

# Establishment of the Metabolite Profile for an *Antrodia cinnamomea* Health Food Product and Investigation of Its Chemoprevention Activity

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**ABSTRACT:** *Antrodia cinnamomea* is an edible fungus endemic to Taiwan that has been attributed with health promotion benefits. An *A. cinnamomea* mycelium health food product, which was produced by solid-state culture, was selected as the target for investigation in this study. Fourteen representative metabolites of *A. cinnamomea* mycelium (EMAC) were selected as index compounds to establish the metabolite profile for evaluation of EMAC product quality. It was also demonstrated that EMAC administration significantly reduced liver inflammation and serum oxidative stress in vivo. 4-Acetylanthroquinonol B obtained by a bioactivity-guided fractionation from EMAC was able to not only inhibit LPS-induced nitric oxide formation in macrophages but also protect against ethanol-induced oxidative stress in liver cells. The results suggest this *A. cinnamomea* product might be a potent antioxidative and anti-inflammatory supplement for chemoprevention.

**KEYWORDS:** *Antrodia cinnamomea*, anti-inflammatory, chemoprevention, 4-acetylanthroquinonol B

## INTRODUCTION

Mushrooms have been used as foods and medicines since antiquity and have an established history of use in traditional therapies. *Antrodia cinnamomea* (syn. *Antrodia camphorata* and *Taiwanofungus camphorata*) is a precious and unique edible fungus that originates from the forests of Taiwan. It has long been used as a folk remedy for treating various diseases including liver diseases, hypertension, abdominal pain, and cancer.<sup>1–3</sup> Owing to its perceived efficacy, *A. cinnamomea* dry fruiting bodies are sold at prices exceeding U.S. \$15000 per kilogram in the local market of Taiwan,<sup>3</sup> and the total market value of *A. cinnamomea* products is estimated to be over U.S. \$1 billion per year. Because of its high commercial value, *A. cinnamomea* has attracted much interest in research and development in Taiwan over the past decade, and so far numerous studies have been conducted on its physiology and biochemical and pharmacological properties;<sup>3–16</sup> many compounds identified from *A. cinnamomea* have been reported to possess health-promoting activities.

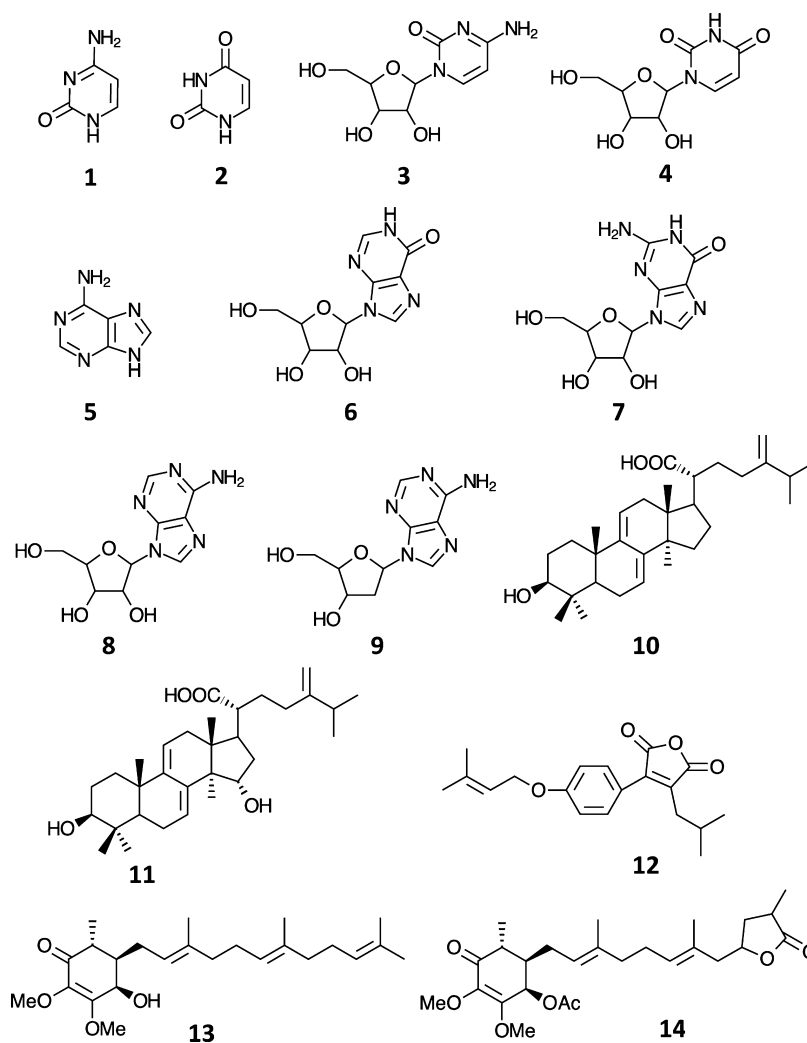
In our previous study, we analyzed a fixed group of compounds including triterpenoids, benzolics, and polyacetylenes and established a suitable, reliable system to evaluate the

