal and commercial values with the demand far exceeds the available supply. Thus, the petri-dish cultured *A. camphorata* (PDCA) is expected to develope as a substitute. In this paper, nineteen triterpenes were isolated from PDCA, and thirteen of them were the unique anthroic acids in *A. camphorata*, including the main content antcin K, which suggested that PDCA produced a large array of the same anthroic acids as the wild one. Furthermore, no obvious acute toxicity was found suggesting the edible safety of PDCA. In mice alcohol-induced liver injury model, triglyceride (TG), aspartate aminotransferase (AST), alanine aminotransferase (ALT), and malondialdehyde (MDA) had been reduced by the PDCA powder as well as the main content antcin K, which indicated that the PDCA could protect alcoholic liver injury in mice model and antcin K could be the effective component responsible for the hepatoprotective activities of PDCA against alcoholic liver diseases.

[KEY WORDS] Petri-dish; *Antrodia camphorata*; Hepatoprotective

[CLC Number] R965 **[Document code]** A **[Article ID]** 2095-6975(2019)01-0033-10

Introduction

Antrodia camphorata, a well-known and highly valued edible medicinal mushroom, known as "Niu-chang-chih", "Chang-chih" or "Niu-chang-ku", is considered as a present from heaven in Taiwan. It is commonly used as Chinese folk medicine to preserve human vitality and promote longevity, which is claimed as "ruby in mushroom" locally. *A. camphorata* possesses a variety of functional metabolites including triterpenoids, polysaccharides, antroquinonol and adenosines [1], which exhibited a broad spectrum of prominent activities, like hepatoprotective, hangover, anti-cancer, anti-inflammatory, anti- allergy, anti-fatigue and immunomodulatory [1-5]. *A. camphorata* grows naturally inside the Taiwan endemic *Cinnamomum kanehirae* tree which has been protected by the government since it becomes rarer and rarer. Consequently, the demand for *A. camphorata* far exceeds the available supply due to the slowly growth of its wild fruiting body as well as its host specificity and rarity in nature, which led to the rather expensive price [6]. Currently, more and more attention has been paid to the artificial cultivation，which is expected to develop as a substitute. The petri dish cultured *A. camphorata* (PDCA) possesses the advantages of simple culture method, moderate culture cycle and without dependence on the rare host *C. kanehirae*, which shows powerful market value and has been exploit to meet the market requirement.

[Received on] 30-Oct.-2018
[Research funding] This work was supported by Fundamental Research Funds for the Central Universities (Nos. 20720160117 and 20720150204) and Xiamen Science and Technology program grant (Nos. 3502Z20161235 and 3502Z20173021).
[*Corresponding author] Tel: 86-592-2881180, E-mail: linting@xmu.edu.cn (LIN Ting); Tel: 86-592-2187225, E-mail: haifeng@xmu.edu.cn (CHEN Hai-Feng)
[Δ]These authors contributed equally to this work.
These authors have no conflict of interest to declare.

Alcoholic liver disease, one of the most common causes of chronic liver disease in the world, is mainly caused by the excessive intake of alcohol, with a broad spectrum of morphological features such as steatosis, steatohepatitis, fibrosis, and cirrhosis. Moreover, approximately 15% of patients with established alcoholic cirrhosis developed to liver cancer. Currently, developing a potent functional food for the prevention of alcoholic liver disease and investigating it's effective constituents are of great significance. *A. camphorata* was originally used by the aboriginal tribes in Taiwan for the treatment of discomforts caused by alcohol drinking. Furthermore, several reports have shown that the fruiting bodies of *A. camphorata* exhibited the activities of preventing and ameliorating liver diseases, including hepatoprotective activities by preventing ethanol or CCl_4 induced liver injury. However, the material basis of the hepatoprotective activities of PDCA towards alcoholic liver disease were still unclear. Thus, in this paper, the isolation and structure elucidation of the chemical constituents of the PDCA were carried out. The hepatoprotective activities towards ethanol induced liver injury of the PDCA and its main content antcin K were investigated. Besides, the acute toxicity of the PDCA was evaluated preliminary in order to demonstrate its edible safety.

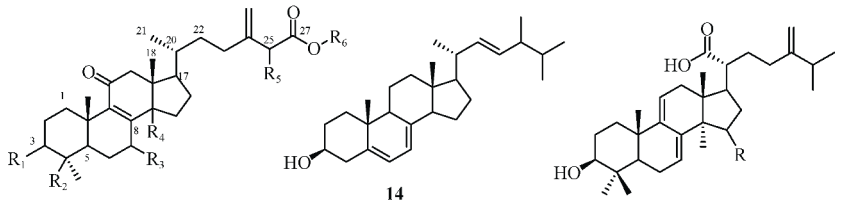
Results and Discussion

Study on the secondary metabolites of PDCA

Nineteen compounds, (25S)-Antcin K (**1**), (25R)-Antcin K (**2**), (25S)-Antcin C (**3**), (25R)-Antcin C (**4**), Antcin B (**5**), Antcin G (**6**), Antcamphins E (**7**), (25S)-Camphoratins C (**8**), Camphoratins D (**9**), Antcamphins F (**10**), (25S)-Methyl antcinate K(**11**), (25R)-Camphoratins C (**12**), (25R)-Methyl antcinate K (**13**), Ergosterol (**14**), Dehydroeburicoic acid (**15**), Eburicoic acid (**16**), Dehydrosulphurenic acid (**17**), Sulphurenic acid (**18**), and Versisponic acid D (**19**) were isolated from the PDCA (Fig. 1). Their structures were elucidated by means of spectroscopic analyses (ESI-MS, ^{1}H and ^{13}C NMR spectra) and comparing to the literature. The ESI-MS and NMR data can be seen in the supplemental material. Compounds **1**−**13** are the characteristic anthroic acids in *A. camphorate*. Among them, (25S)-antcin K (**1**) and (25R)-antcin K (**2**), a pair of epimers, were the main content of the PDCA (Fig. 2). Thus, the mixture of (25S)-antcin K and (25R)-antcin K, named antcin K, were used for the following mice experiment to investigate whether they were the effective components responsible for the hepatoprotective activities of the PDCA against alcoholic liver diseases.

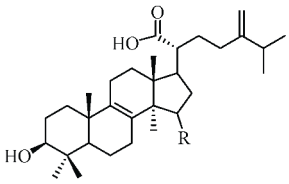
Study on the acute toxicity of PDCA

The effect of the PDCA on body weight of mice has been investigated. As showed in Figs. 3 and 4, the body weight of the mice was raised after the administration of PDCA. Compared with female group, the male group grew faster. In the same gender, the weight growth trends were similar between the experimental and control group. There was no statistically significant difference ($P > 0.05$) between these two groups. The above analysis suggested that PDCA had no effect on the body weight of mice.



