

Hepatoprotective Effect of an *Antrodia cinnamomea* Product Via a Novel Process on Carbon Tetrachloride-Induced Hepatotoxicity in Rats

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Abstract: *Antrodia cinnamomea*, causing a brown heart rot of *Cinnamomum kanehirai* Hay. endemic to Taiwan, was reported to have several biological activities for treating liver diseases, inflammation, tumors, *et al.* It was believed that wild or wood-cultured *A.cinnamomea* on *C.kanehirai* Hay. was better than by other means. However, *C.kanehirai* Hay. was rare and expensive, that resulted in a higher price of wild or wood-cultured *A.cinnamomea*. Hence, a novel process was developed to spray solid-state-cultured *A.cinnamomea* extracts on wood-cultured *A.cinnamomea* powder to make a high quality and low price product. The purpose of the study was to evaluate its hepatoprotection against carbon tetrachloride-induced hepatotoxicity in rats. Results revealed that aspartate aminotransferase (AST) and alanine aminotransferase (ALT) of Sprague Dawley rats administered 20% carbon tetrachloride (CCl₄) twice a week, when the rats was also administered 413.4 or 1033.5 mg/kg body weight (BW) *A.cinnamomea* daily for 8 weeks, were significantly reduced in serum. Administration of 1033.5 mg/kg BW *A.cinnamomea* daily would not only reduce the rats' liver and spleen swelling, liver fibrosis, and level of hydroxyproline, but increase activities of superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, and glutathione. In conclusion, the *A.cinnamomea* product via the novel process at 1033.5 mg/kg BW had hepatoprotective effects on carbon tetrachloride-induced hepatotoxicity in rats.

Keywords: *Antrodia cinnamomea*, Hepatoprotection, Novel Process, Carbon Tetrachloride

1. Introduction

The chronic liver diseases and cirrhosis was one of the ten leading causes of death in Taiwan [1]. In the worldwide, liver cirrhosis was ranked 23rd according to a systematic analysis for the Global Burden of Disease Study 2010 [2]. The mortality from liver cirrhosis accounted for a growing and substantial disease burden worldwide each year, and the global liver cirrhosis deaths increased from 676 thousands in 1980 to over 1 million in 2010 [3]. The etiology of liver cirrhosis varied in different countries, but either alcoholic liver disease or viral hepatitis was the commonest cause [4-6].

Antrodia cinnamomea, a polypore fungus endemic to

Taiwan, was reported to have extensive biological activity and effectiveness in treating liver injury [7]. However, studies revealed *A.cinnamomea* products from different cultivation had different effects on inhibition of human hepatoma cell proliferation [8, 9], inhibition of inducible nitric oxide synthase expression [10], and antioxidant activities [9], and the solid-state-cultured *A.cinnamomea* had more effectiveness than the liquid-state-cultured one [8, 10]. Wild and wood-cultured *A.cinnamomea* products were rarely discussed because of expensive cost and rareness of *Cinnamomum kanehirai* Hayata, but it was believed that the wild and wood-cultured *A.cinnamomea* products were more

effectiveness in general.

Hence, a novel process was developed in the study to spray solid-state-cultured *A.cinnamomea* extracts on wood-cultured *A.cinnamomea* powder to make a high quality and low price product. Whether the *A.cinnamomea* product via the novel process had the same hepatoprotection was the purpose of the study.

2. Material and Methods

2.1. Microorganism and Culturing Condition

Antrodia cinnamomea was obtained from the Greenrays International Co., Ltd. (Chiayi, Taiwan). In solid-state-cultured condition, the mycelia were cultured using mushroom grow bags. Each bag contained corn cob, rice bran, wheat bran, and corn powder in the ratio 15.8:2.6:2.6:1 (w/w), adding 0.1% magnesium sulfate (MgSO_4) and 0.1% zinc sulfate (ZnSO_4), with water content of 53.94%. After sterilizing by autoclaving at 120°C for 2 hours, the bag was cooled down to 20°C and then inoculated with the mycelia. After cultivation at 25°C for 3 months, the grow bag was removed and reverse osmosis water was sprayed to induce primordia. The fruiting bodies were selected and harvested after 6 months, and the harvested mycelia with substrates were dried with air and were crushed. In wood-cultured condition, stout camphor (*Cinnamomum kanehirai* Hayata) trunks were inoculated with the mycelia and were placed in growth chambers equipped with a Supa Fine HF-096 cool mist humidifier (Shivn Feng Enterprise Co., Ltd., New Taipei City, Taiwan) running at 7–9 AM and 8–9 PM. The fruiting bodies were selected and harvested after 6 months. The solid-state-cultured and wood-cultured fruiting bodies both were dried and were powdered into 100–150 meshes.