quality of ethanol extract from *A. cinnamomea* fruiting bodies.<sup>17</sup> People in Taiwan believe that only those growths on the aromatic tree *Cinnamomum kanehirai* Hayata specifically possess the health promotion properties;<sup>18–20</sup> however, to conserve natural resources, excessive felling of *C. kanehirai* forest is prohibited by the government of Taiwan. To mass produce *A. cinnamomea* mycelium, it is necessary to investigate the bioactivities of *A. cinnamomea* from growth conditions other than *C. kanehirai*, as well as to cultivate its fruiting bodies for use in *A. cinnamomea* products. Different manufacturing processes have been developed, such as *A. cinnamomea* mycelium fermented in either liquid or solid states, which are important products in the market. Nowadays, although hundreds of *A. cinnamomea* products are sold, only three products have been awarded a “National Health Food” certificate by Taiwan’s Department of Health. For this study, we selected one certified *A. cinnamomea* mycelium product,

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**Figure 1.** Index compounds of *A. cinnamomea* mycelium: 1, cytosine; 2, uracil; 3, cytidine; 4, uridine; 5, adenine; 6, inosine; 7, guanosine; 8, adenosine; 9, deoxyadenosine; 10, dehydroeburicoic acid; 11, dehydrosulfurenic acid; 12, 3-isobutyl-4-[4-(3-methyl-2-butenyloxy)phenyl]furan-2,5-dione; 13, antroquinonol; 14, 4-acetylanthroquinonol B.

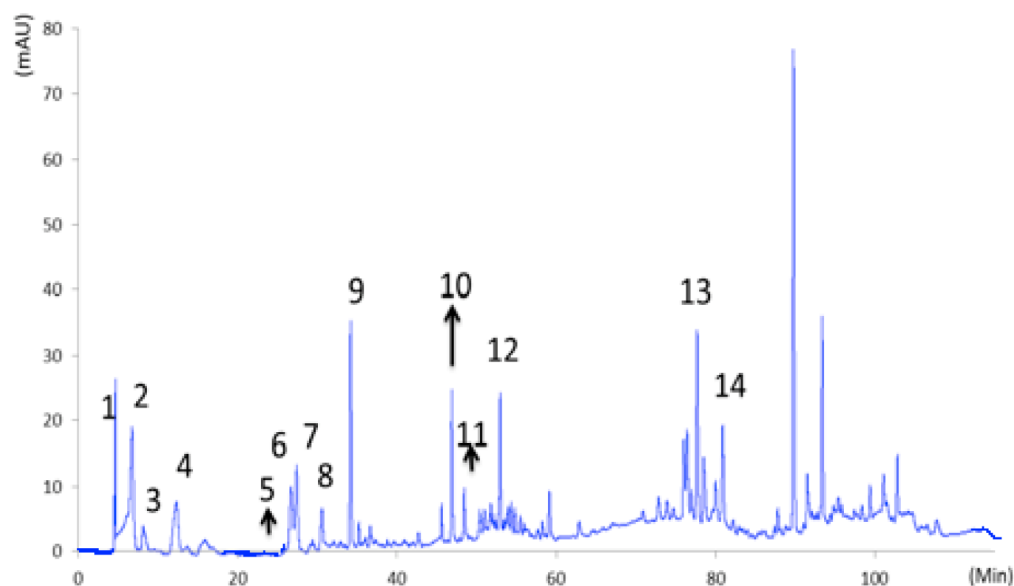
which was produced by solid-state fermentation, as study material. According to our previous study, this mycelium product has a potent hepatoprotective effect, and one of its derivative compounds, antroquinonol, significantly inhibits ethanol-induced aspartate aminotransferase (AST), alanine aminotransferase (ALT), reactive oxygen species (ROS), nitric oxide (NO), malondialdehyde (MDA) production, and glutathione (GSH) depletion in HepG2 cells.<sup>21</sup> In this follow-up study, the metabolite profile of a large-scale manufacturing mycelium of *A. cinnamomea* cultured in solid state was established by using HPLC, and 14 selected index compounds, which are the dominant and represented metabolites, were quantified. The *in vivo* antioxidant (*in vivo* photochemiluminescence assay) and anti-inflammation activity (*in vivo* and *in vitro* nitric oxide production inhibitory activity assay) of the mycelium as well as a representative compound, 4-acetylanthroquinonol B, in the ethanolic extract were evaluated.

