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ANTRODIA CINNAMOMEA EXTRACT ATTENUATES CARBON TETRACHLORIDE-INDUCED CHRONIC LIVER FIBROSIS IN SPRAGUE-DAWLEY RATS

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Abstract

Background: *Antrodia cinnamomea* (AC) mycelia have been traditionally used by majority of the indigenous populace in Taiwan for symptoms including treating alcohol intoxication. Other beneficial effects have been studied at some point. The present study evaluated the hepato-protection effects in Sprague-Dawley rats.

Methods: The model used carbon tetrachloride (CCl_4) to induce a chronic liver injury in male rats. Animals were treated with silymarin 200 mg/kg and AC mycelia at doses of 206, 619 and 1,032 mg/kg. The effects of AC on hepatic enzyme markers alanine and aspartate aminotransferase (ALT and AST) and other biochemical parameters were measured in the CCl_4-induced rats.

Results: AC demonstrated a hepato-protective effect by decreasing ALT and AST levels and increasing albumin levels in CCl_4 treated rats. The effects of AC on the activity of antioxidant enzymes were evaluated. AC administration restored the activities of catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GrD). The degree of liver fibrosis was significantly reduced by AC administration in CCl_4-treated rats.

Conclusion: These results suggest that AC could protect the hepatocytes from CCl_4-induced liver injury likely via an antioxidant mechanism.

Keywords: *Antrodia cinnamomea*, hepatoprotective, carbon tetrachloride

Abbreviations: AC, *Antrodia cinnamomea*; BW, Body Weight; Sprague-Dawley, SD

Introduction

Liver diseases have been widely-seen as “silent killers,” and one of the most prevalent types is liver fibrosis. This wound-healing response to liver injury triggers remodeling of the extracellular matrix (Bataller and Brenner, 2005). The primary cells that produce this matrix, such as collagen, are the hepatic stellate cells, normally known as the body’s store for vitamin A (Geerts, 2001). Upon receiving activation signals, they become collagen-secreting cells and participate in tissue remodeling. This dynamic process eventually results in liver cirrhosis, in which the architecture organization is distorted and liver function is impaired due to disrupted blood flow (Bataller and Brenner, 2005, Friedman, 2003). The common causes of liver cirrhosis include viral hepatitis, alcoholic liver disease and non-alcoholic fatty liver disease.

Currently there are limited number of medications that offer hepato-protection, and a prolonged use of these medications are associated with side effects, thus leading to more attention because focused on alternative therapies over the past few years (Krishnappa et al,. 2014). For example, silymarin isolated from milk thistle (*Silybum marianum*) is now a hepato-protective drug and acts as a free radical scavenger (Flora et al., 1998). *Antrodia cinnamomea* (AC) is a Taiwanese medicinal fungus widely used by the indigenous population for treating alcohol intoxication, as

well as for headaches, skin allergies and gastrointestinal conditions. AC is now a popular nutraceutical in Asian countries. More than seventy bioactive compounds have been extracted from AC, and these include, but are not limited to. polysaccharides, triterpenoids, benzenoids, benzoquinones, succinic and maleic acids in AC (Geethangili and Tzeng, 2011). The wild fruiting bodies can take up to one year to grow, and *C. kanehirai* has been listed as a grade 1 conservation wood, which significantly limits the supply of wild AC fruiting bodies. These factors have pushed the AC market price to USD$15,000–25,000 per kg (Lu et al., 2013). Mycelia are alternatives to meet the increasing demand for AC fruiting bodies, due to the significantly shorter cultivation time. Moreover, illegal logging of *C. kanehirai* can be avoided, hence promoting *C. kanehirai* conservation.

CCl_4 is widely used for inducing liver injury in rats. Hepatotoxicity is generated by free radicals produced via the reductive dehalogenation, which is catalyzed by P-450 cytochrome. The free radicals, including trichloromethyl species, initiate lipid peroxidation, which eventually results in inhibition of enzymatic activities and subsequent liver damage (McLean et al., 1969, Rao and Recknagel, 1968). This study thus examined the potential of AC in hepato-protection against chronic fibrosis induced by CCl_4.