2.2. Extraction, Concentration, and Spray Granulation

Six hundred kilograms powder of mycelia with substrates was extracted 3 different extraction solvents. First, the powder was extracted with 6,000 kg of 55% ethanol at 50°C for 18 hours. Second, the filter residue was extracted with 6,000 kg of reverse osmosis water at 90°C for 18 hours. Finally, the second filter residue was extracted with 6,000 kg of reverse osmosis water under high pressure at 120°C for 18 hours. These three filtrates were then concentrated separately under vacuum at 45–55°C [11]. The concentrates were used for spray-drying granulation with the powdered fruiting bodies as starter cores at 65–70°C in reversed order, and thereafter powdered into 100 meshes again.

2.3. Animal Experiment

Sixty male Sprague Dawley rats aged 7–8 weeks were purchased from BioLASCO Taiwan Co., Ltd. (Taipei, Taiwan) and tested in Super Laboratory Co., Ltd. (New Taipei City, Taiwan). Two rats in each cage were grown under a 12-h light/dark cycle at $22 \pm 3^\circ\text{C}$ and relative

humidity of $55 \pm 15\%$. The rats were given *ad libitum* access to food and reverse osmosis water. After one-week acclimation and quarantine period, they were randomly divided into six groups: (1) Control group, olive oil + reverse osmosis water; (2) RO group, 20% CCl_4 + reverse osmosis water; (3) Silymarin group, 20% CCl_4 + 20 mg/mL silymarin solution; (4) AC2067 group, 20% CCl_4 + 20.67 mg/mL *A.cinnamomea* solution; (5) AC4134 group, 20% CCl_4 + 41.34 mg/mL *A.cinnamomea* solution; (6) AC10335 group, 20% CCl_4 + 103.35 mg/mL *A.cinnamomea* solution. Two mL/kg body weight (BW) of olive oil (solvent) or 20% CCl_4 was administered via oral gavage every Tuesday and Friday for 8 weeks, and 10 mL/kg BW of reverse osmosis water (solvent), silymarin, or *A.cinnamomea* solution was administered every day for 8 weeks. The doses of *A.cinnamomea* were 206.7, 413.4, 1033.5 mg/kg BW, respectively. The body weight of every rat was measured once a week while the weekly food intake was recorded. At the eighth week, carbon dioxide (CO_2) was used for the humane euthanasia of the rats. Thereafter, the liver and spleen were excised and weighed.

2.4. Serum Biochemical Assays

Blood samples were collected from caudal vein at the first, third, and sixth weeks, and a final blood sample was taken from the heart of rat. The serum was obtained from blood by centrifuging at 4°C at 3,000 g for 15 min. Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were tested using the ADVIA® 1800 Clinical Chemistry System (Siemens Healthcare GmbH, Erlangen, Germany) at the 1st, 3rd, 6th, and 8th weeks. Total protein, albumin, globulin, triglyceride, and cholesterol were examined using the same equipment at the 8th week.

2.5. Biochemical Assays and Histology of Liver

The liver tissue was processed according to the method of Xia *et al.* [12], and enzyme activities of superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase (GRd) were determined using Randox kits (Randox Laboratories, Ltd., Crumlin, UK). Catalase activity was determined according to the method of Aebi [13].

The determination of glutathione (GSH) content was modified from the method of Hissin and Hilf [14]. After 0.5 g of liver tissue adding 5 mL of 1.15% potassium chloride (KCl) was homogenized, 1 mL of the solution mixed with 1 mL of 10% trichloroacetic acid (CCl_3COOH) was centrifuged at 4°C at 3,000 g for 15 min. To 0.01 mL of the supernatant, 0.18 mL of the phosphate-EDTA buffer and 0.01 mg/mL of σ -phthalaldehyde diluted in methanol (CH_3OH) were added. After thorough mixing, the glutathione (GSH) of the solution was determined at 420 nm with the activation at 350 nm.

