Amelioration of carbon tetrachloride-induced hepatic injury by emulsified Antrodia extract

Wei-Chih Chang 1, Chang-Tsen Hung 2, Yuh-Shuen Chen 3, Chih-Chi Hsieh 4, Chien-Wei Hou 4*, Horng-Liang Lay 5

1 Graduate Institute of Bioresources, National Pingtung University of Science and Technology, Pingtung, Taiwan
2 Department of Health and Leisure Management, Yuanpei University of Medical Technology, Hsinchu, Taiwan
3 Department of Plant Industry, National Pingtung University of Science and Technology, Pingtung, Taiwan
4 Department of Biotechnology and Pharmaceutical Technology, Yuanpei University of Medical Technology, Hsinchu, Taiwan
5 Department of Health and Leisure Management, Yuanpei University of Medical Technology, Hsinchu, Taiwan

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A B S T R A C T
Objective(s): Antrodia cinnamomea (AC) is found with anti-inflammatory and immunomodulatory biological activities. In this study, we investigated the anti-hepatitis effect of the emulsified AC extract from RO water or supercritical fluid CO2 with ethanol co-solvent extract methods of AC preparations.

Materials and Methods: Five groups of eight to ten weeks male rats with a count of ten for each group were studied to evaluate the protection of two kinds of AC extract from hepatic injury. A total liver injury of rats was induced by injecting 40% carbon tetrachloride (CCl4) 1 mg/kg intraperitoneally. Positive and negative control groups rats were perfused with CCl4 or isotonic saline, respectively. Experimental groups received oral administration once/day of AC preparations before CCl4 treatment: water AC extract (WAE group), or emulsified AC extract from supercritical fluid extraction (EAE group) for 5 days, and sacrificed on the 6th day and the blood and liver samples were collected under chloral hydrate anesthesia. The anti-inflammatory, antioxidant markers, and relevant signaling pathways were measured (AST, ALT, ROS, IL-1, IL-6, NO, and COX-2, MAPKs, and caspase-3).

Results: EAE at 50 mg/kg significantly decreased the serum AST, ALT, ROS, IL-1, IL-6, NO, and COX-2 levels. Both extracts reduced the activation of p-ERK in the liver samples, but EAE inhibited COX-2 and caspase-3 protein expression better than WAE. The EAE ameliorated CCl4-induced hepatic injury significantly; as compared with WAE and the positive control.

Conclusion: The hepatoprotection of EAE could be attributed to the antioxidant and anti-inflammatory effects of Antrodia.

Introduction
The liver is an important organ for uptake, metabolism, conjugation, and excretion of various endogenous and foreign substances. Chronic hepatitis or exposure to toxins leads to liver fibrosis and necrosis. This process eventually leads to hepatoma (1, 2). Antrodia cinnamomea (AC), is a unique mushroom species for indigenous people in Taiwan to ameliorate liver disorders from excessive alcohol consumption. Currently, AC is known to have diverse biological activities, including anti-hypertensive, anti-hyperlipid, anti-inflammatory, antioxidative, anti-tumor, and immunomodulating effects (3).

Active components of AC have been isolated from the mushroom or cultivated fruiting bodies and fermented mycelia including benzenoids, diterpenes, triterpenoids, steroids, maleic/succinic acid derivatives, and polysaccharides (4-7). Anthronoquinonol and ethanolic extracts of AC mycelia, enhance HO-1 and Nrf-2 activation via MAPKs in vitro and in vivo (4). Anthron, a steroid-like compound isolated from the AC fruiting bodies, protects hepatic cells from free radical-induced oxidative stress and cell death via Nrf2-dependent induction of antioxidant genes in vitro and in vivo. (5). Antrodan, a protein-bound polysaccharide isolated from AC mycelia, exhibits significant anti-inflammatory bioactivity in vitro (7). These studies show that AC antioxidants ameliorate acute hepatic injury in various models.

