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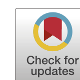
Combination of cut-log cultivated fruiting body and solid-state cultured mycelia of *Taiwanofungus camphoratus* ameliorates CCl_4-induced liver injury in rats



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ABSTRACT

Taiwanofungus camphoratus, a medicinal mushroom indigenous to Taiwan, possesses various pharmacological functions. The most recognized ethnopharmacological relevance of *T. camphoratus* is hepatoprotection since it was traditionally used for treating liver disorders by Taiwan aborigines. The aim of this study is to evaluate the hepatoprotective effect of the combination of fruiting body and solid-state cultured mycelia of *T. camphoratus* (LDAC) on carbon tetrachloride (CCl_4)-induced chronic liver damage in rats. We treated Wistar rats daily with low, medium and high [87.5, 175 and 437.5 mg/kg body weight (bw), respectively] doses of LDAC for 9 weeks. After the first week of treatment, rats were administered 20% CCl_4 (0.5 mL/0.3 kg bw) twice a week to induce liver damage until the treatment ended. The results showed that administration of LDAC by oral gavage significantly reduced the absolute weight of the liver and the serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in CCl_4-treated rats. The activities of the antioxidant enzymes glutathione peroxidase (GPx), glutathione reductase (GRd) and catalase (CAT) were increased by LDAC treatment. Moreover, LDAC improved CCl_4-induced hepatic vacuolization, necrosis and fibrosis in a dose-dependent manner, and no adverse effects were observed in the LDAC-treated groups. Based on the results, LDAC is a promising hepatoprotective agent for preventing and ameliorating CCl_4-induced chronic liver injury, and this effect might be exerted through activation of the antioxidant defense system.

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1. Introduction

Chronic liver disease and cirrhosis are recognized as major global health issues accompanied by unmet medical and supportive care needs.[1] In Taiwan, chronic liver disease and cirrhosis have been among the top ten leading causes of death for decades, and worldwide, cirrhosis-related deaths have exceeded one million since 2010.[2] Currently, preventive medicine is rising in popularity, and people tend to seek functional foods or herbal medicines for health promotion and disease prevention. In particular, herbal medicinal products with science-based information are becoming highly accepted by people who pursue health promotion or have medical needs. Patients with a chronic disease or cancer most commonly use herbal medicine as complementary and alternative medicine (CAM) because of its multilevel, multitarget and coordinated intervention effects.[3] Available evidence indicates that folk remedies, including edible herbal formulations and Chinese herbal medicine, have therapeutic potential for treating chronic liver disease and liver fibrosis.[4–6] Hence, there is an imperative need to pay more attention to the discovery of promising therapeutics from folk medicine.

Taiwanofungus camphoratus (syn. *Antrodia cinnamomea*, *Antrodia camphorata*) is a medicinal mushroom that is indigenous to Taiwan and that grows only in the inner cavity of *Cinnamomum kanehirae* Hayata (Lauraceae). Taiwan aborigines commonly use the fruiting body of *T. camphoratus* as folk medicine for health promotion and for the treatment of liver disease, drug and food intoxication, diarrhea and cancer.[7–9] Recently, *T. camphoratus* has

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List of abbreviations

ALT	alanine aminotransferase
AST	aspartate transferase
bw	body weight
CAT	catalase
CCl_4	carbon tetrachloride
CMC	carboxymethyl cellulose
GPx	glutathione peroxidase
GRd	glutathione reductase
GSH	glutathione
H&E	hematoxylin and eosin
HPLC	high-performance liquid chromatography
LDAC	the combination of cut-log cultivated fruiting body and solid-state cultured mycelia of *Taiwanofungus camphoratus*
SOD	superoxide dismutase
TC	total cholesterol
TG	triglyceride

been found to possess a variety of biological activities, including anticancer,[10–12] liver protective,[13,14] anti-inflammation,[15,16] and immune-regulation effects.[17,18] Additionally, many bioactive components of *T. camphoratus* have been identified, such as polysaccharides, terpenoids, benzenoids and nucleic acids.[8,9] The extracts of *T. camphoratus* mycelia and its components, such as polysaccharides, antroquinonol and antrodin B, were demonstrated to protect hepatic cells from alcohol-induced liver damage and fibrosis.[19–21] A previous study also showed that the fruiting body of *T. camphoratus* enhanced the liver antioxidant capacity against chronic alcohol damage.[22] Antcin K and antcin H, which are abundant in the fruiting body of *T. camphoratus,* also have hepatoprotective effects through the action of antioxidants in the liver.[23,24] However, the fruiting body of *T. camphoratus* is rare in nature and usually needs 1–2 years for growth by cut-log cultivation. In contrast, the mycelia of *T. camphoratus* take only 4 months for production by solid-state cultivation. Therefore, to meet the market demand, combining the cut-log fruiting body and solid-state cultivated mycelia of *T. camphoratus* would be a good strategy for saving time and reducing costs. To the best of our knowledge, there are no investigations yet on the hepatoprotective activity of the combination.

In the present study, the hepatoprotective activity of the combination of fruiting body and solid-state cultured mycelia of *T. camphoratus* (LDAC) was examined by using carbon tetrachloride (CCl_4)-induced chronic liver injury in rats. Chronic liver injury was chemically induced by administering CCl_4 to rats twice weekly for eight weeks. LDAC was gavaged once per day for nine weeks (before one week of CCl_4 treatment). Then, the blood biochemical parameters, liver biopsy and liver antioxidant enzymes were assessed to evaluate the hepatoprotective properties of LDAC.