14

15 R = H
16 R = OH



17 R = H
18 R = OH
19 R = $OCOCH_3$

1 R_1 = α-OH, R_2 = β-OH, R_3 = β-OH, R_4 = H, R_5 = α-CH_3, R_6 = H
2 R_1 = α-OH, R_2 = β-OH, R_3 = β-OH, R_4 = H, R_5 = β-CH_3, R_6 = H
3 R_1 == O, R_2 = H, R_3 = β-OH, R_4 = H, R_5 = α-CH_3,R_6 = H
4 R_1 == O, R_2 = H, R_3 = β-OH, R_4 = H, R_5 = β-CH_3, R_6 = H
5 R_1 == O, R_2 = H, R_3 = O, R_4 = H, R_5 = α-CH_3, R_6 = H
6 R_1 == O, R_2 = H, R_3 = OCOCH3, R_4 = H, R_5 = CH_3, R_6 = H
7 R_1 == O, R_2 = β-OH, R_3 = β-OH, R_4 = H, R_5 = α-CH_3, R_6 = H
8 R_1 == O, R_2 = β-OH, R_3 = O, R_4 = H, R_5 = α-CH_3, R_6 = H
9 R_1== O, R_2 = H, R_3 = β-OH, R_4 = α-OH, R_5 = CH_3, R_6 = H
10 R_1 == O, R_2 = β-OH, R_3 = β-OH, R_4 = H, R_5 =β-CH_3, R_6 = H
11 R_1 = α-OH, R_2 = β-OH, R_3 = β-OH, R_4 = H, R_5 = α-CH_3, R_6 = CH_3
12 R_1 == O, R_2 = β-OH, R_3 = O, R_4 = H, R_5 = β-CH_3, R_6 = H
13 R_1 = α-OH, R_2 = β-OH, R_3 = β-OH, R_4 = α-OH, R_5 = α-CH_3, R_6 = CH_3

Fig. 1 Index compounds of PDCA: (25S)-antcin K (1), (25R)-antcin K (2), (25S)-antcin C (3), (25R)-antcin C (4), antcin B (5), antcin G (6), antcamphins E (7), (25S)-camphoratins C (8), camphoratins D (9), antcamphins F (10), (25S)-methyl antcinate K (11), (25R)-camphoratins C (12), (25R)-methyl antcinate K (13), ergosterol (14), dehydroeburicoic acid (15), eburicoic acid (16), dehydrosulphurenic acid (17), sulphurenic acid (18), versisponic acid D (19)

Effects of the PDCA on food and water intake were studied. As a result, the male group had much more food intake amount than the female group, while the experimental and control groups had the similar food intake amount in male mice (Fig. 4). Besides, the experimental group had more food intake than the control group in female mice with no statistically significant difference ($P > 0.05$). As shown in Fig. 5, experimental groups had more water intake amount than the control groups ($P > 0.05$). The results indicated that PDCA had no influence on the food and water intake of mice.

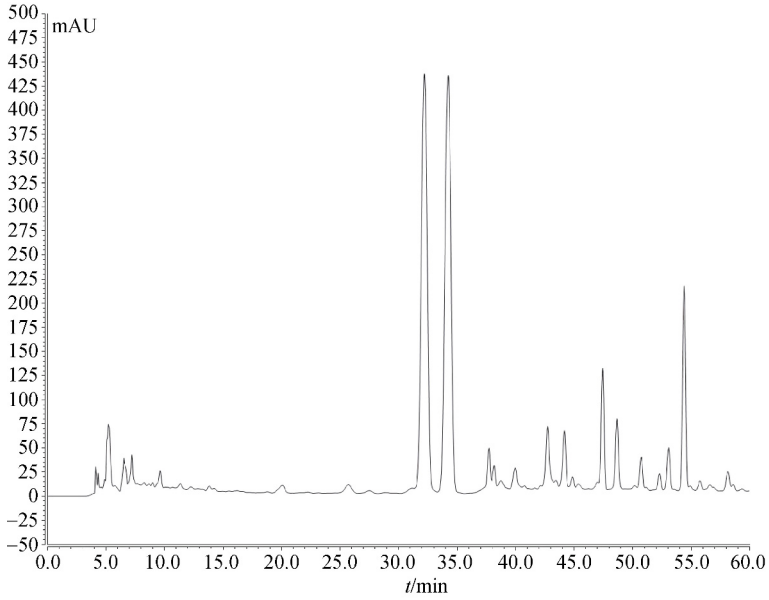


Fig. 2 The HPLC spectrum of the PDCA

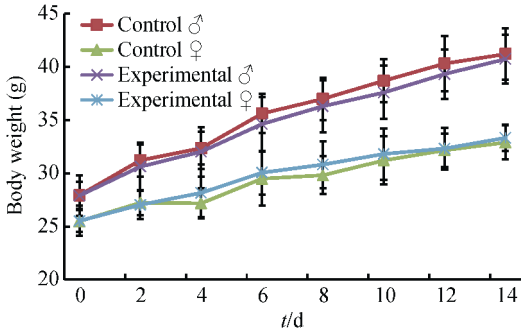


Fig. 3 Effects of the PDCA on body weight of mice in different groups

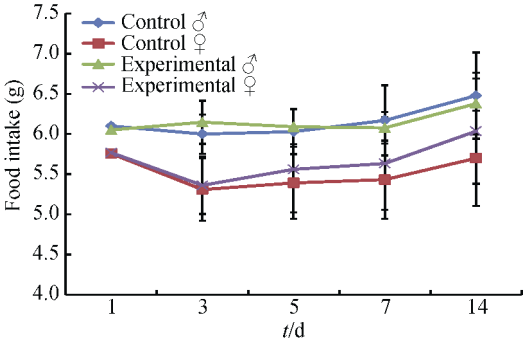


Fig. 4 Effects of the PDCA on food intake of mice

After treated PDCA, all the experimental mice exhibited bright and clean hair color with normal diet, activity and excretion. No abnormal secretions have been observed for their eyes, nose and mouth. Besides, no unfavorable condition or death was observed. The color and shape of the heart, liver, spleen, lung and kidney were not abnormal. The livers of mice were sectioned and stained with HE and then observed under optical microscope (Fig. 6). The results showed that the liver tissue and liver cells of the control group and the experimental group exhibited no significant difference. Clear and integrity liver tissue structure with no degeneration and necrosis could be observed and the liver cells were in neat orders with the normal size.