Materials and Methods

Test materials and preparation

Strains of AC were provided by the Research and Development Center (New Bellus Enterprises Co., Ltd, Tainan, Taiwan). Extracts were prepared as previously described (Cheng et al., 2014). For high performance liquid chromatography (HPLC) analysis, dried AC was dissolved in ethanol (100 mg/ml) and extracted for 60 minutes in a water bath at 28℃. The ethanol extract was passed through a 45 μm filter. The filtrate was then separated by a Hitachi HPLC equipped with a UV detector. The Mightysil RP-18 GP column (4.6mm×250mm, 5 μm) was used for separation. The gradient elution consisted of 0.03% phosphoric acid (A) and methanol (B). The gradient profile was as follows: 0-5 min, A:B = 25:75; 5-15 min, A:B = 25:75; 15-28 min, A:B = 0:100; 28-28.1, A:B = 0:100; 28-28.1 min, A:B = 25:75; 28-35 min, A:B = 25:75 with a flow rate of 1.0 ml/min.

Animals and study design

Sprague-Dawley (SD) rats were purchased from BIOLasco, Taiwan Co., Ltd. (Taipei, Taiwan) with a weight range of 200-260g. All animals were housed in plastic cages within a room temperature of about 22 ±3°C with 12 hour light/dark cycles. The animals were fed with ALtromin Diet 1324-20A (Germany) and sterilized reverse osmosis water *ad libitum*. The study was approved by the Institutional Animal Care and Use Committee (IACUC).

Animals were randomly assigned into six groups (n=10 per group). Liver fibrosis was induced by CCl_4 injections. All groups except the blank control group received an intra-peritoneal injection of 0.2 ml/100g kg CCl_4 in an olive oil vehicle mixture, twice a week for eight consecutive weeks, whereas the blank control was administered olive oil only. The blank and the negative controls were given 0.5% carboxymethylcellulose sodium salt (10 ml/kg) *via* gavage for eight weeks. The positive controls were gavaged with silymarin (200 mg/kg). All the other rats were administered 206, 619 and 1,032 mg/kg of AC *via* gavage for eight weeks. The rats were anaesthetised with diethyl ester and the blood was immediately collected from the tail vein. At the end of week 8, the rats were anaesthetised and sacrificed via cervical dislocation and the blood was drawn from the tail vein. The liver and spleen were quickly removed from the rat, washed with ice cold saline, dried and weighed. The largest lobe of the tissue was divided into two specimens. One specimen was preserved in 10% neural buffered formalin for histo-pathological analysis. The other was dried at 100℃ for hydroxyproline quantitation.

Biochemical analysis

Blood was sampled after anesthesia from the tail vein. The blood was centrifuged at 4,700 rpm for 15 minutes and plasma was collected. A COBAS MIRA Chemistry Analyzer was used to examine the following parameters. During weeks 1, 3 and 4 the aminotransferase (AST) and alanine aminotransferase (ALT) levels were measured, while at the end of week 8 the AST, ALT, albumin, total protein, globulin, cholesterol and triglycerides levels were determined.

Levels of antioxidant enzymes, glutathione and hydroxyproline in hepatic tissue

The liver tissue was processed as described by Xia et al. (1994). The activities of SOD, GPx and GRd superoxide dismutase enzyme were measured with a commercially available Ransod, Ransel, and GR kits (Randox Laboratories Ltd., Crumlin, Antrin, UK), respectively. The catalase activity was estimated as described in Aebi (1984). 0.5 g of liver samples was homogenized in 5 ml of 1.15% KCl solution. 1 ml of homogenate was mixed with 1 ml of trichloroacetic acid 10% and centrifuged at 3,000 g for 15 minutes. 0.18 ml phosphate-EDTA, freshly prepared 0.01 ml σ-phthaldehyde was thoroughly mixed. The measurements were taken with a spectrophotometer (Epoch™, BioTek, Winooski, VT, USA) with an excitation wavelength of 350 nm and emission wavelength of 420 nm, and shown in mole/g liver tissue (Neuman and Logan, 1950). Briefly, the dried liver specimen after hydrolysis was oxidized by H_2O_2 and stained with p-dimethylaminobenzoaldehyde, and absorbance was then measured at 540 nm. The amount of hydroxyproline was expressed as µg/g tissue.