## MATERIALS AND METHODS

**Mycelium of *Antrodia cinnamomea* and Reagents.** Solid-state cultured *A. cinnamomea* mycelia were obtained from the R&D Center of Taiwan Leader Biotechnology Corp. (Taichung, Taiwan). Minimum essential medium (MEM), fetal bovine serum (FBS),

penicillin, and streptomycin were purchased from Gibco/Invitrogen (Carlsbad, CA, USA). Gries reagent, silymarin, 2',7'-dichlorofluorescein diacetate (DCF-DA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), and mouse monoclonal anti- $\beta$ -actin antibody were purchased from Sigma-Aldrich (St. Louis, CA, USA). Mouse monoclonal HO-1 and rabbit polyclonal Nrf-2 antibodies were obtained from Abcam (Cambridge, MA, USA). Cell culture dishes and multiwell plates were purchased from Techno Plastic Products (Trasadingen, Switzerland). Standards for metabolite profiling, including cytosine, uracil, cytidine, uridine, adenine, inosine, guanosine, adenosine, and deoxyadenosine, were purchased from Sigma-Aldrich. Dehydroeburicoic,<sup>22</sup> dehydrosulfurenic acid,<sup>23</sup> 3-isobutyl-4-[4-(3-methyl-2-butenyloxy)phenyl]furan-2,5-dione,<sup>24</sup> antroquinonol,<sup>25</sup> and 4-acetylanthroquinonol B<sup>25</sup> were purified from mycelia of *A. cinnamomea* in our laboratory. The structures of these compounds were elucidated and confirmed by spectroscopic analyses; UV and IR data were acquired on a PerkinElmer 241 polarimeter, a Bio-Tek  $\mu$ Quant MQX200 ELISA reader, and a PerkinElmer Spectrum 100 FT-IR spectrometer, respectively. <sup>1</sup>H NMR, <sup>13</sup>C NMR, HSQC, HMBC, <sup>1</sup>H-<sup>1</sup>H COSY, and DEPT spectra were obtained on Varian Unity Inova-600 and -400 MHz spectrometers using CDCl<sub>3</sub> as the solvent. The purity of the isolated compounds was >99% according to HPLC and <sup>1</sup>H NMR analyses. The structures of index compounds are presented in Figure 1.

**Extract Preparation and Metabolite Profiling.** To prepare extracts of mycelia of *A. cinnamomea*, fresh air-dried mycelia (200 g)



**Figure 2.** HPLC profile of ethanolic extract of *A. cinnamomea* mycelium: 1, cytosine; 2, uracil; 3, cytidine; 4, uridine; 5, adenine; 6, inosine; 7, guanosine; 8, adenosine; 9, deoxyadenosine; 10, dehydroeburicoic acid; 11, dehydrosulfurenic acid; 12, 3-isobutyl-4-[4-(3-methyl-2-butenyloxy)phenyl]furan-2,5-dione; 13, antroquinonol; 14, 4-acetylantroquinonol B.