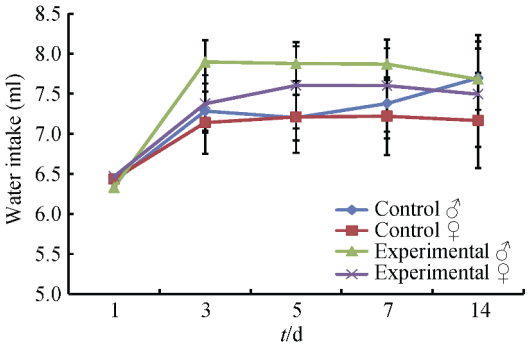


Fig. 5 Effects of the PDCA on water intake of mice

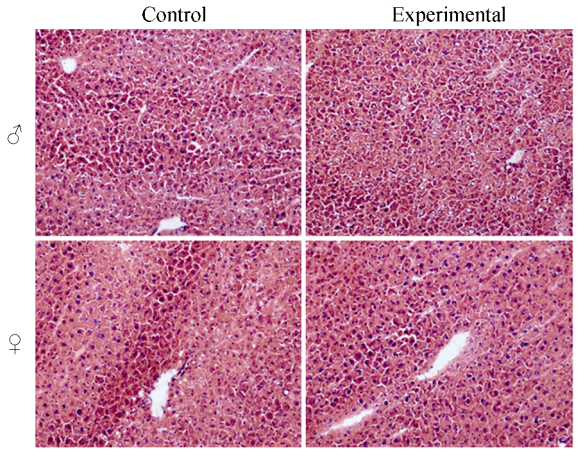


Fig. 6 HE staining of liver sections

The acute toxicity experiment of PDCA demonstrated that the indicators of the mice showed no abnormality in the continuous observation for fourteen days after single administration of the PDCA. The dose used in this experiment (4.8 g·kg^{-1} in mice) was equivalent to 20 times of normal dose in mice converted from adult's (conversion factor of 9.1). The above analysis suggested that PDCA did not exhibit obvious acute toxicity in Kunming mice.

Protective effect of the PDCA on the alcohol induced liver injury

The effects of different doses of the PDCA on body weight of mice were showed in Fig. 7 and Table 1, which suggested that different doses of the PDCA had no effect on the body weight of mice. The weight increment of the model control group was less than other groups, but there was no statistically significant difference.

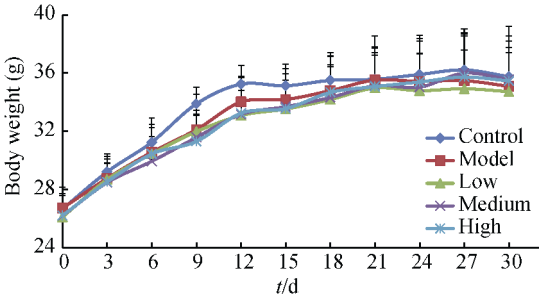


Fig. 7 Effects of different doses of the PDCA on body weight of mice

The liver function indexes TG, AST, MDA, VLDL, ALT and ALDH of the all the groups were detected. (Table 2) Significant difference ($P < 0.05$) could be observed between control and model control groups. Moreover, compared with the model control group, the level of TG, MDA and VLDL as well as the activity of AST significantly does dependent decreased in medium-dose and high-dose groups ($P < 0.05$) (Figs 8−11). Comparing with the model control group, the activity of ALT and the formation of ALDH in low, medium and high dose groups sharply reduced in a dose dependent manner ($P < 0.05$). (Figs. 12 and13).

Table 1 Weight gain of mice after treatment of different dose of PDCA($\bar{x} \pm s$, $n = 12$)

Groups	Initial weight (g)	Final weight (g)	Weight increment (g)
Control	26.67 ± 1.49	35.75 ± 2.44	9.08 ± 1.22
Model control	26.73 ± 0.99	35.09 ± 2.31	8.37 ± 1.41
Low dose	26.11 ± 1.61	34.73 ± 4.46	8.61 ± 3.09
Medium dose	26.21 ± 1.53	35.55 ± 2.19	9.35 ± 1.31
High dose	26.22 ± 1.37	35.46 ± 3.10	9.24 ± 1.83

Under normal circumstances, none or very few lipid droplets could be seen in cells except fat cells. Lipid droplets significantly increased in pathological conditions which could be specifically stained by oil red to salmon pink. The results showed that no obvious lipid droplets formed in the control group. A large numbers of lipid droplets could be observed in the model control group, which demonstrated that the liver cells were fatty degenerated after administration of 50% ethanol. As the dose increment of the PDCA, the lipid droplets formed in liver cells was decreased. Compared with control group, the sizes and densities of lipid droplets formed in low and middle dose groups were smaller, and no obvious lipid droplet formed in the high dose group. The above results indicated that PDCA could inhibit fatty degeneration in liver cells in a dose dependent manner (Fig. 14).

Table 2 Effects of the PDCA on biochemical indicators related liver function ($\bar{x} \pm s$, $n = 12$)

Groups	TG (mmol·L^{-1})	AST	ALT	MDA (nmol·mL^{-1})	VLDL (mmol·L^{-1})	ALDH (ng·mL^{-1})
Control	1.49 ± 0.21	136.10 ± 22.1	69.07 ± 12.93	12.82 ± 0.93	0.87 ± 0.15	8.91 ± 2.00
Model control	2.53 ± 0.73***	214.65 ± 42.03***	115.09 ± 20.41***	20.20 ± 5.90***	1.36 ± 0.30***	14.07 ± 1.95***
Low dose	2.24 ± 0.34	190.73 ± 45.23	101.65 ± 17.91$^{\#}$	16.91 ± 4.16	1.23 ± 0.28	12.28 ± 1.91$^{\#}$
Medium dose	1.97 ± 0.66$^{\#}$	172.10 ± 49.47$^{\#}$	92.68 ± 19.28$^{\#\#}$	14.92 ± 1.66$^{\#\#}$	1.12 ± 0.18$^{\#}$	10.53 ± 1.99$^{\#\#\#}$
High dose	1.76 ± 0.25$^{\#\#}$	153.23 ± 52.44$^{\#\#}$	82.20 ± 12.57$^{\#\#\#}$	13.58 ± 1.03$^{\#\#\#}$	0.98 ± 0.19$^{\#\#\#}$	9.22 ± 1.75$^{\#\#\#}$

Annotation: $^{***}P < 0.001$ *vs* control, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ *vs* model control

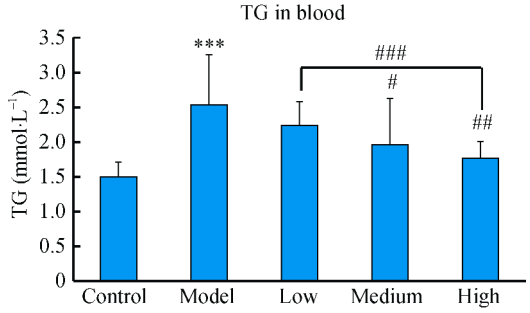


Fig. 8 Content detection of triglyceride in serum. $^{***}P < 0.001$ *vs* control, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ *vs* model control, $n = 12$

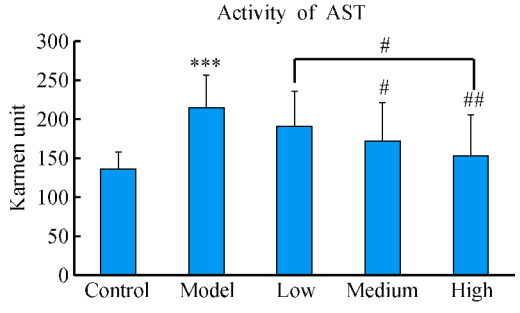


Fig. 9 The activity of Glutamic-oxaloacetic transaminase in serum. $^{***}P < 0.001$ *vs* control, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *vs* model control, $n = 12$