2. Materials and methods

2.1. Test article preparation and analysis

The test article Leader Deluxe *Antrodia cinnamomea* capsule (LDAC) was manufactured by Taiwan Leader Biotech Corp. (Taipei, Taiwan). LDAC is composed of cut-log cultivated fruiting body extract and solid-state culture of *T. camphoratus*. LDAC powder was used to prepare different doses in 0.5% carboxymethyl cellulose (CMC) for the animal experiments. To analyze the index compounds in LDAC, high-performance liquid chromatography (HPLC) was performed. First, 1 g of LDAC powder was sonicated in 10 mL of methanol for 60 min to obtain the extract, which was centrifuged at 3000 rpm for 15 min. The supernatant was then collected and filtered using a 0.45-mm syringe filter, and the filtrate was analyzed using HPLC. Qualitative analysis of the major index compounds (i.e., antcin K and 1,4-dimethoxy-2,3-methylenedioxy-5-methylbenzene) in LDAC was further performed using HPLC (Waters Alliance e2695 Separation Module, MA, USA). The HPLC procedure was carried out on a Cosmosil 5C18-AR-II column (5-mm particle size, 250×4.6 mm inner diameter; Nacalai Tesque Inc., Kyoto, Japan) using a gradient solvent system consisting of 0.1% formic acid (Solvent A) and acetonitrile (Solvent B). The two-solvent system was run as follows: 5% B (0 min), 5% B (1 min), 40% B (13 min), 50% B (18 min), 100% B (25 min), 100% B (30 min), 5% B (32 min), and 5% B (40 min). The peaks were recorded with a photodiode array detector (Waters 2998 PDA detector, MA, USA) at 254 nm, and the solvent flow rate was maintained at 1.0 mL/min.

2.2. Animal treatment

Fifty male Wistar rats (weight: 200–250 g; age: 7 weeks old) were obtained from the Laboratory Animal Center, College of Medicine, National Taiwan University (Taipei, Taiwan). All animals were handled in accordance with the guidelines of the Institutional Animal Care and Use Committee, National Taiwan University. The standard experimental conditions were as follows: temperature, 23 ± 2 °C; relative humidity, 50–70%; and a 12-h light/dark cycle. The rats were fed a rodent chow diet and tap water given *ad libitum*. After 1 week of acclimatization, the rats were divided into 5 groups of 10 rats each, including the normal control (vehicle only); negative control (vehicle + CCl_4); and CCl_4 with low, medium and high doses of LDAC (LDAC 87.5 mg/kg bw/d + CCl_4, LDAC 175 mg/kg bw/d + CCl_4 and LDAC 437.5 mg/kg bw/d + CCl_4, respectively). LDAC was orally administered to the animals once daily for 9 weeks (weeks 0–8). After 1 week of treatment (week 0), rats in the vehicle + CCl_4, LDAC 87.5 mg/kg bw/d + CCl_4, LDAC 175 mg/kg bw/d + CCl_4 and LDAC 437.5 mg/kg bw/d + CCl_4 groups were further orally administered 20% CCl_4 (diluted in olive oil, 0.5 mL/0.3 kg bw) twice per week for 8 weeks to induce hepatic fibrosis, whereas rats in the normal control group were administered equal volumes of vehicle (0.5% CMC and olive oil). Schematic diagrams are shown in Fig. 1, which presents the design for the control and treatment groups. Body weight was measured once per week during the experimental period. At the end of the experiment, the rats were sacrificed, and the blood, liver, kidney and spleen were collected from each rat for analysis.

2.3. Serum biochemical analysis

To assess the liver damage in the rats, rat serum was collected by centrifuging blood samples at 12,000 rpm for 5 min at 4 °C. The biochemical parameters, including alanine aminotransferase (ALT), aspartate aminotransferase (AST), triglyceride (TG), and cholesterol (TC), were analyzed by using commercial test strips (ALT and AST Spotchem II reagent strips, Arkray Inc., Kyoto, Japan) and an automatic blood analyzer (Spotchem EZ automated dry chemistry analyzer).

2.4. Preparation of liver homogenate

Liver tissue (0.5 g) was homogenized in 10 vol of ice-cold liver homogenization buffer (8 mM KH_2PO_4, 12 mM K_2HPO_4 and 1.5% KCl, pH 7.4) at 4 °C. The homogenate was then centrifuged at 10,000 rpm for 30 min at 4 °C. The supernatant was further

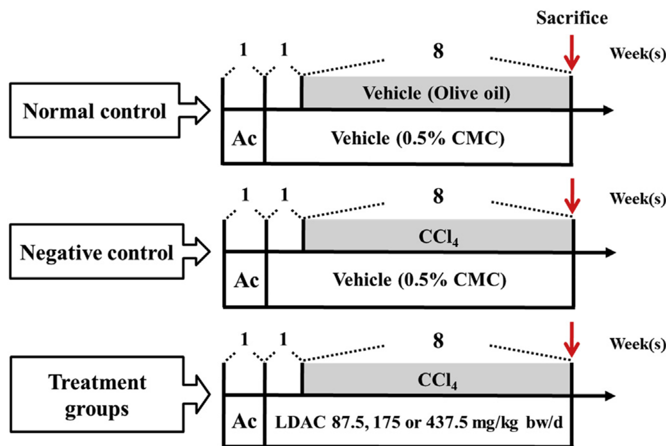

Fig. 1. Schematic diagrams showing the design for studying the protective activity of LDAC against CCl_4-induced liver injury in rats. The treatments of animals are detailed in the "Materials and Methods" section. Ac, acclimatization; CMC, carboxymethyl cellulose; CCl_4, carbon tetrachloride; LDAC, the combination of fruiting body and solid-state cultured mycelia of *Taiwanofungus camphoratus*.

subjected to ultracentrifugation at 32000 rpm for 60 min at 4 °C. The supernatant was stored at −80 °C until further analyses of the liver TG, TC and glutathione content and antioxidant enzyme activities. The protein content in the liver homogenate was estimated spectrophotometrically by measuring the absorbance at 595 nm using the Bio-Rad protein assay kit (Hercules, CA, USA). Triglycerides and total cholesterol of the liver homogenate were estimated by using commercial kits from Cayman (Cayman Chemical, MI, USA).