were immersed in ethanol (2 L) for 5 days and concentrated under vacuum to yield the EtOH extract (24.9 g). The EtOH extract (20 g) was partitioned between EtOAc/H<sub>2</sub>O to give an EtOAc-soluble fraction (10.25 g) and an H<sub>2</sub>O-soluble fraction. The EtOAc-soluble fraction displayed potent inhibitory activity on LPS-induced NO production and was further chromatographed over silica gel (4 × 30 cm; 60–80 mesh; Merck) eluted with *n*-hexane and a gradient of *n*-hexane/EtOAc (100:0; 97.5:2.5; 95:5; 92.5:7.5; 90:10; 85:15; 80:20; 75:25; 70:30; 65:35; 60:40; 50:50; 40:60; 30:70; 20:80; 10:90; 0:100). The eluent was collected in constant volumes (each 500 mL), and 30 subfractions (EA-1–EA-30) were obtained on the basis of the results of thin layer chromatography (TLC) analysis. Among them, EA-9 and EA-11 exhibited potent NO inhibitory activity compared with other subfractions; EA-9 and EA-11 were purified by HPLC (FLOM dual piston pump model 240 series; Shodex RI-101 detector) coupled with a Phenomenex column (5 μm, 250 mm × 10 mm); the elute solvent system was *n*-hexane/ethyl acetate = 80:20 at a flow rate of 3 mL/min to obtain antroquinonol (EA-9; retention time = 14 min) and 4-acetylantroquinonol B (EA-11; retention time = 18 min).

The crude extract was then redissolved in methanol (10 mg/mL) and passed through an SPE cartridge (Sep-Pak C18, Waters, Milford, MA, USA). The pretreated methanolic solution was further separated by HPLC using the Agilent 1100 HPLC system equipped with a UV detector. A Luna C18 column (150.0 × 4.6 mm; Phenomenex, Torrance, CA, USA) was used with a gradient elution of MeOH (A) and 10% aqueous ACN (B) conducted as 100% B at 0–15 min, 100–98% B at 15–20 min, 98–85% B at 20–35 min, 85–65% B at 35–40 min, 65–40% B at 40–50 min, 40–30% B at 50–65 min, 30–15% B at 65–85 min, 15–0% B at 85–95 min, and 0% B at 95–115 min; the flow rate was 1.0 mL/min, and the detector wavelength was set at 254 nm. Fourteen compounds in the EtOH extracts were obtained at retention times of 2.9 min (1), 5.2 min (2), 6.7 min (3), 10.9 min (4), 22.0 min (5), 25.6 min (6), 26.3 min (7), 29.7 min (8), 33.3 min (9), 46.3 min (10), 48.0 min (11), 52.5 min (12), 78.0 min (13), and 81.3 min (14).

**In Vivo Antioxidant Capacity Evaluation.** Male ICR mice (4 weeks old, 25 ± 5 g) were purchased from BioLasco (Taipei, Taiwan). All animal experiments were conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* and Taiwanese laws relating to the protection of animals and were approved by the local ethics committee of National Chung-Hsing University. Mice were divided into six groups consisting of six mice each. Crude extracts of *A. cinnamomea* mycelium (25, 50, and 100 mg/mL) and silymarin (10

mg/mL) were dissolved in 1% DMSO and phosphate-buffered saline (PBS). Groups 1–4 were orally administered various concentrations of *A. cinnamomea* mycelium (250, 500, and 1000 mg/kg/day) or silymarin (100 mg/kg/day) extract. Group 5 control mice received vehicle (1% DMSO + PBS) only, and group 6 was administered *A. cinnamomea* mycelium extract 1000 mg/kg/day. After 10 days, mice in groups 1–4 were orally administered 5 g/kg of alcohol, and groups 5 and 6 were fed PBS with alcohol. Experimental animals were sacrificed by decapitation during anesthesia with ethyl ether 12 h after alcohol treatment. Blood was collected by cardiac puncture in EDTA tubes and centrifuged at 500g for 10 min at 4 °C, and serum antioxidant capacity was determined with photochemiluminescence assay by using a Photochem (Analytik Jena AG, Jena, Germany). In the photochemiluminescence assay, the generation of free radicals was partially eliminated by the reaction with antioxidant present in serum samples, and the remaining radicals were quantified by luminescence generation. Ascorbic acid calibration curves were used to evaluate antioxidant capacity, and the results were expressed as micromoles of equivalents per milliliter of serum. The levels of aspartate aminotransferase (ALT) and alanine aminotransferase (AST) in culture medium were measured using commercially available assay kits (Randox Laboratories, Antrim, UK). Cellular GSH level was measured using a commercially available GSH assay kit (Oxis International, Foster City, CA, USA). Lipid peroxidation was determined by the formation of MDA in cultured cell lysates using a lipid peroxidation assay kit (Oxford Biomedical Research, Rochester Hills, MI, USA) following the supplier's instructions.