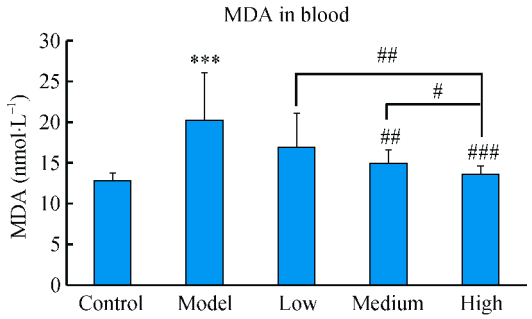


Fig. 10 Content detection of malonaldehyde in serum. $^{***}P < 0.001$ *vs* control, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *vs* model control, $n = 12$

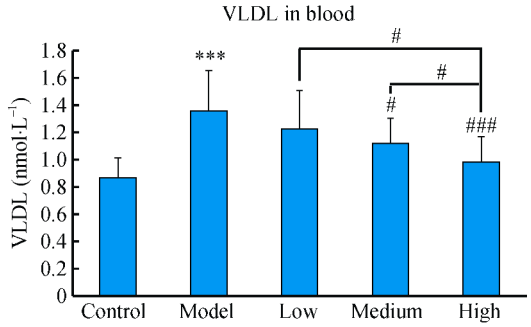


Fig. 11 Content detection of very low density lipoprotein in serum. $^{***}P < 0.001$ *vs* control, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *vs* model control, $n = 12$

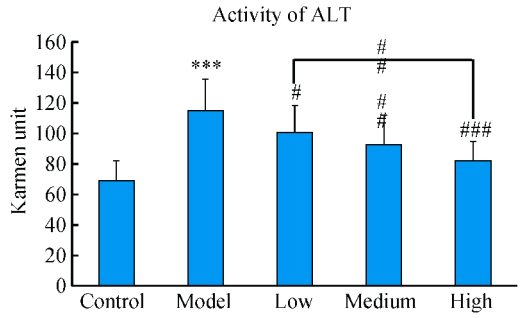


Fig. 12 Glutamic-pyruvic transaminase activity determination in serum. $^{***}P < 0.001$ *vs* control, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ *vs* model control, $n = 12$

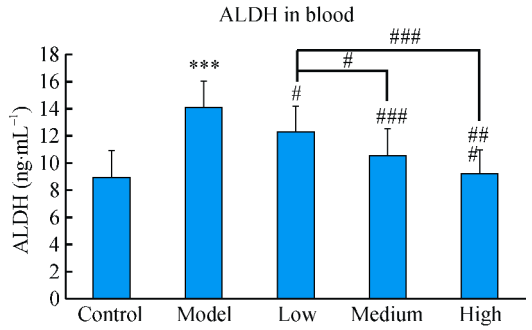


Fig. 13 Content detection of acetaldehyde dehydrogenase in serum. $^{***}P < 0.001$ *vs* control, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *vs* model control, $n = 12$

In sum, the different doses of PDCA had no effect on the weight increment of mice. Treated with 50% alcohol, the liver of mice could be injured with fatty degeneration and increment of related biochemical indicators. PDCA could dose dependently reduce the AST and ALT activity, the content of TG, MDA, VLDL, and ALDH in serum, and hepatic fat accumulation of alcohol injured liver, as well as effectively alleviate the alcohol injury to the liver tissue.

Protective effect of antcin K on the alcohol induced liver injury

To investigated whether the protective effect of PDCA on the alcohol induced liver injury was attributed to its main content antcin K, the effects of antcin K on body weight of mice, biochemical indicators of liver function and the formation of lipid droplets were investigated. As showed in Fig. 15, different doses of antcin K didn't influence the body weight of mice (Table 3). After treatment with 50% alcohol, the biochemical indicators of liver function of mice in model group increased, and were significantly different from that in control group ($P < 0.05$) (Table 4). The content of TG, MDA and the activity of AST in the middle dose, high dose and positive drug groups decreased significantly in a dose dependent manner after the treatment of antcin K, and significant differences could be observed by comparing with the model control group ($P < 0.05$). (Figs. 16−19). Besides, compared with the model control group, the low dose, middle dose, high dose and the positive drug groups dose dependently reduced the activity of ALT with significant differences ($P < 0.05$). As showed in Fig. 20, no or very small amount of lipid droplets in the control group compared to numerous lipid droplets were observed in the model group. After treatment of antcin K, the formation of lipid droplets in hepatocytes decreased in a dose dependent manner, which indicated that antcin K can inhibit the accumulation of fat in liver.

In conclusion, the different doses of antcin K had no effect on body weight, diet and amount of drinking water in mice. Antcin K can reduce the activities of AST and ALT in serum and the contents of TG, MDA, VLDL and ALDH, as well as the accumulation of fatty liver cells, which can effectively alleviate the damage effect caused by alcohol on liver tissue.

MATERIALS AND METHODS

Chemical and reagents

NMR spectra were taken on a Bruker Avance III-600 NMR spectrometer with TMS as an internal standard. HR-FT-MS data were acquired using an Apex ultra 7.0 FT-MS (Bruker). Silica gel (200–300 mesh, Qingdao Marine Chemical, Inc., Qingdao, China), lichroprep reversed-phase RP-18 silica gel (40–63 μm, Merck, Darmstadt, Germany), and Sephadex LH-20 (40–70 μm, Amersham Pharmacia Biotech AB, Uppsala, Sweden) were used for column chromatography (CC). TLC was carried out using glass-precoated silica gel GF254 (Qingdao) and visualized under UV light or by spraying with vanillin (contains 10% H_2SO_4) ethanol reagent.