The detection of hydroxyproline in the supernatant was modified from Neuman and Logan's method [15]. The liver tissue was sliced into strips and dried at 80°C in an oven for 20–22 hours, and then the dried tissue was hydrolyzed by

adding hydrochloric acid (HCl) at 100°C in the oven. Thereafter, the cooled hydrolyzed sample was centrifuged. Into each test-tube containing 1 mL of standard or the supernatant was pipetted in succession 1 mL each of 0.01 M copper sulfate (CuSO_4), 2.5 N sodium hydroxide (NaOH), and 6% hydrogen peroxide (H_2O_2). They were mixed and shaken by Vortex Mixer, and then placed in a water bath at 80°C for 5 minutes. After the tubes were chilled, 2 mL of 4-(dimethylamino) benzaldehyde and 4 mL of 3 N sulfuric acid (H_2SO_4) were pipetted into the tubes in succession. The tubes were placed in a water bath at 70°C for 3 min and cooled in iced water. The detection was done at 540 nm using a spectrophotometer.

The liver tissue was cut into pieces and fixed in 10% formalin. The specimens were dehydrated, cleaned, penetrated with paraffin, and embedded in paraffin. The specimens were sectioned at a thickness of 5 μm using a semi-motorized rotary microtome Leica RM 2145 (Leica Microsystems Nussloch GmbH, Nussloch, Germany) and stained with hematoxylin and eosin (H&E) or sirius red. The specimens were observed using Nikon Optiphot-2 microscope (Nikon Corporation, Tokyo, Japan). Vacuolation and necrosis were semiquantitatively scored. Grade 0 represented normal, and grades 1–4 had slight to marked involvement. Fibrosis was graded as follows: grade 0, normal liver; grade 1, increase of collagen without formation of septa; grade 2, incomplete septa from portal tract to central vein; grade 3, complete but thin septa interconnecting with each other and dividing the parenchyma into separate fragments; grade 4, as grade 3, with thick septa (complete

cirrhosis) [16].

2.6. Statistical Analysis

SAS Studio 3.4 (SAS Institute Inc., Cary, North Carolina, USA) was used for statistical analysis. The body weight, weekly food intake, ALT, and AST were analyzed and compared with the Control, RO, Silymarin, or AC2067 groups for repeated measures analysis using a mixed model. The one-way analysis of variance (ANOVA) followed by post-hoc Dunnett's test was performed for the total protein, albumin, globulin, triglyceride, cholesterol, and biochemical assays and semiquantitative assessments of liver. Statistical significance was defined as $p < 0.05$.

3. Results

3.1. Body Weight and Food Intake

Administration of 20% CCl_4 2 mL/kg BW twice a week for 8 weeks resulted in significantly decreased food intake and body weight in the rats (Figure 1). At the 8th week, the weekly food intake and body weight were 203.7 ± 3.6 g and 436.6 ± 23.3 g in the Control group and 157.7 ± 20.5 g and 364.8 ± 54.2 g in the RO group, respectively. Treatment with the dose of 1033.5 mg/kg BW *A.cinnamomea* every day slightly increased the weekly food intake and body weight to 171.3 ± 11.1 g and 384.7 ± 26.3 g at the 8th week although there was no significance among the groups administered 20% CCl_4 .