**In Vivo Anti-inflammation Experiments.** Mice were divided into six groups consisting of six mice each. Crude extract of mycelium (25, 50, and 100 mg/mL) and curcumin (10 mg/kg) were dissolved in 1% DMSO and administered to mice by intraperitoneal injection with or without various concentrations of extract (250, 500, and 1000 mg/kg) or curcumin (100 mg/kg) 4 h before LPS induction by intraperitoneal injection (5 μg/kg).<sup>26,27</sup> Control mice received vehicle (DMSO) only. Mice were sacrificed by decapitation during anesthesia with ethyl ether at 12 h after LPS injection. Blood was collected by eye bleeding or cardiac puncture in EDTA tubes and centrifuged at 500g for 10 min at 4 °C, and NO concentrations in serum were measured indirectly using the Griess reaction.<sup>28</sup> The livers were quickly removed at 12 h after LPS injection, flash frozen in liquid nitrogen, and milled. Whole proteins were isolated using Mammalian Protein Extraction Reagent (Cayman Chemicals, Ann Arbor, MI, USA), and whole cell extracts were prepared as previously described.<sup>26</sup> The protein content

was quantified according to the Bradford method with absorbance at 595 nm.<sup>27</sup>

**Nitric Oxide Production Inhibitory Activity Assay.** The effect of the compounds isolated from *A. cinnamomea* extracts on NO production was measured indirectly by analysis of nitrite levels using the Griess reaction.<sup>26,28</sup> Briefly, RAW 264.7 cells grown in a 75 cm<sup>2</sup> culture dish were seeded in 96-well plates at a density of  $2 \times 10^5$  cells/well. Cells were cultured at 37 °C in DMEM supplemented with 10% FBS, 100 units/mL penicillin, and 100 µg/mL streptomycin in a 5% CO<sub>2</sub> incubator as recommended by the American Type Culture Collection (ATCC). Adherent cells were then incubated with or without 1 µg/mL of LPS for 24 h, in the presence or absence of *A. cinnamomea* extracts. Nitrite concentration (as an estimate of NO production) was measured in the RAW 264.7 cell culture medium by the Griess reaction.<sup>27</sup> This assay was also conducted during compound isolation procedure to help in recognizing the existence of active compounds in extract fractions, a so-called bioactivity-guided fractional procedure.

**Protein Extraction and Western Blot Analysis.** Cytoplasmic and nuclear fractions were obtained by using commercially available nuclear and cytoplasmic extraction reagents kit (Pierce Biotechnology, Rockford, IL, USA). HepG2 cells ( $1 \times 10^7$  cells/mL) were cultured in 6-cm dishes and incubated with various concentrations of 4-acetylanthroquinol B or silymarin for 1 h. Oxidative stress was then induced by the addition of ethanol (100 mM) to the culture medium for 12 h. At the end of incubation, cells were lysed with cytoplasmic and nuclear extraction reagent. The protein concentration was determined using Bio-Rad protein assay reagent (Bio-Rad Laboratories, Hercules, CA, USA). For Western blot analysis, 20 µg protein fractions were separated by 7–12% SDS–polyacrylamide gel electrophoresis and transferred onto PVDC membrane. Western blots were performed with appropriate antibodies using the ECL Western blotting reagent (Millipore, Billerica, MA, USA), and the image was visualized by a VL Chemi-Smart 3000 (Viogene Biotek, Sunnyvale, CA, USA) imaging device.

## RESULTS

**Determination and Quantification of Index Compounds in *A. cinnamomea* Mycelium.** Nine primary metabolites, cytosine, uracil, cytidine, uridine, adenine, inosine, guanosine, adenosine, and deoxyadenosine, and five representative secondary metabolites, dehydroeburicoic acid, dehydrosulfurenic acid, 3-isobutyl-4-[4-(3-methyl-2-butenyloxy)-phenyl]furan-2,5-dione, antroquinonol, and 4-acetylanthroquinol B (Figure 1), of *A. cinnamomea* mycelium were selected as index compounds (purity > 99.5%) to analyze the ethanolic extract of mycelium produced by solid-state culture. A comprehensive profile of the ethanol extract of *A. cinnamomea* mycelium was established as shown in Figure 2. To measure the content of the index compounds in *A. cinnamomea* mycelium, calibration curves of the index compounds were established using seven dilution standards from 10 to 1000 µg/mL. Table 1 shows the regression parameter and linearity of the proposed HPLC profiling method. The contents of 14 index compounds were determined by the peak area in the HPLC profile and calculated using the calibration curves of index compounds (Table 1). Antroquinonol (1.78 µg/mg) was the most abundant compound in the mycelium extract, followed by inosine (1.61 µg/mg), 4-acetylanthroquinol B (1.48 µg/mg), and 3-isobutyl-4-[4-(3-methyl-2-butenyloxy)phenyl]furan-2,5-dione (1.31 µg/mg). The contents of two lanostane-type triterpenoids, dehydroeburicoic acid and dehydrosulfurenic acid, were 0.93 and 0.91 µg/mg, respectively.