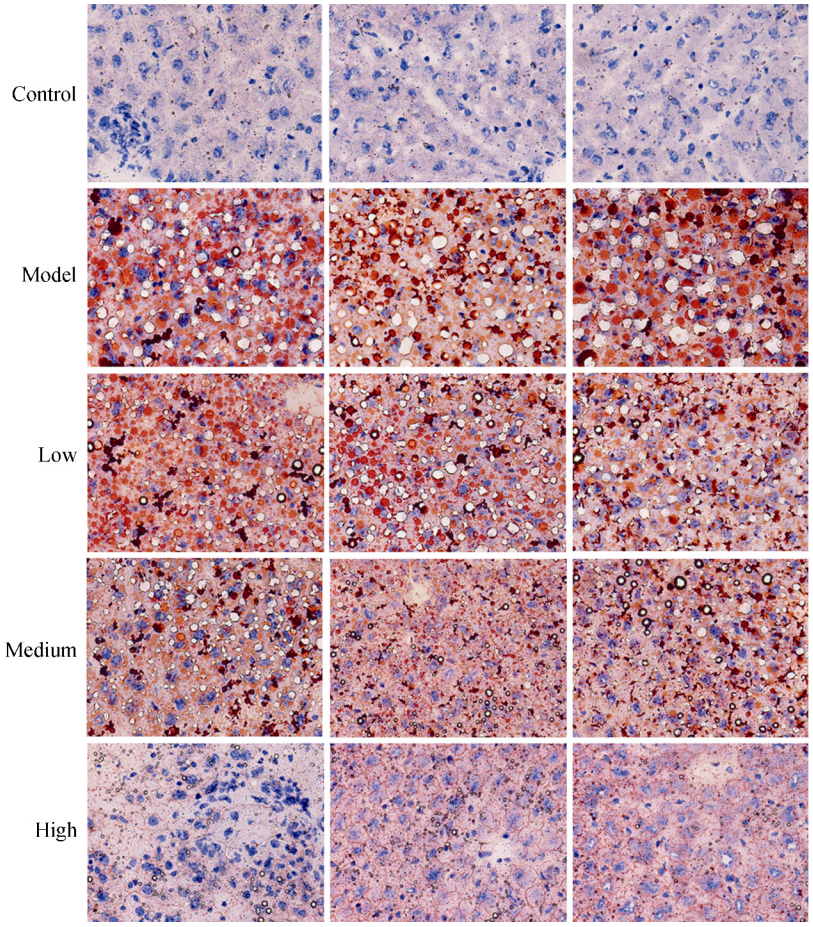


Fig. 14 Oil red staining of liver sections in different groups of mice

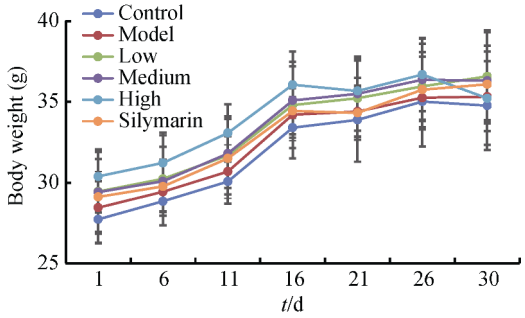


Fig. 15 Effects of different doses of *antcin K* on body weight of mice, *n* = 12

Table 3 Body weight of mice after treatment of antcin K ($\bar{x} \pm s$, *n* = 12)

Groups	Initial weight (g)	End weight (g)	Increment (g)
Control	27.73 ± 1.45	34.10 ± 1.35	6.37 ± 1.22
Model	28.45 ± 2.23	35.32 ± 1.42	6.87 ± 1.41
Low dose	29.45 ± 2.61	36.57 ± 2.76	7.12 ± 1.40
Middle dose	29.42 ± 2.48	36.30 ± 3.11	6.88 ± 3.09
High dose	30.38 ± 1.07	35.22 ± 2.88	4.83 ± 1.31
Silymarin (Postive control)	29.12 ± 0.97	36.08 ± 2.41	6.97 ± 1.83

Table 4 Effects of antcin K on biochemical indicators related liver function (*n* = 12)

Groups	TG (mmol·L^{-1})	AST (U·L^{-1})	ALT (U·L^{-1})	MDA (nmol·mL^{-1})
Control	1.36 ± 0.07	15.71 ± 2.40	8.96 ± 0.99	12.33 ± 3.68
Model	2.44 ± 0.19***	46.08 ± 4.31***	39.56 ± 2.54***	19.69 ± 2.61**
Low dose	2.07 ± 0.02$^{\#\#\#}$	28.00 ± 3.39$^{\#\#\#}$	26.34 ± 3.38$^{\#\#\#}$	15.62 ± 4.35$^{\#}$
Middle dose	1.74 ± 0.22$^{\#\#\#}$	24.16 ± 2.27$^{\#\#\#}$	21.90 ± 3.34$^{\#\#\#}$	14.71 ± 2.90$^{\#\#}$
High dose	1.64 ± 0.19$^{\#\#\#}$	16.08 ± 2.53$^{\#\#\#}$	12.83 ± 0.65$^{\#\#\#}$	11.57 ± 4.11$^{\#\#}$
Postive control	1.62 ± 0.09$^{\#\#\#}$	20.90 ± 2.44$^{\#\#\#}$	8.32 ± 0.82$^{\#\#\#}$	11.95 ± 3.85$^{\#\#}$

$^{***}P < 0.001$ *vs* control, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ *vs* model control

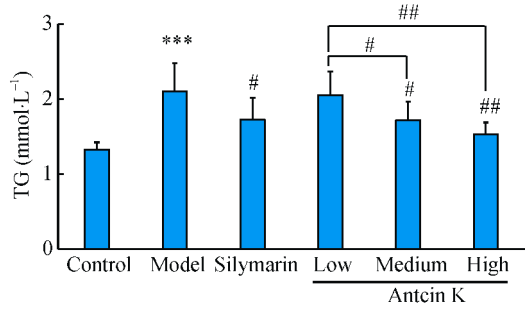


Fig. 16 Content detection of triglyceride in serum. $^{***}P < 0.001$ *vs* control, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *vs* model control, $n = 12$

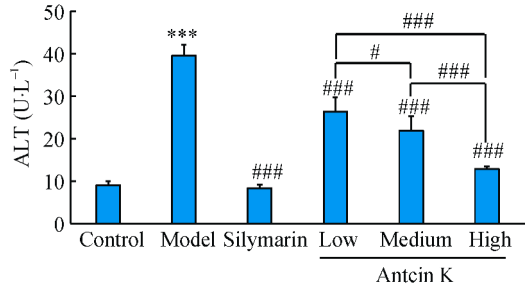


Fig. 17 Glutamic-pyruvic transaminase activity determination in serum. $^{***}P < 0.001$ *vs* control, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *vs* model control, $n = 12$

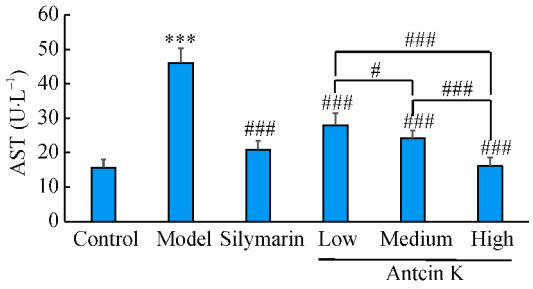


Fig. 18 The activity of Glutamic-oxaloacetic transaminase in serum. $^{***}P < 0.001$ *vs* control, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *vs* model control, $n = 12$

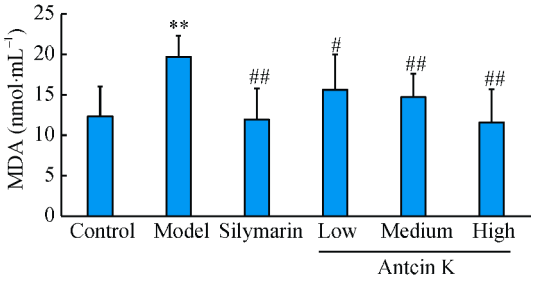


Fig. 19 Content detection of malonaldehyde in serum. $^{***}P < 0.001$ *vs* control, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *vs* model control, $n = 12$

Extraction and isolation

The petri-dish cultured *A. camphorata* was supplied by Arjil Pharmaceuticals L.L.C and identified by Dr. Lo, Jir-Mehng of the Industrial Technology Research Institute in Taiwan.