Histopathological evaluation

Fixed liver tissue was embedded in paraffin, cut into 5 µm thick sections, stained with hematoxylin, eosin stain and Sirius red stain. The degree of liver injury was assessed according to the criteria described in Knodell et al. (1981), whereas the semi-quantitative analysis of liver fibrosis was assessed as described in Ruwart et al. (1989). The sections were evaluated blindly by a veterinary pathologist (LCW).

Statistical analysis

Group means and standard deviations were calculated for the collected data. The data were analyzed by one-way analysis of variance (one-way ANOVA), followed by *post-hoc* Duncan's multiple range testing. A $p<0.05$ value was considered as the minimum level for significance.

Results

Quality control and phytochemical marker of AC

The results of phytochemical profiling by HPLC are shown in Fig. 1. The major constituents are 4-Acetylantroquinonol B, ergosterol, 4-Acetylantroquinonol J and antrodin A. 4-acetylanthroquinonol B served as an index compound for internal control of batch-to-batch differences.

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AC treatment mitigates CCl_4-induced increases in serum AST and ALT levels

Serum ALT and AST levels in the negative control group (CCl_4 control) were elevated, indicative of liver injury at weeks 1, 3, 6 and 8. Treatment of animals with silymarin in the positive control group significantly reduced the levels of ALT and AST at weeks 1 and 6 when compared with those of the negative control group. Treatment with doses 206, 619 and 1, 032 mg/kg of AC markedly reduced the AST levels following CCl_4 treatment at weeks 1, 3, 6 and 8 (Table 1). Treatment with high doses of AC (619 and 1,032 mg/kg) resulted in a significant decrease in ALT levels at 1, 3, 6 and 8 weeks, whereas reduced ALT levels were only observed in weeks 1, 3 and 6 with a low dose of AC (206 mg/kg) (Table 2).

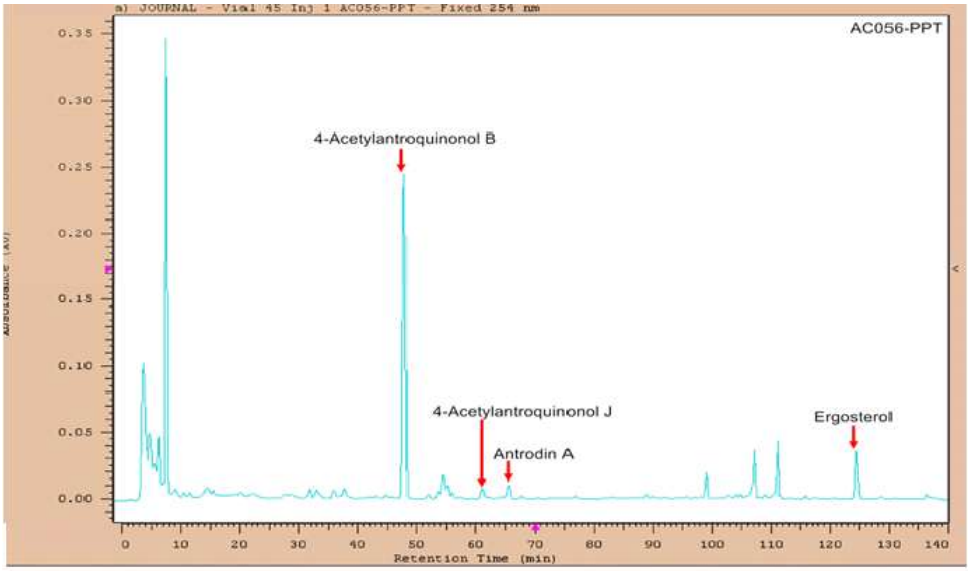

Figure 1: HPLC chromatogram of the compounds in the *Antrodia cinnamomea* mycelia. 4-acetyl antroquinonol B served as an index compound for the quality control purpose.