**Effect of *A. cinnamomea* Mycelium on LPS-Induced NO Production in Mouse Blood Serum.** To evaluate the anti-inflammatory activity in vivo, levels of inflammation were

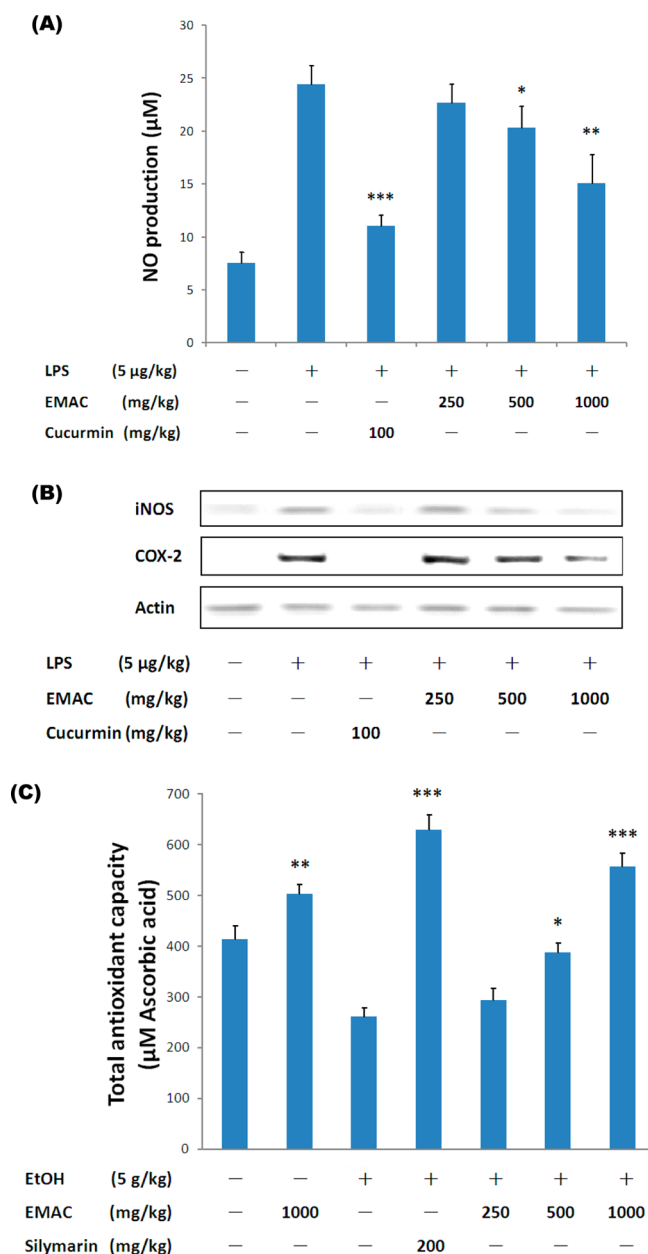
**Table 1. Calibration Curve and Qualification Results of the Compositions in *Antrodia cinnamomea* Mycelium**