The fruiting bodies of the PDCA (400 g) were extracted with methanol (10 L × 12 h × 3 times) at 28 °C. The crude extract (104.3 g) was separated into sixteen fractions (Fr1−Fr16) by a silica gel column (1000 g, 120 mm × 300 mm), eluted with *n*-hexane/ethyl acetate (99 : 1, 98 : 2, 97 : 3, 10 : 1 and 5 : 1, 10 L for each gradient, 1000 mL per collection bottle) and dichloromethane/methanol (1 : 0, 99 : 1, 98 : 2, 97 : 3, 10 : 1, 5 : 1 and 1 : 0, 10 L for each gradient, 1000 mL per collection bottle). Fraction Fr 7 (0.9 g, yield 0.9%) crystallized in methanol and yield compound **14** (90 mg, yield 10%). Fraction Fr 10 (2.3 g, yield 2.2%) was dissolved in dichloromethane-methanol (1 : 1, *V/V*), and divided into two subfractions (Fr 10-a and Fr 10-b) by centrifugation. Fraction Fr 10-b (2.0 g, yield 87.0%) was subjected to silica gel column (elution with petroleum ether- acetone and dichloromethane- methanol, 30 mm × 250 mm, 60 g, 10000 mL) to give five subfractions (Fr 10b-1–Fr 10b-5), Fr 10b-3 (1.3 g, yield 65.0%) was separated by column chromatography on ODS (eluted with methanol-water, 35 mm × 200 mm, 40 g, 6000 mL) to give eight subfractions (Fr 10b3-a – Fr 10b3-h). Fr10b3-c (112.4 mg, yield 8.6%) and Fr 10b3-d (61.8 mg, yield 4.8%) was separated by HPLC (YMC Pack ODS-A column, 5 μm, 250 mm × 10 mm, flow rate: 5 mL·min^{-1}, acetonitrile−water− phosphoric acid, 60 : 40 : 0.1, *V/V/V*, 254 nm) to give compound **5** (33.1 mg, yield 19.0%) and compound **6** (14.5 mg, yield 8.3%), compound **17** and **18** (19.4 mg, yield 11.1%). Fraction Fr 12 (3.5 g, yield 3.4%) was chromatographed on silica gel column (elution with dichloromethane−methanol, 30 mm × 350 mm, 80 g, 15 000 mL) to give four subfractions (Fr 12-a–Fr 12-d). Fr 12-b (1.1 g, yield 31.4%) was separated by column chromatography on ODS (eluted with methanol−water, 35 mm × 200 mm, 40 g, 10 000 mL) to give fourteen subfractions (Fr 12b-1–Fr 12b-14). Fr12b-8 (272.3 mg, yield 24.8%) was separated by HPLC (YMC Pack ODS-A column, 5 μm, 250 mm × 10 mm, flow rate: 5 mL·min^{-1}, acetonitrile−water−phosphoric acid, 50 : 50 : 0.1, *V/V/V*, 254 nm) to give compound **3** (61.9 mg, yield 22.7%) and compound **4** (50 mg, yield 18.4%). Fr12b-12 (113.1 mg, yield 10.3%) was separated by HPLC (YMC Pack ODS-A column, 5 μm, 250 mm × 10 mm, flow rate: 5 mL·min^{-1}, acetonitrile−water−phosphoric acid, 60 : 40 : 0.1, *V/V/V*, 254 nm) to give compound **15** (3 mg, yield 2.7%) and compound **16** (10.1 mg, yield 8.9%). Fraction Fr 13 (5.8 g, yield 5.6%) was chromatographed on ODS column (eluted with methanol-water, 45 mm × 150 mm, 40 g, 10 000 mL) to give eight subfractions (fraction Fr 13-a–Fr 13-h), Fr13-d (506.1 mg, yield 8.7%) was further purified by column chromatography on ODS (eluted with methanol−water, 35 mm × 200 mm, 40 g, 10 000 mL) to give seven subfractions (Fr 13d-1–Fr 13d-7). Fr 13d-3 (82.1 mg, yield 16.2%) was separated by HPLC (YMC Pack ODS-A column, 5 μm, 250 mm × 10 mm, flow rate: 5 mL·min^{-1}, acetonitrile-water−phosphoric acid, 45 : 55 : 0.1, *V/V/V*, 254 nm) to give compound **19** (5.8 mg, yield 7.1%). Fr 13d-4 (259.7 mg, yield 51.3%) was separated by HPLC (YMC Pack ODS-A column,