2.5. Determination of glutathione level

The liver homogenate glutathione (GSH) level was measured using a glutathione assay kit (Cayman Chemical, MI, USA). Briefly, the sulfhydryl group of GSH reacts with DTNB (5,5′-dithio-bis-2-nitrobenzoic acid, Ellman's reagent), producing yellow-colored 5-thio-2-nitrobenzoic acid (TNB). The mixed disulfide GSTNB (between GSH and TNB) that is concomitantly produced is reduced by glutathione reductase (GRd) to recycle the GSH and produce more TNB. The rate of TNB production is directly proportional to this recycling reaction, which in turn is directly proportional to the concentration of GSH in the sample. GSH was quantified by measuring the absorbance of TNB at 405 nm and was expressed as nmol/mg protein.

2.6. Glutathione reductase assay

GRd was determined by using a glutathione reductase assay kit (Cayman Chemical, Ann Arbor, MI). Briefly, 25 μL of liver homogenate was added to 75 μL of potassium phosphate buffer. Then, 900 μL of reaction solution (100 mM potassium phosphate buffer, 1.1 mM $MgCl_2 \cdot 6H_2O$, 5.0 mM GSSG, 0.1 mM NADPH) was added, and changes in the absorbance at 340 nm were measured. GRd activity was expressed as nmol NADPH/min/mg protein.

2.7. Glutathione peroxidase assay

Glutathione peroxidase (GPx) in the liver homogenate was determined by using a glutathione peroxidase assay kit (Cayman Chemical, MI, USA). Briefly, 10 μL of liver homogenate was mixed with 90 μL of potassium phosphate buffer (20 mM, pH 7.0) and then added to 800 μL of reaction solution (pH 7.0, 100 mM potassium phosphate buffer, 1 mM EDTA, 1 mM NaN_3, 0.2 mM NADPH, 1 U/mL GRd and 1 mM GSH) for incubation at room temperature for 5 min. Next, 10 μL of 2.5 mM H_2O_2 was added to the reaction mixture, and changes in the absorbance at 340 nm were measured. GPx activity was expressed as nmol NADPH/min/mg protein.

2.8. Catalase assay

Liver catalase activity (CAT) was measured by using a catalase assay kit (Cayman Chemical, MI, USA). Briefly, the method is based on the reaction of the enzyme with methanol in the presence of an optimal concentration of H_2O_2. The formaldehyde produced is measured spectrophotometrically with 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole (Purpald) as the chromogen. Purpald specifically forms a bicyclic heterocycle with aldehydes, which, upon oxidation, changes from colorless to purple color. Subsequent measurement of the absorbance was performed to calculate the enzyme activity of CAT, which was expressed as nmol/min/mg protein.

2.9. Superoxide dismutase assay

Superoxide dismutase (SOD) in the liver was measured using a superoxide dismutase assay kit (Cayman Chemical, Ann Arbor, MI). SOD activity was assessed by measuring the dismutation of superoxide radicals generated by xanthine oxidase and hypoxanthine spectrophotometrically at 450 nm, and the enzyme activity of SOD

Table 1
Effects of LDAC on body and organ weights in CCl_4-treated rats.

Group[1)]	Body		Liver		Spleen		Kidney	
	Initial weight (g)	Final weight (g)	Absolute weight (g)	Relative weight (g/100 g bw)	Absolute weight (g)	Relative weight (g/100 g bw)	Absolute weight (g)	Relative weight (g/100 g bw)
Normal control	240 ± 14^{a}	368 ± 26^{a}	14.53 ± 2.62^{a}	3.27 ± 0.45^{a}	0.96 ± 0.17^{a}	0.22 ± 0.03^{a}	2.62 ± 0.24^{ab}	0.59 ± 0.03^{a}
Vehicle + CCl_4	240 ± 15^{a}	359 ± 37^{a}	17.63 ± 2.52^{b}	4.07 ± 0.32^{bc}	1.01 ± 0.13^{a}	0.23 ± 0.03^{ab}	2.77 ± 0.31^{b}	0.64 ± 0.03^{b}
LDAC 87.5 + CCl_4	241 ± 6^{a}	376 ± 28^{a}	15.67 ± 1.57^{a}	4.17 ± 0.34^{c}	1.15 ± 0.34^{a}	0.31 ± 0.09^{c}	2.46 ± 0.23^{a}	0.65 ± 0.04^{b}
LDAC 175 + CCl_4	241 ± 6^{a}	365 ± 23^{a}	13.95 ± 1.03^{a}	3.83 ± 0.30^{b}	1.08 ± 0.17^{a}	0.30 ± 0.04^{c}	2.41 ± 0.21^{a}	0.66 ± 0.05^{b}
LDAC 437.5 + CCl_4	245 ± 6^{a}	366 ± 34^{a}	14.18 ± 1.59^{a}	3.87 ± 0.25^{bc}	0.99 ± 0.10^{a}	0.27 ± 0.03^{bc}	2.49 ± 0.32^{a}	0.68 ± 0.08^{b}

All values are presented as the mean ± standard deviation (n = 10). Values with different superscripts within the same column are significantly different among groups according to one-way analysis of variance coupled with the Duncan multiple comparison test ($p < 0.05$).
[1)] Normal control, 0.5% CMC and olive oil; vehicle + CCl_4, 0.5% CMC +20% CCl_4; LDAC 87.5 + CCl_4, LDAC 87.5 mg/kg bw/d + 20% CCl_4; LDAC 175 + CCl_4, LDAC 175 mg/kg bw/d + 20% CCl_4; LDAC 437.5 + CCl_4, LDAC 437.5 mg/kg bw/d + 20% CCl_4; bw, body weight; CMC, carboxymethyl cellulose; CCl_4, carbon tetrachloride; LDAC, the combination of fruiting body and solid-state cultured mycelia of *Taiwanofungus camphoratus*.