It has been reported that the drug formulated in a self-micromulsifying drug delivery system (SMEDDS), can self-improve its water solubility, dissolution rate, and bioavailability (8), it contacts with gastrointestinal fluid and forms an emulsion with the aid of gastrointestinal motility. The preparation of emulsified AC extract (EAE) from supercritical fluid extraction would be better for hepatoprotection than the water extract of AC (WAE), presumably. Therefore, both extracts were compared in the carbon tetrachloride (CCl4)-induced

*Corresponding author: Chien-Wei Hou, Department of Biotechnology and Pharmaceutical Technology, Yuanpei University of Medical Technology, Hsinchu, Taiwan. N0. 306, Yuanpei St, Hsinchu City, Taiwan. Tel:+886-3-5381163#8154; Fax:+886-3-6102312; Email: rox.hou@mail.ypu.edu.tw
Acute liver injury. The protective mechanism was further compared for cytokines, reactive oxygen species (ROS) or reactive nitrogen species (RNS) production, as well as related signaling pathways.

**Materials and Methods**

### Reagents

CCL₄ was obtained from Sigma-Aldrich (St. Louis, MO, USA). Anti-phospho-p38, ERK, and JNK MAPKs, COX-2, caspase-3, and β-actin antibodies were purchased from Abcam (Cambridge, UK) and secondary anti-rabbit immunoglobulin G, conjugated to alkaline phosphatase from Jackson ImmunoResearch (Philadelphia, PA, USA).

### Preparation of AC extracts

Supercritical fluid extraction of AC fruiting body from solid-state culture was done, using CO₂ mixed with a constant amount of ethanol co-solvent (10% of CO₂ volume) at temperatures 50°C and pressure of 350 bar. The triterpenoid contents of AC fruiting body extracts were 0.11, 0.30, 0.20, 0.26, and 0.07% for Antcin A, B, C, H, and K, respectively. EAE was prepared as nine grams of paste extract, yielded from 100 g AC after evaporating with ethanol and mixing well with 1 g salad oil (1 mg/ml), and stored at 4°C until it was used. WAE was prepared by autoclaving 40 g AC in 1200 ml RO water at 1.5 atmospheric pressure, 120°C for 2 hr. The extract was filtered, freeze-dried, adjusted to 40 ml with water (10 mg/ml), and stored at 4°C until used.

### Treatment of animals

Male Sprague-Dawley rats (300-400 g), obtained from the National Laboratory Animal Center (Taipei, Taiwan), were maintained in the Animal Center of the Hungkuang University (Taichung, Taiwan). The animal studies were performed following the guidelines of the Guidebook for the Care and Use of Laboratory Animals (2002), published by the Chinese Society of Animal Science in Taiwan. Five groups of rats with a count of ten for each group were used to assess the protection of AC extracts from hepatic injury. Rats were injected intra-peritoneally, with 40% CCl₄ (1 mg/kg) to induce acute liver injury. Positive and normal controls were rats treated with and without CCl₄, respectively. Treated groups received oral administration of A. cinnamomea preparations before CCl₄ injection. Blood and liver samples were collected from animals after administering chloral hydrate (400 mg/kg, IP) anesthesia. Serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), reactive oxygen species (ROS), interleukin (IL)-1, IL-6, nitric oxide (NO) were determined as follows:

### Cytokine and liver function assays

Serum levels of IL-1β, and IL-6 were measured using enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN, USA). Hepatic injury was assessed by measuring serum ALT and AST with an automatic blood analyzer (Hitachi High-Technologies, Tokyo, Japan).

**NO assay**

Nitrite was measured as NO, using the Griess test. Briefly, a serum sample was reacted with an equal volume of Griess reagent (0.1% naphthylethylene diamine and 1% sulfanilamide (1:1) in H₃PO₄) in 96-well plates for 10 min. The absorbance at 540 nm was measured in a microplate reader.

**ROS generation**

ROS was measured with 2,7-dichlorodihydrofluorescein diacetate (H₂DCF-DA). Additionally, H₂DCF-DA was dissolved in methanol and deacetylated in serum mixed with 10 mM H₂DCF for 10 min in the dark. The reaction solution was plated in 96-well plates and monitored on a Fluoroskan Ascent Fluorometer (Labsystems Oy, Helsinki, Finland) using an excitation wavelength of 485 nm and an emission wavelength of 538 nm.