peak	compound	calibration curve	content <sup>a</sup> (µg/mg)
1	cytosine	$Y = 22.6X - 38.2,$ $r^2 = 0.987$	0.21
2	uracil	$Y = 31.4X + 149.6,$ $r^2 = 0.991$	0.15
3	cytidine	$Y = 15.4X - 119.6,$ $r^2 = 0.982$	0.76
4	uridine	$Y = 22.7X + 9.7,$ $r^2 = 0.993$	1.24
5	adenine	$Y = 41.5X + 5.4,$ $r^2 = 0.983$	0.06
6	inosine	$Y = 17.4X - 4.1,$ $r^2 = 0.985$	1.61
7	guanosine	$Y = 20.4X + 129.7,$ $r^2 = 0.996$	0.71
8	adenosine	$Y = 17.9X - 28.9,$ $r^2 = 0.979$	0.61
9	deoxyadenosine	$Y = 15.3X + 377.6,$ $r^2 = 0.972$	1.03
10	dehydroeburicoic acid	$Y = 28.3X - 46.5,$ $r^2 = 0.978$	0.95
11	dehydrosulfurenic acid	$Y = 36.6X - 26.1,$ $r^2 = 0.969$	0.91
12	3-isobutyl-4-[4-(3-methyl-2-butenyloxy)phenyl]furan-2,5-dione	$Y = 24.3X + 91.5,$ $r^2 = 0.982$	1.31
13	antroquinonol	$Y = 23.6X - 27.4,$ $r^2 = 0.978$	1.78
14	4-acetylanthroquinol B	$Y = 32.3X + 108.9,$ $r^2 = 0.972$	1.48

<sup>a</sup>The contents of index compounds are based on the weight of EtOH extract.

determined by detecting the NO concentration in mouse serum challenged with intraperitoneal injection of LPS (5 µg/kg). The result showed preinjection with various concentrations of *A. cinnamomea* mycelium (EMAC) ethanol extract gave significantly dose-dependent inhibition of LPS-induced NO production in mouse serum. As shown in Figure 3A, the concentrations of NO in mouse serum were increased from 7.2 to 26.1 µM after LPS induction, indicating the mouse was suffering from inflammation. However, the NO concentrations were reduced to 23.4, 21.8, and 14.2 µM when the mice received EMAC prior to LPS challenge at doses of 250, 500, and 1000 mg/kg, respectively. NO concentration in the mice that were treated with the anti-inflammatory compound, curcumin, was 10.3 µM at a single dose of 100 µg/kg. This result confirmed that EMAC strongly inhibits endotoxin-induced inflammatory NO production in vivo.

**Effects of *A. cinnamomea* Mycelium on LPS-Induced iNOS and COX-2 Protein Expression in Mouse Liver Tissue.** To further confirm the causes of NO reduction, we then detected the protein expression levels of iNOS and COX-2 in mouse liver tissues (Figure 3B). The result showed iNOS expression paralleled NO production levels (Figure 3A). LPS-challenged mice injected with EMAC at various concentrations showed only very low levels of iNOS protein expression, implying that the reduction of NO concentration in serum might result from a relatively low level of iNOS to catalyze such reaction. The expression of COX-2, another inflammatory response-related protein, was also suppressed in a dose-dependent manner. In particular, mice treated with 500 mg/



**Figure 3.** Anti-inflammatory and antioxidant effects of ethanolic extract of *A. cinnamomea* mycelium in vivo. (A) Effect of EMAC on nitrite production in LPS-induced mice blood serum. ICR mice were treated with EMAC (250–1000 mg/kg) or curcumin for 4 h, and then inflammation was induced by treatment with 5 µg/kg LPS. Mice were sacrificed by decapitation 12 h after LPS injection, and samples were collected. (B) Western blot result shows iNOS and COX-2 protein expression levels in mouse liver tissue after the same treatment as above. (C) Effects of ethanolic extract of EMAC on total antioxidant capacity in EtOH-induced oxidative damaged mice blood serum. ICR mice were treated with EMAC (250–1000 mg/kg) or silymarin for 10 days, and then oxidative damage was induced by treatment with 5 g/kg EtOH. Mice were sacrificed by decapitation 12 h after EtOH treatment, and samples were collected. Values represent the mean  $\pm$  SD of six mice. (\*)  $P < 0.05$ , (\*\*)  $P < 0.01$ , and (\*\*\*)  $P < 0.001$  indicate significant differences from the LPS- or EtOH-treated group.