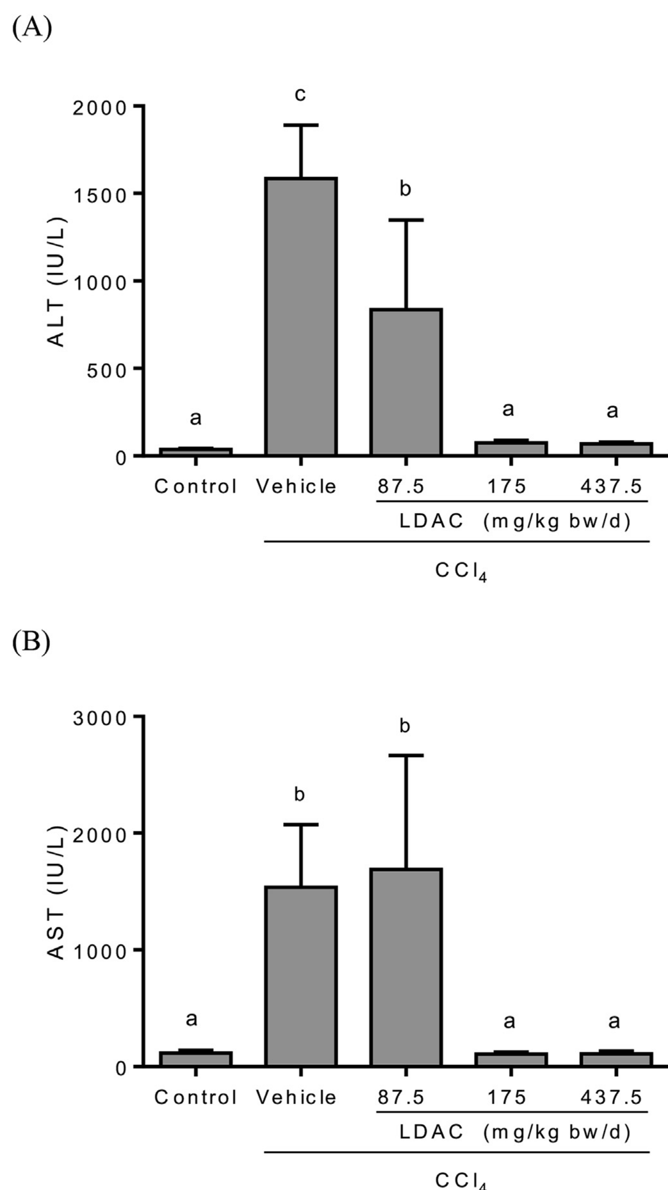

Fig. 2. Effects of LDAC on serum (A) alanine aminotransferase (ALT) and (B) aspartate transferase (AST) levels in CCl_4-treated rats. Data are presented as the mean ± standard deviation (n = 10). Values with different superscripts are significantly different among groups according to one-way analysis of variance coupled with the Duncan multiple comparison test ($p < 0.05$).

Table 2
Effects of LDAC on triglyceride (TG) and total cholesterol (TC) in CCl_4-treated rats.

Group (mg/kg) [1)]	Serum (mg/dL)		Liver (mg/g)	
	TG	TC	TG	TC
Normal control	95 ± 35^c	72 ± 14^b	64 ± 8^b	108 ± 15^a
Vehicle + CCl_4	36 ± 23^a	47 ± 16^a	60 ± 17^b	149 ± 20^b
LDAC 87.5 + CCl_4	58 ± 16^b	58 ± 13^a	59 ± 13^b	136 ± 25^b
LDAC 175 + CCl_4	81 ± 17^c	79 ± 17^{bc}	55 ± 7^{ab}	119 ± 9^a
LDAC 437.5 + CCl_4	78 ± 14^c	88 ± 10^c	48 ± 7^a	105 ± 13^a

All values are presented as the mean ± standard deviation (n = 10). Values with different superscripts within the same column are significantly different among groups according to one-way analysis of variance coupled with the Duncan multiple comparison test ($p < 0.05$).
[1)] Abbreviations are as in Table 1.

was expressed as U/mg protein.

2.10. Histopathological analysis of liver

For the histological examination, the anterior portions of the right lateral lobe of the rat livers were sectioned, fixed in 10% neutral buffered formalin, embedded in paraffin, and sliced into 5-μm sections. The sections were then stained with hematoxylin and eosin (H&E) and Sirius red. A blinded histological assessment of the liver sections was then performed by a veterinary pathologist at the Graduate Institute of Veterinary Pathobiology of the National Chung Hsing University, Taiwan.

2.11. Statistical analysis

All the experimental data are represented as the mean ± standard deviation (SD). Statistical analysis was performed by one-way analysis of variance followed by the Duncan multiple comparison test using SPSS software. A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. HPLC chromatogram of LDAC

Previous studies have indicated that antcin K and 1,4-dimethoxy-2,3-methylenedioxy-5-methylbenzene are the active components of *T. camphoratus* with hepatoprotective and antitumor activities.[24–26] Therefore, in this study, we performed HPLC analysis of the fingerprint and identified the two index compounds in LDAC by comparing the retention times of the peaks with those of the reference standards (Supplement 1).

3.2. Changes in body and organ weights in rats

As shown in Table 1, the final body weight in the vehicle + CCl_4 group (359 ± 37 g) was marginally lower than that in the normal control group (368 ± 26 g), indicating that induction with CCl_4 tended to lower the body weight gain in rats. Moreover, the absolute and relative liver weights in the vehicle + CCl_4 group (17.63 ± 2.52 g and 4.07 ± 0.32 g/100 g bw, respectively) were significantly higher than those in the normal control group (14.53 ± 2.62 g and 3.27 ± 0.45 g/100 g bw, respectively) ($p < 0.05$), providing direct evidence of the toxic injury to the rat liver. By contrast, compared with the animals in the vehicle + CCl_4 group, the animals in the groups treated with LDAC showed significantly decreased CCl_4-induced elevation in absolute liver weight ($p < 0.05$). In addition, administration of CCl_4 slightly increased the absolute and relative spleen and kidney weights in rats. Supplementation with LDAC significantly lowered the CCl_4-induced absolute weight gain of the kidney but not the spleen.