**Western blot**

Rat liver tissues were homogenized in ice-cold lysis buffer (1:10, weight/volume), containing 20 mmol/l 4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid (pH 7.2), 1% Triton X-100, 10% glycerol, 1 mM PMSF (phenylmethylsulfonyl fluoride), 10 mg/ml leupeptin, and 10 mg/ml aprotinin. This solution was centrifuged at 10,000 × g for 30 min at 4°C. Thereafter, 50 mg of protein was run on an 8% or 10% sodium dodecyl sulfate polyacrylamide gel and transferred onto nitrocellulose membranes (NEN Life Sciences, Boston, MA, USA) at 1.2 A for 3 hr. The membranes were blocked in 5% milk in Tris buffer saline with Tween-20. The membrane was then incubated with each polyclonal rabbit antibodies (p-p38, p-ERK, and p-JNK MAPKs; COX-2, caspase-3, β-actin) and diluted 1:1000 in blocking buffer. Membranes were incubated with secondary anti-rabbit immunoglobulin G, conjugated to alkaline phophatase (1:3000) and detected with alkaline phosphatase substrate solution (5-bromo-4-chloro-3-indolyl-phosphate/nitroblue tetrazolium; Kirkegaard & Perry, Baltimore, MD, USA).

**Antcin assay**

Antcin was determined by liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) method for the Antcins A, B, C, H, and K in the extract of AC fruiting body. Quantitative LC-MS/MS analysis was performed using an Agilent 1100 HPLC system (Agilent Technologies), coupled to an API 4000 triple quadrupole tandem mass spectrometer (Applied Biosystem, Foster City, CA, USA). Chromatographic separation was performed on a Poroshell 120 C18 column (100 × 3.0 mm i.D., 2.7 μm; Agilent, CA, USA).
The mobile phase consisted of 0.2% formic acid aqueous solution (solution A), and acetonitrile (solution B), and a gradient elution program was set as follows: solution A, 70-50% (0-7.5 min), 50-40% (7.5-10.8 min), 40-0% (10.8-19 min), 0-40% (19-21 min), 40-50% (21-25 min), and 50-70% (25-30 min). The column temperature was fixed at 22 °C, the flow rate was set 0.5 mL min−1, and the injection volume was 10 μL. The electrospray negative mode was selected as an ion source for Antcins A, B, C, H, and K detection. The positive electrospray mode was selected as an ion source for 4,7-dimethoxy-5-methyl-1,3-benzodioxole detection. The quantification was performed in multiple reactions monitoring (MRM). The optimized ESI source parameters were as follows: ion spray voltage, −4500 V for negative mode and 4500 V for positive mode; nitrogen nebulizer gas pressure, 12 psi; nitrogen curtain gas pressure, 10 psi; heater temperature, 450 °C, and collision activated dissociation (CAD) gas, 6 psi. The precursor-to-product ion transitions were m/z 453/409, m/z 467/408, m/z 469/425, m/z 485/413, and m/z 487/407 for antcin A, antcin B, Antcin C, antcin H, and antcin K respectively. All data acquisition and processing were performed using Analyst 1.4.1 software (AB SCIEX, Concord, ON, Canada). Antcin assay was done by ABM International Lab Inc (Pingtung Taiwan).

Histopathology

Liver tissues were fixed with a 10% formaldehyde solution overnight and hematoxylin and eosin (H&E) stained for examination.

Statistical analysis

All data were expressed as mean ± SD. For single variable comparisons, Student t-test was used. For multiple variable comparisons, data were analyzed with a one-way analysis of variance, using Dunnet’s test. A *P<0.05 was considered statistically significant.

Results

Serum AST and ALT concentrations

Since antioxidants ameliorate acute hepatic injury in various models (4-6), we thus compared the effect of two AC preparations on the acute hepatic injury model. Serum AST and ALT concentrations in response to CCl4 stress and Antrodia cinnamomea (AC) extract treatment. The serum AST and ALT concentrations of rats subjected to 40% carbon tetrachloride (CCl4, 1 mg/kg), were measured by the presence of two kinds of AC extract from hepatic injury. The result showed that the EAE at 50 mg/kg significantly reduced serum ALT and AST levels from CCl4-induced rats. Data were presented as the mean±SE. *P<0.05 as compared to the CCl4 group.

Measurement of IL-1 and IL-6 levels in serum

We further evaluated the effect of AC extract on the induced acute liver injury of rats. The inflammatory cytokine, IL-1 and IL-6 levels in the serum were determined using ELISA kits (R&D Systems). The EAE, reduced serum IL-1 and IL-6 levels in a dose-dependent manner, in CCl4-induced rats (Figure 2).
Antrodia extract against CCl₄-induced hepatitis

**Figure 3.** Effect of the *Antrodia cinnamomea* (AC) extract on serum nitric oxide level in rats with and without CCl₄ stress. Levels of NO increased with CCl₄-induced liver injury but were reduced with WAE or EAE. Data are expressed as the mean±SE. "*"P<0.05 as compared to the CCl₄ group.