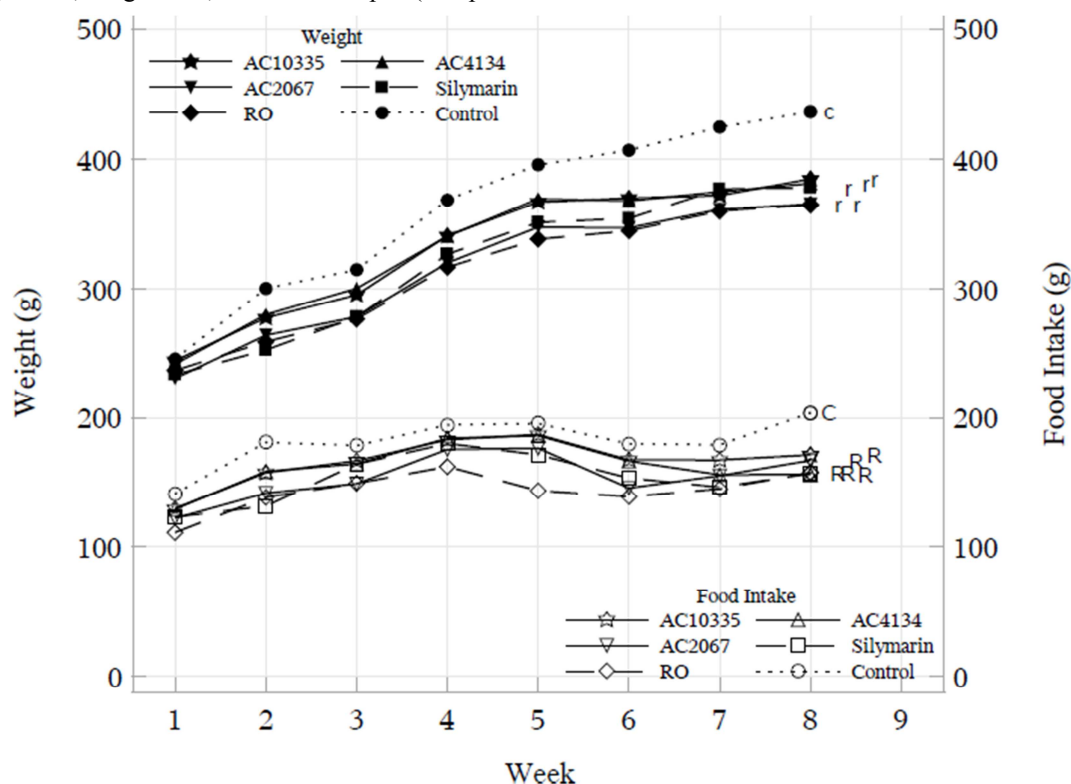


Figure 1. Body weight and weekly food intake of carbon tetrachloride-induced rats treated for 8 weeks.

3.2. Serum Biochemical Assays

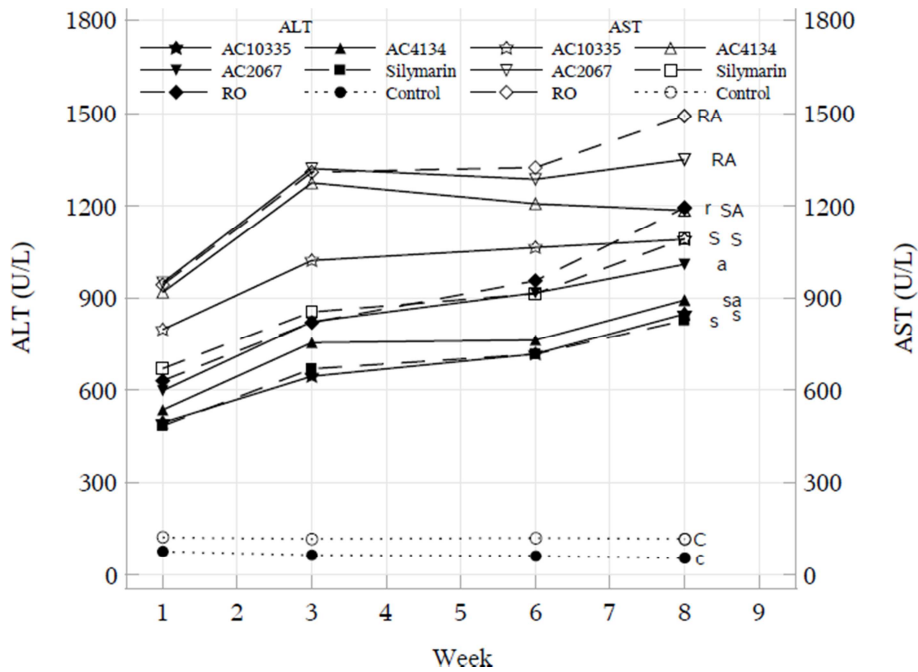


Figure 2. Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities of carbon tetrachloride-induced rats treated for 8 weeks.

Twenty percent of CCl₄ could induce hepatotoxicity, and ALT and AST were 1193.8 ± 257.3 U/L and 1491.3 ± 328.3 U/L in the RO group at the 8th week, respectively. Treatment with the dose of 413.4 mg/kg BW *A.cinnamomea* every day significantly reduced ALT and AST levels to 892.5 ± 120.6

U/L and 1185.3 ± 264.5 U/L, respectively (Figure 2). However, there were no significant difference for total protein, albumin, globulin, triglyceride, and total cholesterol in the serum among the groups administered 20% CCl₄ for 8 weeks (Figure 3).