3.2. Serum biochemical observations

The serum ALT and AST levels in rats are shown in Fig. 2. Both the ALT and AST levels in the vehicle + CCl_4 group (1586 ± 305 IU/L and 1535 ± 538 IU/L, respectively) were markedly higher than those in the normal control group (37 ± 6 and 117 ± 21 IU/L, respectively) ($p < 0.05$). The animals treated with medium and high doses of LDAC showed extraordinarily decreases in ALT and AST levels (LDAC 175 mg/kg bw/d + CCl_4: 74 ± 15 IU/L and 106 ± 18 IU/L, respectively; LDAC 437.5 mg/kg bw/d + CCl_4: 69 ± 11 IU/L and 109 ± 26 IU/L, respectively) and no significant difference from the animals in the normal control group (37 ± 26 IU/L and 117 ± 21 IU/L, respectively).

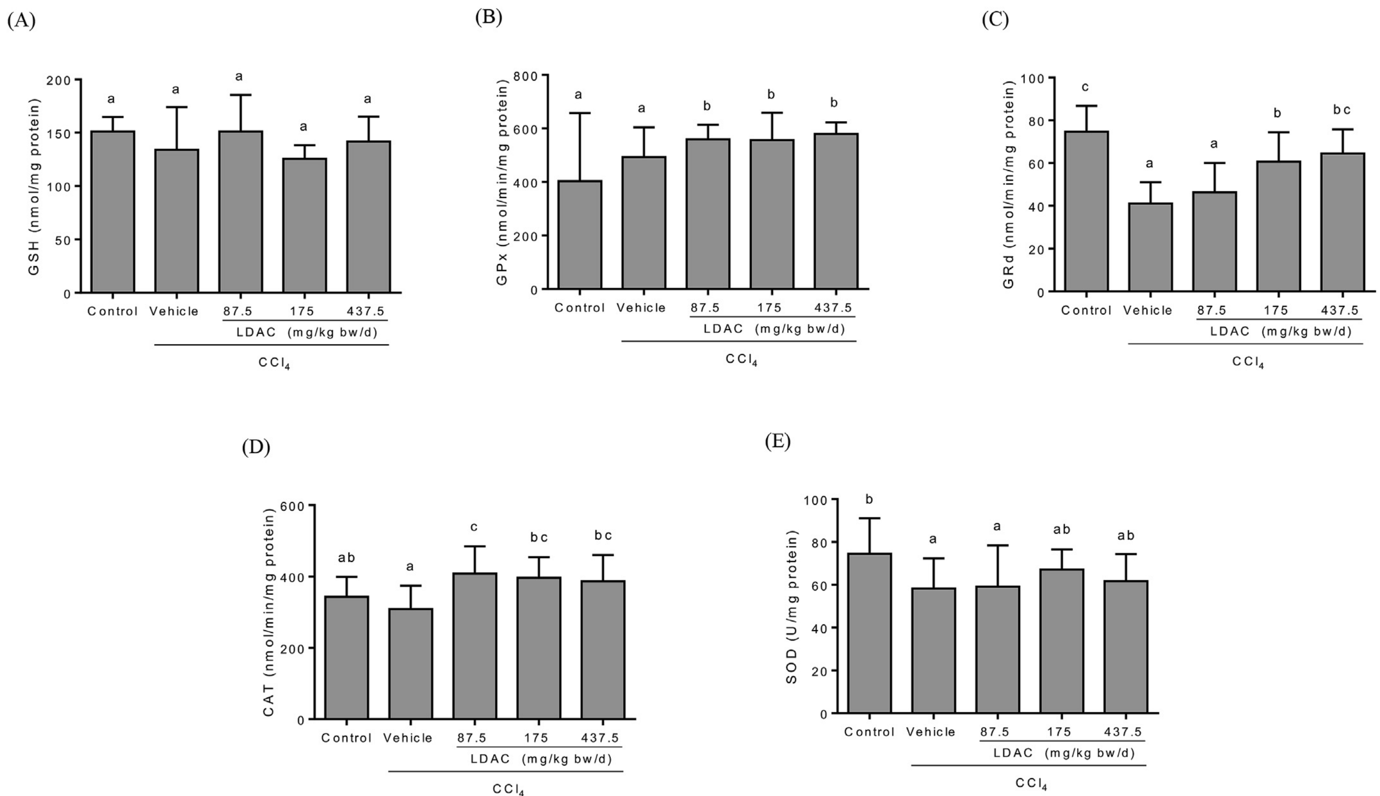

Fig. 3. Effects of LDAC on liver glutathione levels and antioxidant enzymes in CCl_4-treated rats. Data are presented as the mean ± standard deviation (n = 10). Values with different superscripts are significantly different among groups according to one-way analysis of variance coupled with the Duncan multiple comparison test ($p < 0.05$). CAT, catalase; GPx, glutathione peroxidase; GRd, glutathione reductase; GSH, glutathione; SOD, superoxide dismutase.

The serum and liver TG and TC levels in rats are shown in Table 2. The TG content in the vehicle + CCl_4 group (36 ± 23 mg/dL) was significantly lower than that in the normal control group (95 ± 35 mg/dL) ($p < 0.05$). Moreover, the TG levels were significantly higher in the groups treated with low (58 ± 16 mg/dL), medium (81 ± 17 mg/dL) and high (78 ± 14 mg/dL) doses of LDAC than in the vehicle + CCl_4 group ($p < 0.05$), and the medium- and high–dose-treated groups had no significant difference from the normal control group. The serum TC content in the vehicle + CCl_4 group (47 ± 16 mg/dL) was significantly lower than that in the normal control group (72 ± 14 mg/dL) ($p < 0.05$). Furthermore, the TC levels in the medium (79 ± 17 mg/dL) and high (88 ± 10 mg/dL) dose LDAC-treated groups were significantly higher than those in the vehicle + CCl_4 group ($p < 0.05$). The liver TG content showed no obvious difference among all groups except the high-dose LDAC-treated group, which, compared to the vehicle + CCl_4 group, showed a significant decrease. Compared to the vehicle + CCl_4 group, the medium- and high-dose LDAC-treated groups showed significantly decreased liver TC content ($p < 0.05$).