**Figure 4.** Effect of the *Antrodia cinnamomea* (AC) extract on serum ROS level under CCl₄ stress. EAE was able to scavenge serum ROS generation of CCl₄-induced rats. But, there is no scavenging activity with WEA. Data are expressed as the mean±SE. "*"P<0.05 as compared to the CCl₄ group.

**Figure 5.** Protective effect of the *Antrodia cinnamomea* (AC) extract on CCl₄-induced hepatic injury. Histopathology of liver slices from rats from (A) no treatment control group; (B) the CCl₄ group; (C) the EAE group, and (D) the WAE group. The photographs show the liver section with 200× magnification.

**NO assay**

Levels of NO increased with CCl₄-induced liver injury but were lower with the two kinds of AC extract treatment, suggesting that AC extract may protect rats from CCl₄-induced liver injury. These results are consistent with the previous findings that; the reduction of IL-1, IL-6 and NO has a protective effect against CCl₄-induced liver injury in animals.

**ROS scavenging effect of EAE**

ROS is necessary for normal physiological functions but also contribute to liver injury. We found that EAE was able to scavenge 40～50% of CCl₄-induced serum ROS (Figure 4; "*"P<0.05), because ROS signals can trigger the intrinsic apoptosis pathway, EAE might reduce the apoptosis of hepatocytes under CCl₄ stress by scavenging these free radicals. Our results showed that EAE could protect from liver injury by attenuating the increased serum ROS in the CCl₄-induced rats.

**Histopathology analysis**

Under CCl₄ stress, liver cell degeneration increased significantly, in comparison with the control group in the histopathology of rats. Cell atrophy, irregular arrangement with degeneration were observed in the liver section of the CCl₄ group (Figure 5B), but not in the control group (Figure 5A). The EAE group (Figure 5C) showed less cell degeneration and spotty necrosis than the PL group in 200 microscopic fields of the liver section. However, the WAE group (Figure 5D) showed much cells atrophy and hepatocellular degeneration than EAE group. The protection from liver injury was evident by attenuating the serum levels of IL-1, IL-6, NO, and ROS (Figs. 2-4; "*"P<0.05). These results were consistent with histopathology data; that EAE significantly reduced the CCl₄-induced lesion in the liver (Figure 5).

**EAE inhibited CCl₄-induced p-ERK MAPK, COX-2 and caspase-3 activation**

The effects of AE preparations were further examined on CCl₄-induced signaling pathways by Western blot assay. The result showed that both extracts reduced the p-ERK activation, and that EAE could inhibit COX-2, and caspase-3 levels significantly in the liver samples. EAE (50 mg) reduced the expression of the following proteins p-ERK (70 %), MAPK, caspase-3 (50%), and COX-2 (50%) respectively to the CCl₄-induced liver cells in rats. This result is better than that in which, WAE (50 mg) reduced expression of p-ERK (50 %) MAPK, caspase-3 (20%), and COX-2 (20%), respectively (*"P<0.05; Figure 6).

**Discussion**

The present results demonstrated that the EAE treatment (50 mg/kg), significantly decreased the serum AST, ALT, IL-1, IL-6, NO, and ROS levels. These data were consistent with the histopathology results. Both extracts reduced the activation of p-ERK.
Table 1. Comparison of Antcins concentration between EAE/ WAE treatments

| Antcin C (ppm) | Antcin H (ppm) | Antcin K (ppm) |
|----------------|----------------|----------------|
| Liver          |                |                |
| Con            | EAE            | WAE            |
| 0              | 1.1±0.4        | 0              |
| Intestine      |                |                |
| 0              | 2.0±0.3        | 1.0±0.1        |
| Stomach        |                |                |
| 0              | 1.0±0.1        | 0              |
| Brain          |                |                |
| 0              | 0              | 1.0±0.1        |

Con: control; emulsified AC extract: 50 mg/kg; Water extract of AC: 50 mg/kg

Figure 6. AC extract inhibited CCl4-induced phospho-ERK MAP kinase, COX-2 and caspase-3 under CCl4 stress. EAE and WEA both extracts reduced the p-ERK activation, and EAE inhibit COX-2, and caspase-3 levels better than WEA in the liver samples. Data are expressed as mean ± SEM of three independent experiments. *P<0.05 as compared to CCl4 control

signaling pathway, and only EAE inhibited COX-2, and caspase-3 protein levels. These results agreed with other reports and confirmed that active compounds such as Antcins from EAE treatment contributed to the hepatoprotection of the liver.