Effects of AC administration on biochemical measurements

CCl_4 treatment resulted in reduced albumin levels and total cholesterol levels when compared to the blank control. Silymarin administration had no effect on these two parameters. High doses of AC (619 and 1,032 mg/kg) resulted in significantly restored albumin levels. In contrast, AC administration did not restore CCl_4-induced reduction of cholesterol levels (Table 3).

Effects of AC administration of the levels of SOD, catalase, GPx, GrD, GSH and hydroxyproline in hepatic tissue

In the fibrotic liver induced by CCl_4, the levels of the antioxidant enzymes SOD, catalase, GPx, GrD and GSH were significantly reduced when compared to the blank control. Treatment of silymarin (positive control) in the CCl_4 treated rats significantly increased levels of SOD, catalase and GSH. Following treatment with AC (206, 619 and 1,032 mg/kg) catalase, GPx and GrD levels increased significantly. Elevated levels of hydroxyproline are indicative of liver fibrosis, and were observed in the rats treated with CCl_4, while silymarin significantly reduced the levels of hydroxyproline when compared to the blank control. AC administration decreased hydroxyproline levels in a dose-dependent manner. Treatment with high doses of AC (619 and 1,032 mg/kg) had a better effect to that of silymarin on hydroxyproline levels ($p<0.05$) (Table 4).

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Table 1: Effects of AC on AST in SD rats with CCl_4-induced liver injury.

	AST（U/L）					
	Control	CCl_4 + H_20	CCl_4 + Silymarin (200 mg/kg)	CCl_4 + AC (206 mg/kg)	CCl_4 + AC (619 mg/kg)	CCl_4 + AC (1,032 mg/kg)
Week 1	67.9±9.4^{a}	217.1±80.0^{c}	130.5±72.7^{b}	121.4±18.8^{b}	143.4 ±57.1^{b}	118.6 ±30.2^{b}
Week 3	68.8±10.0^{a}	505.2±250.1^{d}	472.4±295.8^{cd}	334.3±238.8^{bcd}	305.4±154.2^{bc}	227.6±110.8^{ab}
Week 6	68.7±10.3^{a}	1606.2±1026.3^{c}	744.4±544.2^{b}	847.2±266.6^{b}	669.0±354.0^{b}	959.4±595.5^{b}
Week 8	72.0 ±11.5^{a}	2075.5± 345.0^{c}	1439.9±994.9^{bc}	1047.9±613.1^{b}	1042.3±592.2^{b}	1106.7±607.9^{b}

Values are expressed as the means ± standard errors, n=10. Values with the same letter are not statistically different (p>0.05).

Table 2: Effects of AC on ALT in SD rats with CCl_4-induced liver injury.

	ALT（U/L）					
	Control	CCl_4 + H_20	CCl_4 + Silymarin (200 mg/kg)	CCl_4 + AC (206 mg/kg)	CCl_4 + AC (619 mg/kg)	CCl_4 + AC (1,032 mg/kg)
Week 1	40.2 ±6.3^{a}	111.9 ±26.1^{c}	78.9 ±33.9^{b}	81.0 ±19.3^{b}	78.6 ± 26.4^{b}	72.8 ±15.0^{b}
Week 3	42.1 ±8.2^{a}	419.6 ±150.2^{d}	335.5 ±168.4^{cd}	234.2 ±125.9^{bc}	217.0 ± 122.8^{b}	200.6 ±94.3^{b}
Week 6	40.1 ±5.9^{a}	1000.7 ± 397.5^{c}	639.2 ±444.5^{b}	612.1 ±248.7^{b}	523.6 ± 232.9^{b}	614.8 ± 425.6^{b}
Week 8	43.3 ±6.9^{a}	1600.9 ± 546.1^{c}	1150.8 ± 800.7^{bc}	1349.6 ± 599.6^{bc}	983.5 ± 596.8^{b}	1010.9 ± 562.9^{b}

Values are expressed as the means ± standard errors, n=10. Values with the same letter are not statistically different (p>0.05).