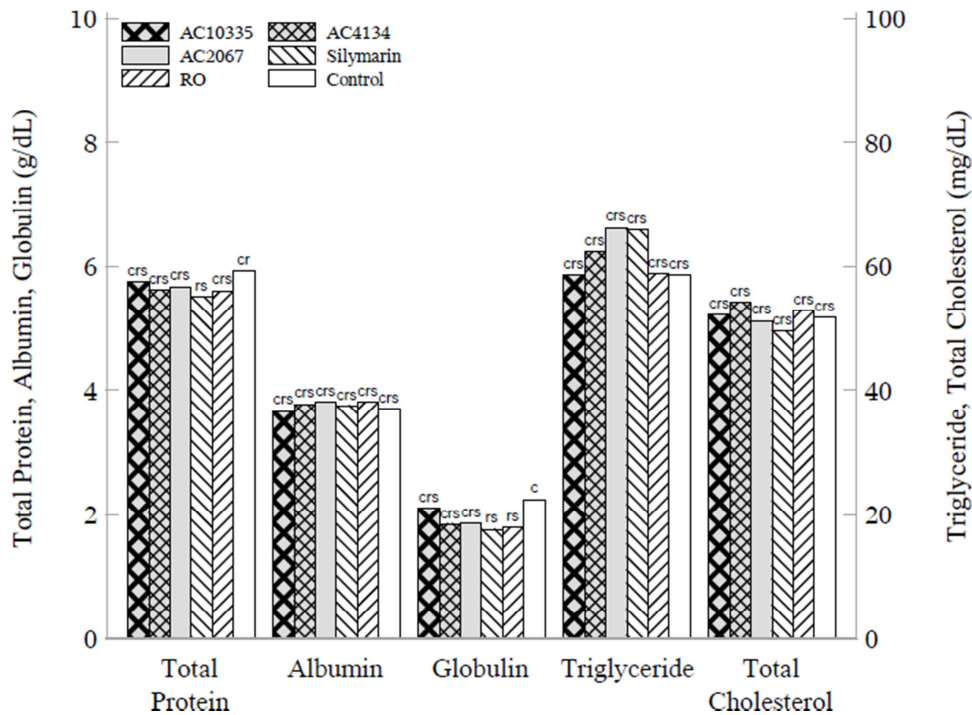


Figure 3. Total protein, albumin, globulin, triglyceride, and total cholesterol of carbon tetrachloride-induced rats after treated for 8 weeks.

3.3. Biochemical Assays and Histology of Liver

The liver and spleen were both enlarged after administration

of 20% CCl₄ for 8 weeks. The liver weight and spleen weight were 13.8 ± 2.5 g and 1.31 ± 0.26 g in the RO group, respectively, while they were 10.4 ± 1.0 g and 0.75 ± 0.14 g in

the Control group, respectively. The dose of 413.4 mg/kg BW *A.cinnamomea* reduced the spleen size and liver size, and so did

the dose of 1033.5 mg/kg BW *A.cinnamomea* (Figure 4).