Water extract of AC fruiting bodies has been shown to protect CCl4-induced chronic liver injury in ICR mice (10). Hsiao et al. (2003), reported that AE (250-1250 mg/kg) ameliorated the increase in plasma AST and ALT levels, and restored superoxide dismutase (SOD) activities, glutathione content and catalase activity of hepatic tissues dose-dependently (10). Water extract of AC mycelia, significantly inhibited a free radical initiator, 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH)-induced apoptotic cell death in the endothelial cells, with evidence shown by reduced DNA fragmentation, cytochrome c release, caspase-3 activation, and dysregulation of Bcl-2 and Bax. It also prevented the AAPH-induced reductions in SOD activity and protein levels (11). Thus, the water extract of AC fruiting bodies could prevent CCl4-induced chronic hepatotoxicity by scavenging the free radical formation or restoring antioxidant enzyme. However, our present result with low dose of WAE was not significant as compared with EAE in CCl4-induced acute hepatic injury.

EAE had several advantages over WAE preparation for better hepatoprotection. This may be explained by a report that showed that SNEDDS of CoQ10 improves its water solubility, dissolution rate, and bioavailability on rat liver cirrhosis model as compared with CoQ10 powder (12). Upon administration, self-nanoemulsifying system comes in contact with gastrointestinal fluid and forms o/w nano-emulsion with the aid of gastrointestinal motility. This provides a large surface area for enhancing the drug release and absorption. The bioavailability of CoQ10 SNEDDS was increased in 2.1-fold compared with CoQ10 suspension after oral administration. ALT, AST, alkaline phosphatase, total protein, and albumin were significantly improved.

Biological membranes play critical roles in the homeostasis of all organisms because they segregate important activities between and within cells and tissues. Small hydrophobic molecules can partition across biological membranes, down to a concentration gradient but hydrophilic molecules generally require some sort of selective transport system to cross the lipid bilayer. Studies show that certain compounds can facilitate the transport of polar molecules across biological membranes (13, 14). Ergostane and lanostane tetracyclic triterpenoids are the major bioactive compounds in AE (15). Pure ergostanes such as antcins B, C, and H can easily pass through the intestinal Caco-2 cell monolayer, while lanostanes are not (16). Antcins H and K have a high polarity as compared with antcins C and B. However, all ergostanes from AE (antcins B, C, H, and K), show favorable permeability than the pure compounds. The self-dispersing lipid formulations (SDLFs), is one of the promising approaches to
overcome the formulation difficulties of various hydrophobic/lipophilic drugs, and to improve the oral bioavailability of poorly absorbed drugs (17). Our results were consistent with these concepts that high concentrations of antcins C, H, and K were detected in the liver and other tissues from EAE-treated animals (Table 1).

Recently, antcin K was reported to protect the N-nitrosodiethyamine (DEN)-induced liver inflammation, fibrosis and carcinogenesis in rats. The inhibition of DEN-enhanced hepatocellular inflammation is achieved through suppressing NF-κB, scavenging ROS activity and upregulating antioxidant defense mechanisms (18). Antcin C has been reported to protect hepatic cells from AAPH-induced cell death through the inhibition of ROS, lipid peroxidation, ALT/AST and GSH depletion (5). It was correlated with induction of antioxidant genes and SOD via transcriptional activation of Nrf2. In addition, antcin C down-regulates pro-apoptotic factors including Bax, cytochrome c, caspase-9, -4, -12, -3, and PARP. These evidence indicate that antcins C and K can protect liver cells from oxidative stress and cell death via anti-oxidative and anti-inflammatory mechanisms.

Conclusion

The presented data showed that emulsified AC preparation was more effective than WAE to ameliorate CCl₄-induced acute hepatic injury by inhibition of cytokines, ROS, and NO through anti-oxidative and anti-inflammatory effects in rats. The protective mechanism was partially attributed to antcins through the suppression of p-ERK signaling pathway, and COX-2 and caspase-3.

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Conflict of interest

The authors declare that they have no competing interests.

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