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Table 3: Effects of AC on the biochemical measurements in SD rats with CCl_4-induced liver injury.

	Control	CCl_4 + H_20	CCl_4 + Silymarin (200 mg/kg)	CCl_4 + AC (206 mg/kg)	CCl_4 + AC (619 mg/kg)	CCl_4 + AC (1,032 mg/kg)
Total protein (g/dL)	6.26 ±0.22[a]	6.14 ±0.26[a]	6.15 ±0.21[a]	6.05 ±0.47[a]	6.33 ±0.31[a]	6.27 ±0.31[a]
Albumin (g/dL)	3.87 ±0.16[b]	3.44 ±0.44[a]	3.64 ±0.32[ab]	3.59 ±0.47[ab]	3.83 ±0.22[b]	3.88 ±0.22[b]
Globulin (g/dL)	2.38 ±0.17[a]	2.70 ±0.56[a]	2.51 ±0.27[a]	2.47 ±0.31[a]	2.50 ±0.29[a]	2.39 ±0.26[a]
Triglycerides (mg/dL)	33.2 ±13.8[a]	23.8 ±13.2[a]	40.6 ±34.2[a]	19.6 ±11.0[a]	19.8 ±11.9[a]	23.3 ±11.5[a]
Total cholesterol (mg/dL)	66.8 ±15.4[b]	53.1 ±10.6[a]	47.9 ±10.9[a]	53.5 ±12.5[a]	50.8 ±7.0[a]	52.5 ±12.0[a]

Values are expressed as the means ± standard errors, n=10. Values with the same letter are not statistically different (p>0.05).

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Table 4: Effects of AC on antioxidant enzyme, glutathione and hydroxyproline levels in SD rats with CCl_4-induced liver injury.

	Control	CCl_4 + H_20	CCl_4 + Silymarin (200 mg/kg)	CCl_4 + AC (206 mg/kg)	CCl_4 + AC (619 mg/kg)	CCl_4 + AC (1,032 mg/kg)
SOD (U/mg protein)	6.1 ±0.7^{c}	3.9 ±1.0^{a}	5.1 ±1.5^{b}	4.2 ±1.0^{a}	4.0 ±0.8^{a}	3.9 ±0.9^{a}
Catalase (U/mg protein)	13.9 ±4.0^{c}	7.0 ±2.5^{a}	12.1 ±3.3^{bc}	9.6 ±1.7^{b}	9.8 ±1.6^{b}	9.8 ±2.0^{b}
GPx (U/mg protein)	0.90 ±0.13^{c}	0.57 ±0.12^{a}	0.67 ±0.14^{ab}	0.69 ±0.10^{b}	0.71 ±0.12^{b}	0.75 ±0.10^{b}
GRd (mU/mg protein)	43.7 ±8.1^{c}	32.3 ±8.6^{a}	34.7 ±7.5^{ab}	42.4 ±7.8^{bc}	41.0 ±8.1^{bc}	42.3 ±9.1^{bc}
GSH (µmol/g tissue)	7.5 ±3.9^{c}	4.2 ±0.4^{a}	6.7 ±2.4^{bc}	4.9 ±1.8^{ab}	5.8 ±1.8^{abc}	5.7 ±0.4^{abc}
Hydroxyproline (µg/ g tissue)	98.3 ±18.5^{a}	236.3 ±113.7^{c}	199.6 ±94.1^{bc}	201.0 ±23.0^{bc}	170.8 ±28.8^{b}	159.8 ±24.9^{b}

Values are expressed as the means ± standard errors, n=10. Values with the same letter are not statistically different (p>0.05).