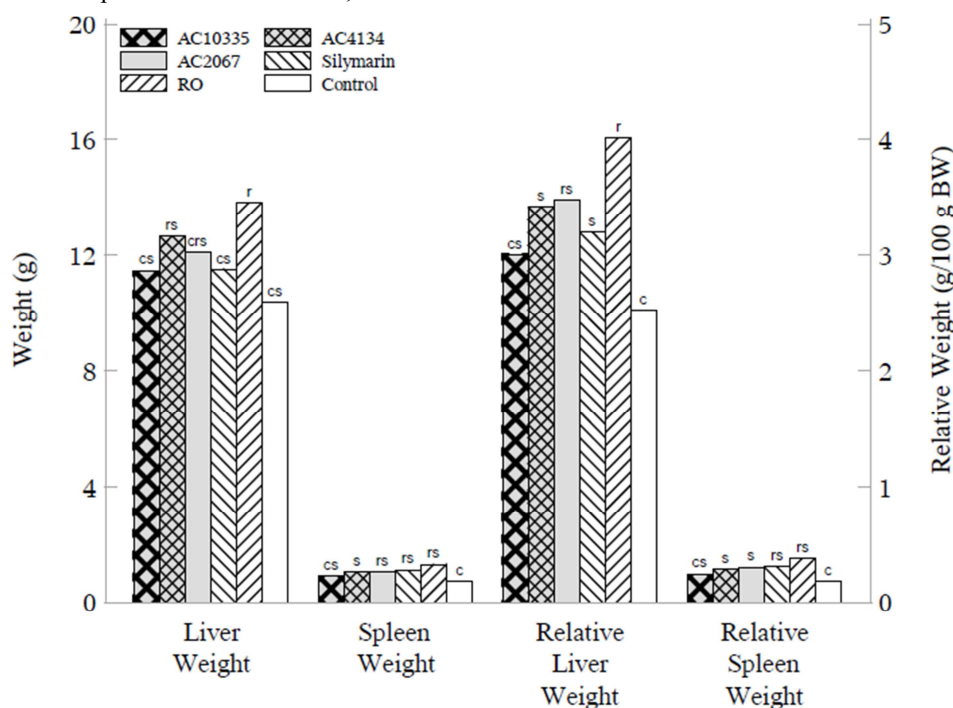


Figure 4. Liver weight, spleen weight, and their relative weights of carbon tetrachloride-induced rats after treated for 8 weeks.

Table 1. Activities of antioxidants in liver of carbon tetrachloride-induced rats after treated for 8 weeks.

Antioxidants	Control	RO	Silymarin	AC2067	AC4134	AC10335
SOD (U/mg protein)	212.6±7.8c	140.9±26.2r	168.6±15.6s	143.1±17.5r	159.5±24.6rs	177.7±19.9s
Catalase (nmol/min/mg protein)	1542.1±132.4cs	896.5±257.7rs	1225.3±269.1cs	992.2±278.7rs	1051.1±387.1rs	1297.4±348.0cs
GPx (mU/mg protein)	1610.7±165.8c	893.2±263.6rs	1155.7±243.4rs	986.7±267.5rs	1082.7±401.6rs	1234.2±348.6s
GRd (mU/mg protein)	17.6±1.9cs	12.9±2.6rs	15.7±3.7cs	12.2±2.3r	14.8±3.5cs	16.4±1.8cs
GSH (μmol/mg tissue)	16.2±1.9c	5.1±1.8r	8.4±2.1s	5.1±3.0r	7.7±2.4rs	8.6±2.4s

The same letter indicated no significant difference ($p > 0.05$) compared with the Control (c), RO (r), or Silymarin (s) groups for Dunnett's test, respectively.

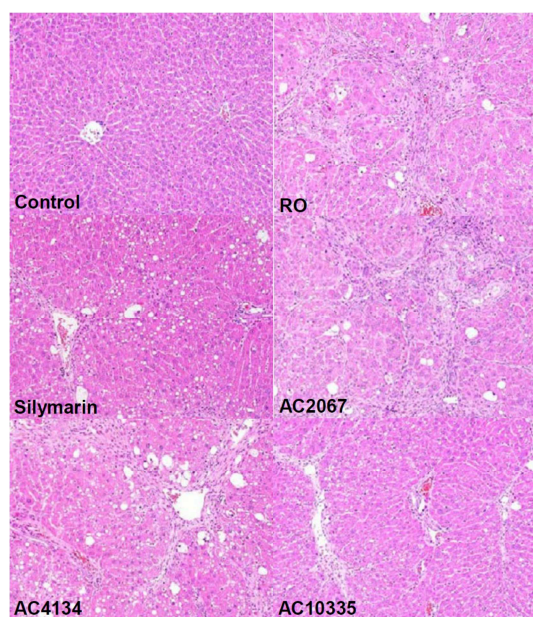


Figure 5. Liver histology observed by hematoxylin and eosin (H&E) staining at 200x magnification in carbon tetrachloride-induced rats after treated for 8 weeks.

After administration of 20% CCl_4 for 8 weeks, activities of SOD, catalase, GPx, GRd, and GSH in the liver were all significantly decreased. The dose of 1033.5 mg/kg BW *A.cinnamomea* could increase the activities of SOD (177.7 ± 19.9 U/mg protein), GPx (1234.2 ± 348.6 mU/mg protein), and GSH (8.6 ± 2.4 μmol/mg tissue), and could recover the activities of catalase (1297.4 ± 348.0 nmol/min/mg protein) and GRd (16.4 ± 1.8 mU/mg protein) (Table 1).