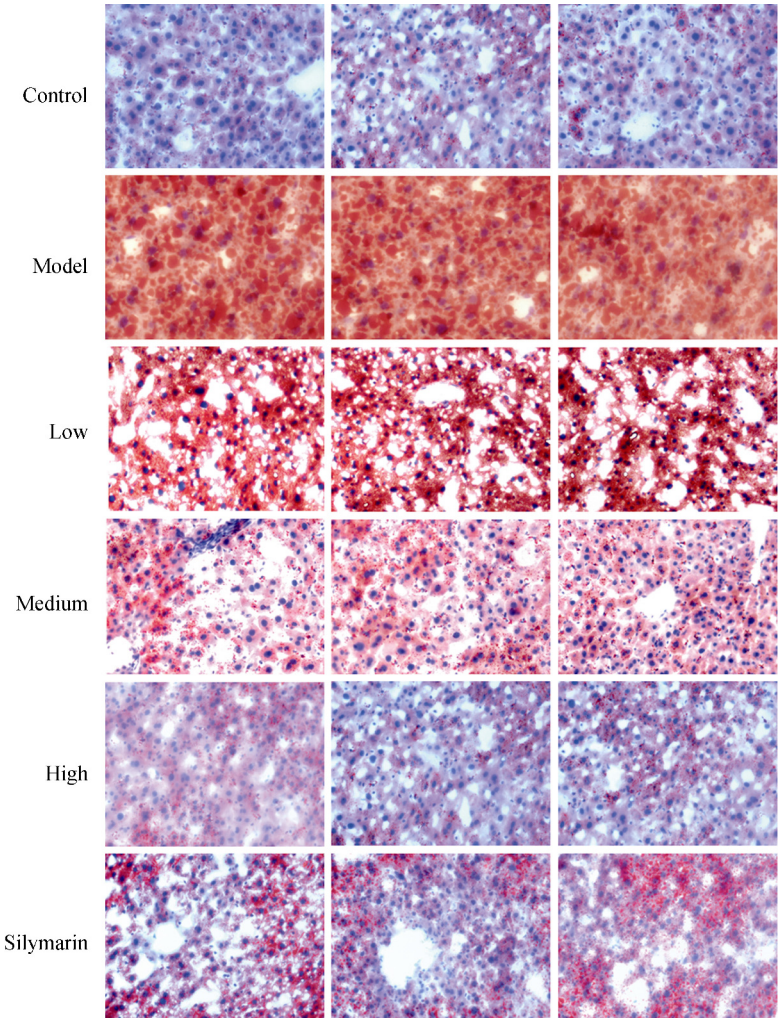


Fig. 20 Oil red staining of liver sections in different groups of mice

5 μm, 250mm × 10 mm, flow rate: 5 mL·min^{-1}, acetonitrile–water–phosphoric acid, 45 : 55 : 0.1, *V/V/V*, 254 nm) to give a pair of epimers **7** and **10** (5.8 mg, yield 2.2%) together with another pair of epimers **11** and **13** (23.5 mg, yield 9.0%). Fr 13d-3 (82.1 mg, yield 16.2%) was separated by HPLC (YMC Pack ODS-A column, 5 μm, 250 mm × 10 mm, flow rate: 5 mL·min^{-1}, acetonitrile–water–phosphoric acid, 45 : 55 : 0.1, *V/V/V*, 254 nm) to give compound **9** (5.8 mg, yield 7.1%). Fr 13d-5 (91.5 mg, yield 18.1%) was separated by HPLC (YMC Pack ODS-A column, 5 μm, 250 mm × 10 mm, flow rate 5 mL·min^{-1}, acetonitrile-water-phosphoric acid, 45 : 55 : 0.1, *V/V/V*, 254 nm) to give a pair epimers **8** and **12** (12 mg, yield 13.1%). Fraction Fr 14 (5.1 g, yield 4.9%) was chromatographed on ODS column (eluted with methanol–water, 46 mm × 160 mm, 40 g, 10000 mL) to give ten subfractions (Fr 14-a–Fr 14-j). Fr 14-j (2.1 g, yield 41.2%) was purified by HPLC (YMC Pack ODS-A column, 5 μm, 250 mm × 10 mm, flow rate: 5 mL·min^{-1}, acetonitrile–water–phosphoric acid, 35 : 65 : 0.1, *V/V/V*, 254 nm) to give compound **1** (12.1 mg, yield 0.6%) and compound **2** (15.1 mg, yield 0.7%).

HPLC analysis

The air-dried fruiting bodies of PDCA (100 mg) was minced and extracted with methanol (10 mL) at ambient temperature. After ultrasonic extraction of 30 min, the crude EtOH extract (10 μL) was analyzed by HPLC (Thermo U-3000) equipped with a UV detector. A welch Ultimate XB-C_{18} column (4.6 mm × 250 mm, 5μm) was employed with three solvent systems, H_2O + 0.2% acetic acid (A), acetonitrile + 0.2% acetic acid (B) and MeOH + 0.2% acetic acid (C). The gradient elution profile was as follows: 0–30 min, 50%–44% A, 25%–28% B, 25%–29% C, 0.5 mL·min^{-1}; 30–60 min, 36%–22% A, 38%–63% B, 26%–15% C, 0.5–0.7 mL·min^{-1}; and the detector wavelength was set at 254 nm with the column temperature 40 ºC.

Acute toxicity of PDCA

The PDCA (supplied by Arjil Pharmaceuticals L.L.C) is crushed and the powder was mixed with water containing 0.3% CMC-Na (Shantou Xilong Chemical Factory, Guangdong, China) to form a 0.12 g·mL^{-1} suspension. Twenty-four mice were randomly divided into four groups (male control group, female control group, male experimental group and

female experimental group) and six mice in each group. The mice were fasting and given water normally for 12 h. PDCA suspension at a dose of 40 mL·kg^{-1} (4.8 g·kg^{-1}) was administered to the experimental groups for once, while the control group was administered with water (0.3% CMC-Na) (Shantou Xilong Chemical Factory, Guangdong, China). After administration, all groups of mice were supplied free diet every day and observed the body weight as well as the food and water intake every two days for fourteen consecutive days. At the end of the experiment, the mice were executed and dissected to observe if there was any lesion in the viscera.

Protective activity of PDCA on alcohol induced liver injury.

Sixty male kunming mice were randomly divided into five groups, which were control, model control, low dose, medium dose and high dose groups, twelve mice for each group. The control and model groups were intragastric filled with water (0.3% CMC-Na) (Shantou Xilong Chemical Factory, Guangdong, China) per day. Besides, the PDCA powder at doses of 0.3, 0.6 and 1.2 g·kg^{-1} were administered daily to the low dose, medium dose and high dose groups, respectively. After continuous administration for 30 days, the alcohol induced hepatic injury model was established for the model group and drug treatment groups by 50% ethanol at the dosage of 12 mL·kg^{-1} [18]. Meanwhile, the control group was given the same amount of water. After fasting 16 h, the blood of each mouse was extracted from the eyeball and its plasma level of triglyceride (TG), aspartate aminotransferase (AST), alanine aminotransferase (ALT), malondialdehyde (MDA), very low density lipoprotein (VLDL) and aldehyde dehydrogenase (ALDH) were detected (All test kits above were purchased from Nanjing Jiancheng Bioengineering Institute). At the same time, the liver tissues were observed after stained with oil red (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

Protective activity of antcin K on alcohol induced liver injury.

Thirty-six male Kunming mice were divided into six groups, which were control, model control, low dose, medium dose and high dose groups. Similarly, the control and model groups were given water contenting 0.3% CMC-Na each day, 60, 30 and 10 mg·kg^{-1}·d^{-1} of antcin K were administered to the low dose, medium dose and high dose groups, respectively. Besides, silymarin (Sigma-Aldrich, Shanghai, China) (100 mg·kg^{-1}·d^{-1}) was given to the positive control group. After 30 days, the alcohol induced hepatic injury model were established for the model group [18], treatment groups and positive control groups by administration of 50% ethanol (12 mL·kg^{-1}), the control group was given with the same amount of water. Then fasting the mice for 16 h, the blood of each mouse was extracted and the level of TG, AST, ALT, MDA in blood was detected, then the liver tissues were observed after stained with oil red.

Plasma parameters

The blood of each mouse was collected in EDTA-coated tubes. Plasma was separated by centrifugation at 4 000 g for 12 min and stored at −80 °C. Plasma alanine triglyceride (TG), transaminase (ALT), aspartate aminotransferase (AST) and malondialdehyde (MDA) were measured using comercialized assay kit [18] (Nanjing Jiancheng Bioengineering Institute) following the manufacturer's instructions.