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Table 5: Effects of AC on the pathological score of CCl_4-induced liver injury in SD rats.

	Vacuolization						Necrosis						Fibrosis					
	0	1	2	3	4	average	0	1	2	3	4	average	0	1	2	3	4	average
Control	10	0	0	0	0	0.0 ± 0.0^{a}	10	0	0	0	0	0.0 ± 0.0^{a}	10	0	0	0	0	0.0 ± 0.0^{a}
$CCl_4 + H_20$	0	0	6	2	2	2.6 ± 0.8^{b}	0	2	5	3	0	2.1 ± 0.7^{b}	0	2	1	0	7	3.2 ± 1.3^{d}
CCl_4 + Silymarin (200 mg/kg)	0	1	6	3	0	2.2 ± 0.6^{b}	0	1	8	1	0	$2.0 \pm 0.a5^{b}$	0	1	3	3	3	2.8 ± 1.0^{cd}
CCl_4 + AC (206 mg/kg)	0	2	2	6	0	2.4 ± 0.8^{b}	0	1	5	4	0	2.3 ± 0.7^{b}	0	1	6	3	0	2.2 ± 0.6^{bc}
CCl_4 + AC (619 mg/kg)	0	0	4	6	0	2.6 ± 0.5^{b}	0	1	8	1	0	1.8 ± 0.4^{b}	0	2	6	1	1	2.1 ± 0.9^{bc}
CCl_4 + AC (1,032 mg/kg)	0	0	6	4	0	2.4 ± 0.5^{b}	0	0	7	3	0	2.3 ± 0.5^{b}	0	4	4	1	1	1.9 ± 1.0^{b}

Values with the same letter are not statistically different ($p>0.05$).