The liver histology in the RO group showed slight to severe vacuolation, enlarged liver cell, necrosis, liver cell regeneration, bile duct recanalization (Figure 5). The average scores of vacuolation and necrosis were 2.1 ± 1.0 and 2.0 ± 0.5 , respectively (Table 2). Slight and severe liver fibrosis was revealed in the RO group, and thick interconnecting septa could be observed (Figure 6). The average score of fibrosis was 3.6 ± 0.7 (Table 2). Treatment with 20 mg/mL silymarin and 206.7–1033.5 mg/kg BW *A.cinnamomea* could not reduce liver vacuolation and necrosis after administration of 20% CCl_4 for 8 weeks, but silymarin and 1033.5 mg/kg BW *A.cinnamomea* could reduce liver fibrosis, 2.4 ± 1.1 and 2.3 ± 0.9 , respectively. Hydroxyproline in the liver was also significantly increased (79.1 ± 15.9 μg/100g tissue) after

administration of 20% CCl₄ for 8 weeks, and treatment with 20 mg/mL silymarin and 1033.5 mg/kg BW *A.cinnamomea* also significantly reduced the hydroxyproline in the liver, 52.8

± 12.5 $\mu\text{g}/100\text{g}$ tissue and 56.2 ± 9.3 $\mu\text{g}/100\text{g}$ tissue, respectively (Table 2).

Table 2. Liver assessment of carbon tetrachloride-induced rats after treated for 8 weeks.

Liver assessment	Control	RO	Silymarin	AC2067	AC4134	AC10335
Vacuolation	0.0 \pm 0.0c	2.1 \pm 1.0rs	2.4 \pm 0.7rs	1.6 \pm 0.5r	1.7 \pm 0.7rs	2.2 \pm 0.8rs
Necrosis	0.0 \pm 0.0c	2.0 \pm 0.5rs	1.6 \pm 0.7rs	1.8 \pm 0.6rs	1.6 \pm 0.5rs	1.7 \pm 0.5rs
Fibrosis	0.0 \pm 0.0c	3.6 \pm 0.7r	2.4 \pm 1.1s	3.4 \pm 0.8r	3.1 \pm 0.7rs	2.3 \pm 0.9s
Hydroxyproline ($\mu\text{g}/100\text{g}$ tissue)	19.6 \pm 5.8c	79.1 \pm 15.9r	52.8 \pm 12.5s	72.7 \pm 18.4r	64.7 \pm 16.2rs	56.2 \pm 9.3s

The same letter indicated no significant difference ($p > 0.05$) compared with the Control (c), RO (r), or Silymarin (s) groups for Dunnett's test, respectively.

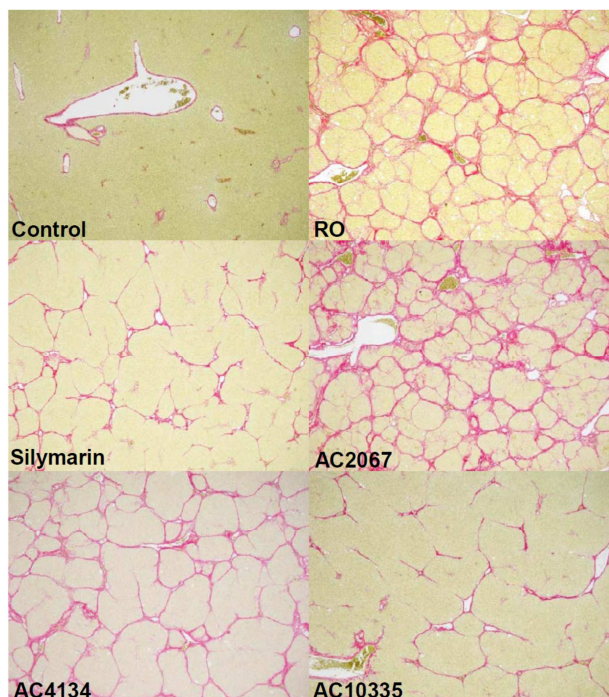


Figure 6. Liver fibrosis observed by sirius red staining at 40x magnification in carbon tetrachloride-induced rats after treated for 8 weeks.

4. Discussion

The activities of ALT and AST were relatively high in a normal adult liver tissue homogenate, 44000 and 142000 U/g wet tissue homogenate, respectively, while their activities in serum were only 16 and 20 U/g wet tissue homogenate, respectively [17]. In Sprague Dawley rats, reference values of ALT and AST were 44 ± 1 and 106 ± 1 U/L, respectively [18]. When liver got damaged, it would lead to releases of ALT and AST from damaged liver cells into bloodstreams and result in impressively elevated levels of ALT and AST in serum [17]. The carbon tetrachloride-induced elevated levels of ALT and AST were significantly reduced when administration of the *A.cinnamomea* product at 413.4 and 1033.5 mg/kg BW for 8 weeks, while those levels were significantly reduced when administration of wheat-based solid-state fermented *A.cinnamomea* at doses greater than or equal to 540 mg/kg BW [19], or administration of *A.cinnamomea* at 3150 mg/kg BW [20].