ELISA

The levels of VLDL and ALDH were detected by ELISA following the manufacturer's instructions (Nanjing Jiancheng Bioengineering Institute). Briefly, standards, serum samples, antibodies combined with biotin and Streptavidin-HRP were added into the plate and incubated 60 min at 37 °C. Washed the plate five times and added Chromogen solutions A and B, incubated for 10 min at 37 °C away from light. Then, the stop solution was added to stop the reaction and measured the OD under 450 nm within 10 min using a microplate spectrophotometer, Multiskan MK3 (Thermo, Waltham, Massachusetts, USA).

Oil red O staining

Fresh frozen liver sections were cut at 5−10 μm thick and mounted on sliders. Then the sliders were air dried for 30 minutes at room temperature and placed in Oil Red solution for 10−15 min, following by rinsing in 2 changes of distilled water. Then the samples were stained in hematoxylin for 3 min and washed thoroughly by running water for 30 sec, following by mounting with aqueous mounting medium and observing under an optical microscopy (Olympus BX51, Japan).

Conclusion

A. camphorata, a well-known edible medicinal mushroom with highly medicinal and commercial values, has intriguing activities of liver protection and has been traditionally used for the treatment of alcoholic liver disease. The petri-dish cultured *A. camphorata* (PDCA) is expected to develop as a substitute to meet the demand of *A. camphorata*. This article carried out the study on the secondary metabolites, acute toxicity, as well as hepatoprotective activities of the PDCA and its main contents antcin K. The results indicated that PDCA contains similar characteristic anthroic acids that had been found in the wild *A. camphorata*. The PDCA did not exhibited obvious acute toxicity in mice at the twenty times normal dosage. Compared with the PDCA, antcin K showed commensurate activities with reducing AST and ALT activities, downregulating the level of TG, MDA, VLDL, ALDH in serum, and suppressing the accumulation of hepatic fat in alcohol injury mice model. Consequently, the PDCA was edible safety and showed hepatoprotective activity towards ethanol induced liver injury mice. Even though the whole PDCA has been reported to have hepatoprotective activity, its main content antcin K was demonstrated as the effective components responsible for the hepatoprotective activity against alcoholic liver diseases for the first time.

References

[1] Cheng JJ, Chao CH, Lu MK. Large-scale preparation of sulfated polysaccharides with anti-angionenic and anti-inflammatory properties from *Antrodia cinnamomia* [J]. *Int J Biol Macromol*, 2018, **113**(1): 1198-1205.

[2] Li ZW, Kuang Y, Tang SN, Li K, *et al*. Hepatoprotective

activities of *Antrodia camphorata* and its triterpenoid compounds against CCl_4-induced liver injury in mice [J]. *J Ethnopharmacol*, 2017, **206**: 31-39.

[3] Yen IC, Lee SY, Lin KT, *et al*. *In vitro* anticancer activity and structural characterization of ubiquinones from *Antrodia cinnamomea* mycelium [J]. *Molecules*, 2017, **22**(5): 747-758.

[4] Lin WC, Deng JS, Huang SS, *et al*. Evaluation of antioxidant, anti-inflammatory and anti-proliferative activities of ethanol extracts from different varieties of *Sanghuang* species [J]. *RSC Adv*, 2017, **7**(13): 7780-7788.

[5] Liu Y, Yang A, Qu Y, *et al*. Ameliorative effects of *Antrodia cinnamomea* polysaccharides against cyclophosphamide-induced immunosuppression related to Nrf2/HO-1 signaling in BALB/c mice [J]. *Int J Biol Macromol*, 2018, **116**: 8-15.

[6] Kao ST, Kuo YH, Wang SD, *et al*. Analogous corticosteroids, 9A and EK100, derived from solid-state-cultured mycelium of *Antrodia camphorata* inhibit proinflammatory cytokine expression in macrophages [J]. *Cytokine*, 2018, **108**: 136-144.

[7] Shen CC, Kuo YC, Huang RL, *et al*. New ergostane and lanostane from *Antrodia camphorata* [J]. *J Chin Med*, 2003, **14**(4): 247-258.

[8] Cherng IH, Chiang HC, Cheng MC, *et al*. Three new triterpenoids from *antrodia cinnamomea* [J]. *J Nat Prod*, 1995, **58**(3): 365-371.

[9] Du YC, Wu TY, Chang FR, *et al*. Chemical profiling of the cytotoxic triterpenoid-concentrating fraction and characterization of ergostane stereo-isomer ingredients from *Antrodia camphorat* [J]. *J Pharm Biomed Anal*, 2012, **58**: 182-192.

[10] Cherng IH, Wu DP, Chiang HC. Triterpenoids from *antrodia cinnamomea* [J]. *Phytochemistry*, 1996, **41**(1): 263-267.

[11] Huang Y, Lin X, Qiao X, *et al*. Antcamphins A-L, ergostanoids from *Antrodia camphorata* [J]. *J Nat Prod*, 2014, **77**(1): 118-124.

[12] Wu SJ, Leu YL, Chen CH, *et al*. Camphoratins A-J, potent cytotoxic and anti-inflammatory triterpenoids from the fruiting body of *Taiwanofungus camphoratus* [J]. *J Nat Prod*, 2010, **73**(11): 1756-1762.

[13] Shen CC, Wang YH, Chang TT, *et al*. Anti-inflammatory ergostanes from the basidiomata of *Antrodia salmonea* [J]. *Planta Med*, 2007, **73**(11): 1208-1213.

[14] Kwon HC, Zee SD, Cho SY, *et al*. Cytotoxic ergosterols from Paecilomyces sp. J300 [J]. *Arch Pharm Res*, 2002, **25**(6): 851-855.

[15] Yang SW, Shen YC, Chen CH. Steroids and triterpenoids of *Antodia cinnamomea*-a fungus parasitic on *Cinnamomum micranthum* [J]. *Phytochemistry*, 1996, **41**(5): 1389-1392.

[16] Xia WU, Yang JS, Dong YS. Chemical constituents of *Fomes officinalis* (Ⅰ) [J]. *Chin Tradit Herb Drugs*, 2005, **6**: 812-814.

[17] Li YT, Pang DR, Zhu ZX, *et al*. Chemical constituents from the fruits of *Vitex negundo* var. cannabifolia and their biological activities *in vitro* [J]. *Chin J Chin Mater Med*, 2016, **41**(22): 4197-4203.

[18] Cai Z, Song L, Qian B, *et al*. Understanding the effect of anthocyanins extracted from purple sweet potatoes on alcohol-induced liver injury in mice [J]. *Food Chem*, 2018, **245**: 463.

Cite this article: WU Yu, TIAN Wen-Jing, GAO Shuo, LIAO Zu-Jian, WANG Guang-Hui, LO Jir-Mehng, LIN Pei-Hsin, ZENG De-Quan, QIU Da-Ren, LIU Xiang-Zhong, ZHOU Mi, LIN Ting, CHEN Hai-Feng. Secondary metabolites of petri-dish cultured Antrodia camphorata and their hepatoprotective activities against alcohol-induced liver injury in mice [J]. *Chin J Nat Med*, 2019, **17**(1): 33-42.