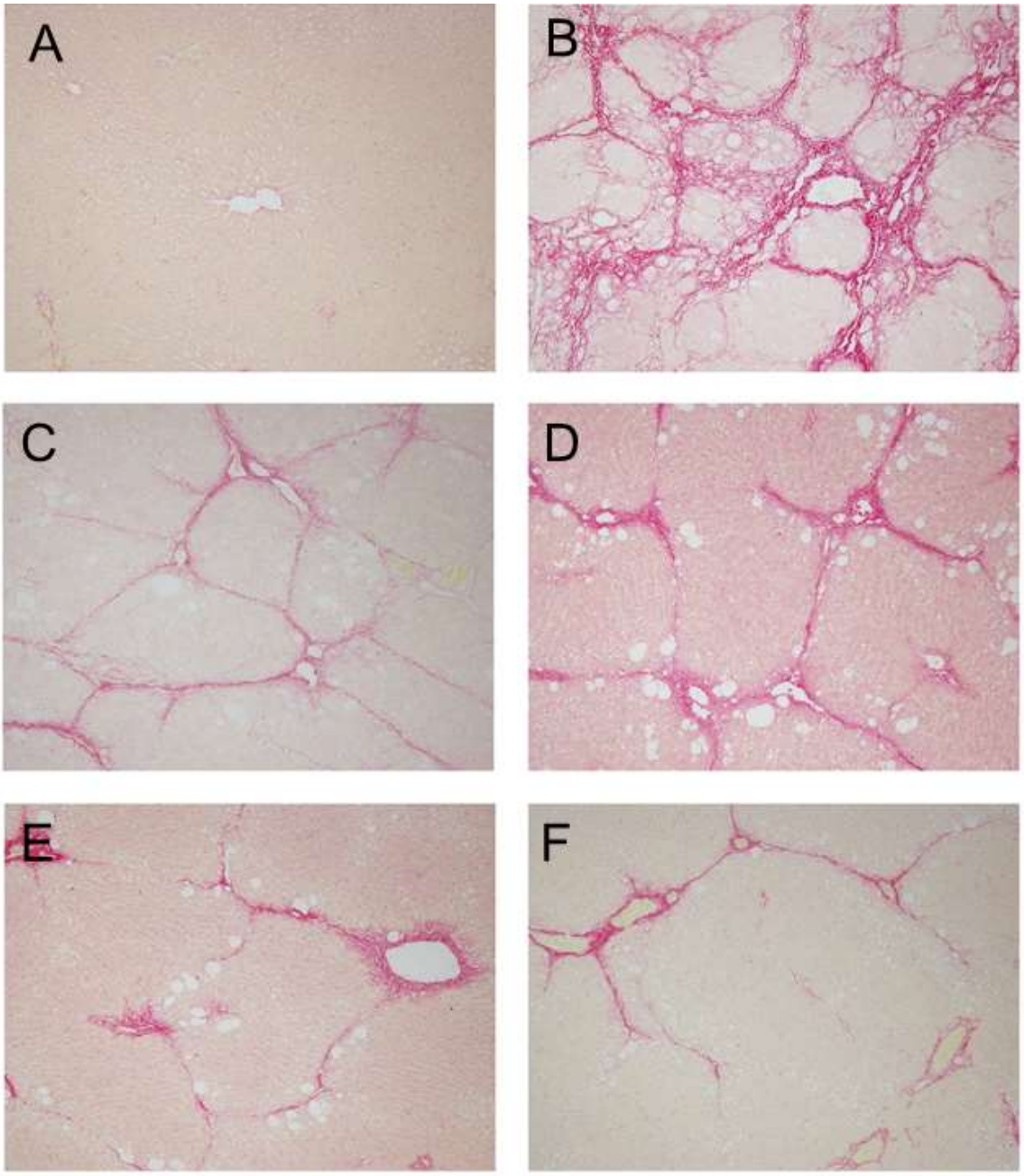

Figure 2: Representative images of liver tissue sections stained with Sirius Red for collagen (A) No fibrosis was evident in the livers of the control group. (B) Rats develop pronounced fibrosis following CCl_4 treatment. Liver tissue from rats treated with (C) 200 mg/kg of silymarin, (D) 206 (E) 619 and (F) 1,032 mg/kg of AC had reduced fibrosis.

AC administration improved liver fibrotic histology

Vacuolization, necrosis and fibrosis were observed in H&E stained liver sections from the rats treated with CCl_4. Treatment with silymarin did not improve any of these features. Liver tissue from the rats treated with AC (206, 619 and 1,032 mg/kg) had a lower overall score for fibrosis, but not for vacuolization and necrosis (Table 5). Sirius red staining for collagen determination was used to confirm the H&E findings. A reduction in the extent of fibrosis was observed in the liver tissue of the AC-administrated rats (Figure 2).

Discussion

Oxidative stress is the main contributing factor to the generation of liver fibrosis in the CCl_4 models. Free radical $CCl_3^{\cdot}$ are first generated from the metabolism of CCl_4 by cytochrome P-450 in the liver cell endoplasmic reticulum, initiating lipid peroxidation and impairing the functioning of Ca2+-ATPase (Andrabi et al., 1989). An influx of calcium ions and water into the cell results in damage to cellular integrity. CCl_4-induced liver injury increases permeability of the hepatocyte membrane and leakage of cellular contents. The liver enzymes AST and ALT are released into the circulation after damage to cell integrity of the hepatocyte, which leads to an increase in AST and ALT activity (Guéchot et al., 2013). Treatment with high doses (216 and 1,032 mg/kg) of ACT significantly reduced levels of AST and ALT in CCl_4 treated rats, which indicates a protective effect of AC against CCl_4-induced liver damage. These results agree with previous studies in mice and Wistar rats (Lin et al., 2006).