De Ritis, *et al.* indicated that abnormally elevated levels of ALT and AST combined with AST/ALT ratio could be a

sensitive index of an activity of a liver disease [21]. Lower AST/ALT ratios were found in viral hepatitis [21–24], drug-induced liver injury [25, 26], and metabolic syndrome [27], but higher AST/ALT ratios were found in cholestasis [21], nonalcoholic fatty liver disorder [23], liver cirrhosis [22, 24, 28], and even alcoholic liver disorder [23, 24, 29]. For carbon tetrachloride-induced hepatotoxicity, the AST/ALT ratio decreased (1.09–1.16) while compared with control group (1.79–1.88) [19, 20], and so did it in the study (1.25). Administration of *A.cinnamomea* or silymarin would not markedly affect the AST/ALT ratio (0.91–1.32) [19, 20] because the levels of ALT and AST recovered in the same direction. However, the level of ALT was sustainably elevated with the increase of treating time (Figure 2). It might suggest that the liver continuously got hurt.

Sustained liver injury would lead to hepatic stellate cells activating and transdifferentiating from quiescent cells into proliferative, fibrogenic, and contractile myofibroblasts, that resulted in accumulation of abundant fibril-forming extracellular matrix [30] and an elevated level of hydroxyproline [31]. With proper treatment, liver fibrosis would be reversible [32]. The study showed that the level of hydroxyproline and the score of fibrosis significantly decreased to 56.2 ± 9.3 $\mu\text{g}/100\text{g}$ tissue and 2.3 ± 0.9 when administration of the *A.cinnamomea* product at 1033.5 mg/kg BW for 8 weeks, respectively, while the level of hydroxyproline and the score of fibrosis were 79.1 ± 15.9 $\mu\text{g}/100\text{g}$ tissue and 3.6 ± 0.7 at control group, respectively (Table 2). Significantly decreased level of hydroxyproline also existed in previous studies when administration of wheat-based solid-state fermented *A.cinnamomea* at dose of 1080 mg/kg BW [19] and administration of *A.cinnamomea* at 1400 mg/kg BW [20]. It suggested that the improvement of liver fibrosis just happened at administration of *A.cinnamomea* at a dose of higher than 1000 mg/kg BW.

On top of that, administration of *A.cinnamomea* could increase activities of superoxide dismutase, catalase, glutathione peroxidase and reduce malondialdehyde levels, the end-product of lipid peroxidation [33, 34], and even the reduced activities of superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, and glutathione were recovered when administration of the *A.cinnamomea* product at 1033.5 mg/kg BW for 8 weeks (Table 1). However, activities of superoxide dismutase, catalase, glutathione peroxidase, and glutathione were not recovered in rats with administration of wheat-based

solid-state fermented *A.cinnamomea* even at dose of 1080 mg/kg BW [19]. Activities of superoxide dismutase and glutathione peroxidase were also not recovered in rats with administration of *A.cinnamomea* even at 3150 mg/kg BW [20]. It was confirmed that carbon tetrachloride would induce oxidative stress, leading to membrane steatosis and lipid peroxidation [35], and could result in marked reduction in the activities of glutathione peroxidase, glutathione reductase, glutathione, superoxide dismutase, catalase either in liver or in brain [36]. Conversely, the reduced activities of the antioxidants lost sufficient capacity of neutralizing free radicals. It might be the reason why wheat-based solid-state fermented *A.cinnamomea* at a dose of lower than 500 mg/kg BW could not sufficiently reduce the elevated levels of ALT and AST [19, 20].

With the novel process, the cost of the *A.cinnamomea* product was lowered, but it provided even more effective hepatoprotection than those by other means to people with chronic liver diseases. *A.cinnamomea* as a traditional Chinese medicine or a healthy food would be more easily promoted. However, for health promotion, integration of businesses, governments, social organizations, and medical institutions would be needed [37].

5. Conclusion

The results indicated that the novel process was effective and successful. In the past, people with liver problems wanted more effective *A.cinnamomea* products but couldn't afford them. The novel process to combine *A.cinnamomea* mycelia extracts with fruiting bodies would make high quality and low price *A.cinnamomea* products possible. However, the study only focused on the hepatoprotection. For other medical effects, whether the *A.cinnamomea* product via the novel process would be also effective was still unknown, and more studies should be conducted in the future.

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