3.3. Effects of LDAC on antioxidant enzymes in CCl_4-treated rats

As shown in Fig. 3A, the liver GSH content was not significantly different among all groups. The results showed that the GPx activity in the vehicle + CCl_4 group (493 ± 111 nmol/min/mg protein) was slightly higher than that the normal control group (403 ± 254 nmol/min/mg protein) but was not significantly different, while the activities in the groups treated with low (560 ± 54 nmol/min/mg protein), medium (557 ± 102 nmol/min/mg protein) and high (580 ± 44 nmol/min/mg protein) doses of LDAC were significantly higher than those in the normal control group ($p < 0.05$) (Fig. 3B). The liver GRd activity in the vehicle + CCl_4 group (41 ± 10 nmol/min/mg protein) was significantly lower than that in the normal control group (75 ± 12 nmol/min/mg protein) ($p < 0.05$). The animals treated with LDAC showed a dose-dependent increase in liver GRd activity (Fig. 3C). Additionally, the LDAC-treated groups showed significantly increased liver CAT activity ($p < 0.05$) (Fig. 3D), but there were no obvious changes in the SOD level (Fig. 3E).

3.4. Histopathological observations

Hepatic vacuolization, necrosis and fibrosis are common outcomes of liver injury. H&E staining was performed to observe CCl_4-induced physiological changes in the rat liver, and Sirius red staining is a collagen staining method commonly used for the detection of hepatic fibrosis. As shown in Fig. 4 and Fig. 5, the vehicle + CCl_4 group exhibited severe hepatic vacuolization, necrosis and fibrosis, and treatment with LDAC markedly improved these pathological changes in CCl_4-treated rats. The liver portal peripheral vacuoles, necrosis and fibrosis were evaluated and scored by a blinded veterinary pathologist. As shown in Table 3, the scores of the liver portal peripheral vacuoles, necrosis and fibrosis in the vehicle + CCl_4 group (3.5 ± 0.5, 2.3 ± 0.5 and 3.0 ± 0.9, respectively) were significantly higher than those in the normal control group (0, 0 and 0, respectively) ($p < 0.05$). The scores in the LDAC treatment groups were all obviously lower than those in the vehicle + CCl_4 group and were even close to those in the normal control group. The results indicated that the medium- and high-dose LDAC-treated groups had no hepatic vacuolization and necrosis and that the fibrous connective tissue in those groups was highly inhibited.

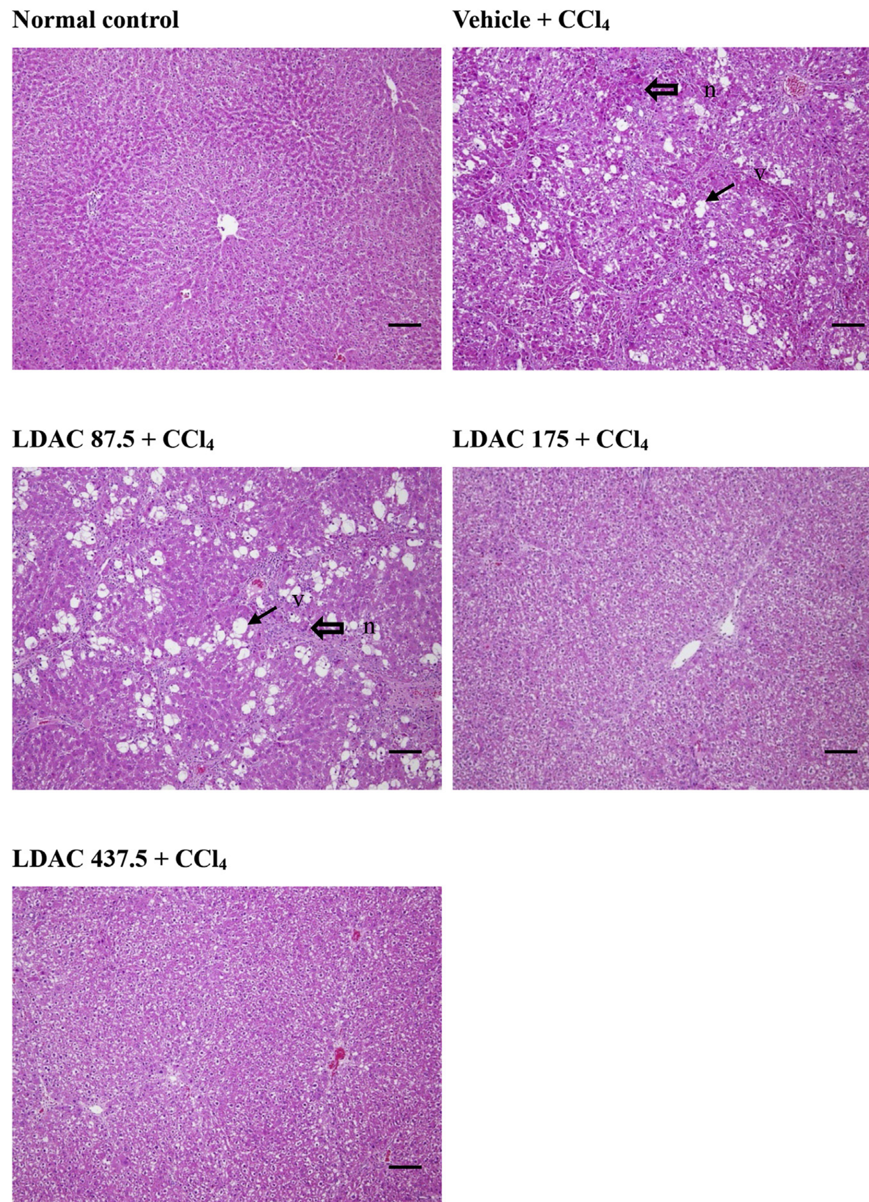

Fig. 4. Histopathological images of CCl_4-induced hepatic vacuolization (arrow) and necrosis (open arrow) in rats. Livers were stained with hematoxylin and eosin and visualized at 100x magnification. Abbreviations of groups are as in Table 1. Bar = 100 μm. v: vacuolization; n: necrosis.