Antioxidant enzymes constitute one of the cellular mechanisms against reactive oxygen species. For example, SOD converts superoxide to H_2O_2, which is further converted to water by CAT and GPx. Catalase levels are high in the liver and kidney and significantly down-regulated in hepatitis and hepatocarcinoma. CCl_4 treatment reduced the activities of SOD, CAT and GPx by oxidative modifications of lipid peroxides (Augustyniak et al., 2014). Our results demonstrated that AC administration (206, 619 and 1,032 mg/kg) effectively restored some of the CAT, GPx and GrD activities in a chronic model of CCl_4-induced liver fibrosis. Enhanced antioxidant activities can prevent formation of excessive free radicals and may be the possible hepto-protective mechanism of AC. Song and Yen (2002) reported that pretreatment with AC increased GrD and GPx activities in an acute model of CCl_4-induced liver injury (Song and Yen, 2002). Indeed, other antioxidant agents, such as vitamin E, have also demonstrated a hepto-protective role in acute and chronic models of CCl_4-induced injury (Federico et al., 2006, Harrison et al., 2003).

Albumin is mainly synthesized in the liver, and impaired hepatic functioning causes a reduction in serum albumin levels. Moreover, CCl_4 has also been shown to induce hypomethylation, which results in the inhibition of protein synthesis (Recknagel et al., 1989). As expected, total protein and albumin levels were significantly reduced in CCl_4-induced rats when compared to the blank control. However, in the present work AC administration restored albumin levels in a dose-dependent manner. Lin et al., (2006) also reported similar findings in Wistar rats.

Liver fibrosis is a wound healing process that presents the liver's response to injury, and, like any other such processes, this leads to the deposition of collagen and other extracellular matrix components. This ongoing process eventually results in liver cirrhosis, in which the tissue organization is disrupted, as is the blood flow through the liver. The main histological features of CCl_4-induced injury found in this study were vacuolization, necrosis and fibrosis. AC administration at all doses led to a significant improvement in the overall histology score for fibrosis, and the results of the H&E stain were confirmed by Sirius red staining for collagen. In the CCl_4-treated liver, the amount of hydroxyproline, a major component of collagen, was elevated. In the livers of rats administrated with high doses of AC (619 and 1,032 mg/kg), the amounts of hydroxyproline were reduced.

Antrodia cinnamomea has demonstrated a hepto-protective effect in an acute model of CCl_4 -induced injury. Similarly, the protective effect was also observed in a chronic model in Wistar rats. There are strain and sex differences in the extent of CCl_4-induced injury in Wistar and Sprague-Dawley rats (Calabrese et al., 1996). Males exhibit a greater susceptibility to CCL_4 hepatotoxicity than females. The mechanisms that explain the sex-difference in this susceptibility are also different for Wistar and Sprague-Dawley rats. Slower hepatic tissue repair accounts for the sex-difference in Wistar rats, whereas greater

susceptibility to hepatic damage accounts for this in Sprague-Dawley rats. Lin et al., (2006) examined that the effects of AC administration in Wistar rats treated with CCl_4. Two AC doses 500 and 1,000 mg/kg were examined, similar to the high doses (619 and 1,023 mg/kg) used in the current study. Only at the highest dose of 1,000 mg/kg was the albumin level improved. Similarly, the hydroxyproline concentration was reduced, as was fibrosis, in rats administrated with 1,000 mg/kg. The present study confirms that AC's capability to reduce fibrosis, as reported by Lin et al., (2006), and provides new insights on the anti-oxidant role of AC in the chronic CCl_4 model.

In conclusion, we have demonstrated that *Antrodia cinnamomea* mycelia provide significant protection in an animal model of chronic liver injury, with a comparable efficacy to that of silymarin. It is likely that the protective effects observed with *Antrodia cinnamomea* mycelia are in part attributed to its antioxidant activities.

Conflicts of interest: Some of the authors (CCL and CCH) are employed by the company that produces *Antrodia cinnamomea* mycelia for functional foods. This manuscript was written as part of their normal employment.

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