4. Discussion

Hepatoprotective activity is the most recognized function of *T. camphoratus*. Several studies have revealed that the fruiting body and fermented mycelia of *T. camphoratus* showed protective effects on liver injury induced by a variety of chemicals, including CCl_4, alcohol and N-nitrosodiethylamine.[13,22,24,26,27] However, the fruiting body of *T. camphoratus* grows very slowly and is rare in nature. To meet market demand, the development of solid-state and cut-log cultivation methods for producing the mycelia and fruiting bodies of *T. camphoratus*, respectively, is a worthwhile task for saving time. In this study, we investigated the liver protective effect of LDAC, a composition of cut-log cultivated fruiting body and solid-state cultured mycelia of *T. camphoratus* by using a CCl_4-induced chronic liver injury model in rats. LDAC at doses of 175 and 437.5 mg/kg bw/d markedly improved CCl_4-induced hepatic inflammation (vacuolization), necrosis and fibrosis without causing body weight loss in rats. No adverse effects were observed during the LDAC treatment period, which can be considered as preliminary safety evaluation of LDAC for human consumption.

Chronic administration of CCl_4 increased the absolute liver weight in rats, but LDAC could significantly reduce the absolute liver weight. Serum ALT and AST are indicators commonly used to assess liver injury because they are highly released into the blood in response to liver damage. The results showed that LDAC at doses of 175 and 437.5 mg/kg bw/d significantly reduced serum ALT and AST levels and that there was no significant difference between the levels in LDAC-treated and normal control groups. These results suggested that LDAC could restore CCl_4-induced ALT and AST elevation to normal levels. Additionally, serum TG and TC levels were markedly decreased in rats treated with CCl_4. It has been reported that CCl_4 (acute, once) could impair liver secretion of lipoproteins into the blood and lead to TG accumulation in liver.[28] We found that serum TG and TC levels significantly decreased in

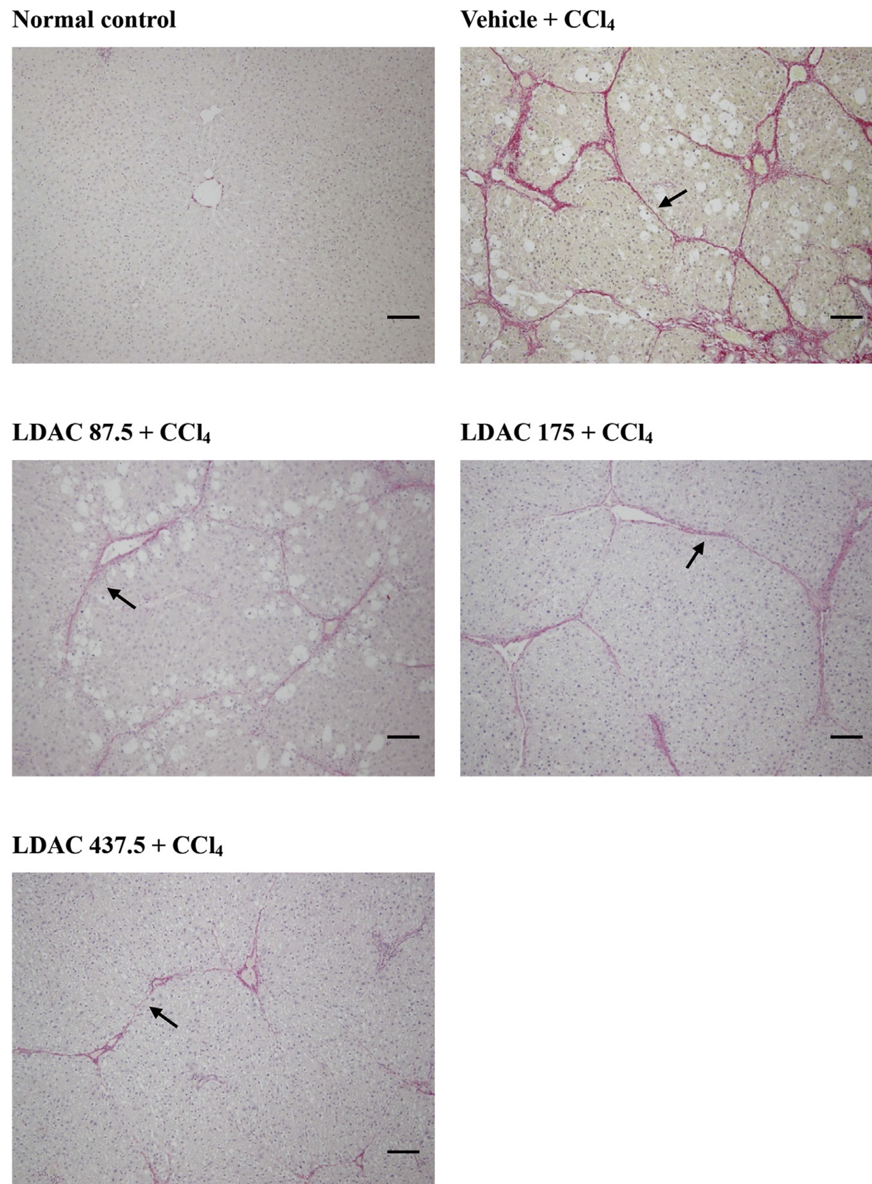

Fig. 5. Histopathological photographs of CCl_4-induced hepatic fibrosis (arrow) treated with LDAC in rats. Livers were stained with Sirius red and visualized at 100x magnification. Abbreviations of groups are as in Table 1. Bar = 100 μm.

the vehicle + CCl_4 group, whereas the liver TG and TC contents in the vehicle + CCl_4 group were not significantly different from those in the normal control group. According to histopathological findings, the vehicle + CCl_4 group showed severe hepatocyte necrosis and fibrosis. This effect might be because chronic exposure to CCl_4 causes severe liver damage and the deposition of much collagen fiber or connective tissue in the liver. Therefore, the lipid content could not be deposited in the liver in the late stage of liver injury. In contrast, LDAC could restore serum TG and TC levels to close to those in the normal control group, and compared to the vehicle + CCl_4 group, the group treated with a high dose of LDAC exhibited decreased TG and TC accumulation in the liver. Furthermore, LDAC dramatically improved CCl_4-induced hepatic vacuolization, necrosis and fibrosis. These results showed that LDAC had potent effects on protecting the liver from CCl_4-induced toxicity.

A previous report has demonstrated that the wheat-based solid-state culture of *T. camphoratus* at a dose of 1080 mg/kg bw/d could ameliorate hepatic fibrosis and necrosis induced by CCl_4 in rats.[26] In this study, we found that the effective dose of LDAC, which was reduced 6-fold to 175 mg/kg bw/d, could restore CCl_4-induced liver damage to nearly normal status. In addition, Li et al. have reported that cultivation techniques remarkably affect the hepatoprotective activity of *T. camphoratus*. The wild-growing or cutting wood-cultured fruiting bodies of *T. camphoratus* showed better liver protection ability than the mycelia. The fruiting body contained abundant triterpenoids, especially ergostane, that endowed it with a more potent effect.[13] Likewise, LDAC composed of fruiting body extracts and solid-state cultured mycelia of *T. camphoratus* showed more potent hepatoprotective activities than wheat-based solid-state cultured *T. camphoratus*. According to these studies, we inferred that fruiting body extracts exerted the hepatoprotective effects of LDAC and that this effect might be contributed by ergostane triterpenoids.

Oral or intraperitoneal injection of CCl_4 can be used to induce acute or chronic liver injury, as CCl_4 is metabolized by cytochrome P450 2E1 (CYP2E1) to generate free radicals such as

Table 3
Scoring of the effect of LDAC on liver vacuolization, necrosis and fibrosis in CCl_4-induced rats.

Group (mg/kg) [1]	Histopathological score of liver		
	Vacuoles[2]	Necrosis[2]	Fibrosis[3]
Normal control	0^a	0^a	0^a
Vehicle + CCl_4	3.5 ± 0.5^c	2.3 ± 0.5^c	3.0 ± 0.9^d
LDAC 87.5 + CCl_4	2.4 ± 0.8^b	1.6 ± 0.7^b	1.8 ± 0.4^c
LDAC 175 + CCl_4	0^a	0^a	1.5 ± 0.5^{bc}
LDAC 437.5 + CCl_4	0^a	0^a	1.2 ± 0.4^b

Histological indices of hepatic vacuolization, necrosis and fibrosis were quantified by a blinded veterinary pathologist based on numerical scoring of liver biopsy specimens. The final numerical score was calculated by dividing the sum of the number per grade of affected rats by the total number of examined rats. All values are presented as the mean ± standard deviation (n = 10). Values with different superscripts within the same column are significantly different among groups according to one-way analysis of variance coupled with the Duncan multiple comparison test ($p < 0.05$).

[1] Abbreviations are as in Table 1.

[2] The histological indices of hepatic vacuolization and necrosis were quantified based on the method of Knodell et al. (1981). The liver damage was graded 0–4 as follows: none (0); slight (1); mild (2); moderate (3); and remarkable (4).

[3] The hepatic fibrosis was graded 0–4 according to the method of Ruwart et al. (1989) as follows: none (0), normal liver; slight (1), increase in collagen without formation of septa; mild (2), formation of incomplete septa from portal tract to central vein (septa that do not interconnect with each other); moderate (3), complete but thin septa interconnecting with each other (incomplete cirrhosis); and remarkable (4), with thick septa (complete cirrhosis).

trichloromethyl radical (•CCl_3) and peroxy trichloromethyl radical (•$OOCCl_3$) in the liver. Free radical production stimulates lipid peroxidation, which results in liver injury and hepatic fibrosis, and a similar pathological process is observed in human cirrhosis.[29] *T. camphoratus* and its components have been reported to have antioxidant and anti-inflammatory activities in various studies.[30–32] Antroquinonol, a major component of solid-state-cultured *T. camphoratus* mycelia, showed a liver protective effect through Nrf-2 activation and upregulation of antioxidant gene expression. Eburicoic acid and dehydroeburicoic acid from mycelia have been demonstrated to protect the liver from CCl_4-induced hepatic damage by increasing the activities of antioxidant enzymes, including CAT, SOD and GPx, and decreasing iNOS and COX-2 expression.[20,33,34] Antcin K, antcin B and antcin C, the main triterpenoids found in the fruiting body of *T. camphoratus,* have been demonstrated to have hepatoprotective activity related to free radical scavenging, oxidative stress reduction and anti-inflammatory effects.[13,24,35,36] In addition, previous studies have found that these compounds are major components of cut-log cultivated fruiting body extracts and solid-state-culture mycelia of *T. camphoratus*.[34,35] Therefore, the hepatoprotective effect of LDAC might be contributed by those compounds. This study also showed that LDAC significantly increased the activities of the hepatic antioxidant enzymes GPx, GRd and CAT but did not affect the GSH and SOD levels in the liver of CCl_4-treated animals. Overall, we speculated that the protective effect of LDAC against CCl_4-induced hepatotoxicity is exerted by activating the Nrf-2 pathway or other antioxidant mechanisms and might also be related to suppressing inflammatory mediators. However, the mechanisms need to be clarified in the future.

In conclusion, this study demonstrated that LDAC effectively decreased serum ALT and AST levels, increased the antioxidant ability of the liver and ultimately improved hepatic inflammation, necrosis and fibrosis in CCl_4-treated rats. This report showed that the combination of the fruiting body extract and solid-state-cultured mycelia of *T. camphoratus* had potent liver protective effects against CCl_4-induced chronic liver injury.

Conflict of interest statement

The animal study was supported by a grant from Taiwan Leader Biotech Corp. (Taipei, Taiwan), which was not involved in study design; collection, analysis, or interpretation of data; or writing of the report. The authors take complete responsibility for the integrity of the data and the accuracy of the data analysis.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jtcme.2019.04.008.

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