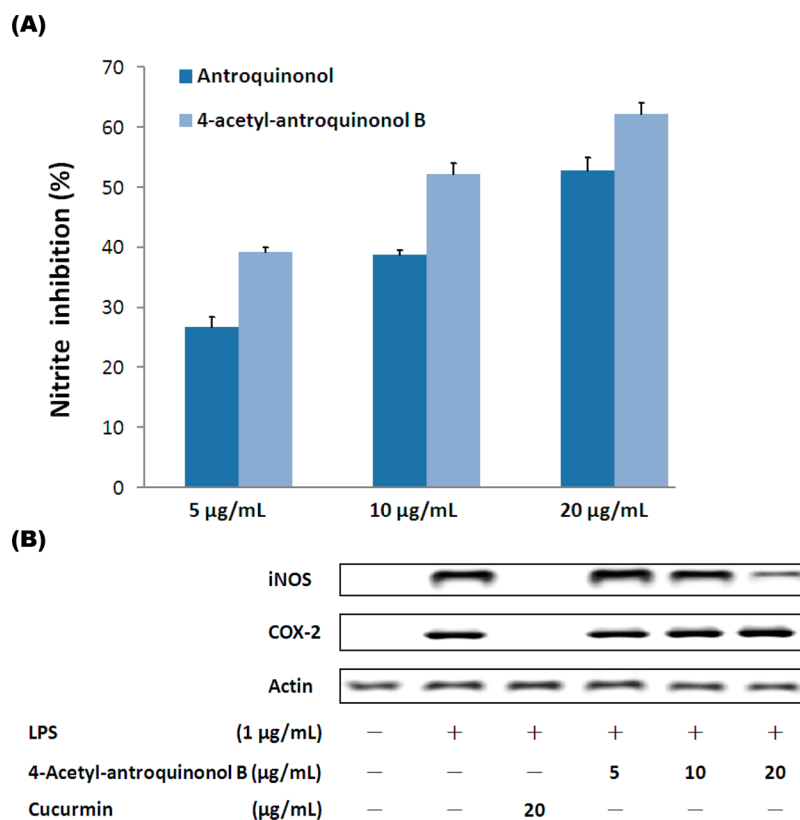
kg EMAC showed a significant effect that was similar to that of the group treated with 100 mg/kg curcumin.

**Antioxidative Activity of Mycelium in Vivo.** To evaluate the antioxidant activity in vivo, mice were orally administered

EMAC for 10 days before being challenged with alcohol (5 g/kg) to induce oxidative stress. Figure 3C shows the effects of EMAC on antioxidant capacity as measured in the serum of mice. The total AC in the serum of mice increased from 414.4 to 503 µM ascorbic acid after mice were fed EMAC (1000 mg/kg). On the other hand, the antioxidant capacity decreased from 414.4 µM ascorbic acid to 216.2 µM after challenge with alcohol, indicating mice were suffering from oxidative stress; however, the antioxidant capacity again increased to 282, 354, and 558 µM ascorbic acid, respectively, when 250, 500, and 1000 mg/kg EMAC were administered. It is noted that the antioxidant capacity of the EMAC treatment (1000 mg/kg) with the alcohol group (558 µM) was significantly higher than that of the group that was administered EMAC without alcohol (503 µM). This result might be due to the synergistic effect of EMAC extract and alcohol in enhancing the antioxidative response system in mice. The reference compound, silymarin, increased the ascorbic acid titer to 630 µM at a dose of 200 mg/kg. Together, these results suggest that the mycelium extract of *A. cinnamomea* can stimulate antioxidant activity in mice.

**Anti-inflammatory Activity of Compounds in Mycelium.** Two compounds, antroquinonol and 4-acetylanthroquinonol B, that possess potent anti-inflammation activity were isolated from EMAC using a bioactivity-guided fractional procedure (Figure 4A). The calculated concentrations of antroquinonol and 4-acetylanthroquinonol B required for inhibiting 50% of LPS-induced NO production ( $IC_{50}$ ) were 17.6 and 12.8 µg/mL, respectively. Because 4-acetylanthroquinonol B possesses better inhibitory activity than antroquinonol, the possible mechanism of 4-acetylanthroquinonol B in anti-inflammation was further studied. We reasoned that the inhibition of LPS-induced pro-inflammatory molecules could be due to suppression of iNOS and COX-2 at the transcriptional level, affecting protein expressions. As shown in Figure 4B, we found the quiescent RAW 264.7 cells expressed low or undetectable levels of iNOS. In contrast, a strong iNOS expression was observed due to treatment with 1 µg/mL LPS. 4-Acetylanthroquinonol B suppressed LPS-induced iNOS expression in a dose-dependent manner. However, 4-acetylanthroquinonol B did not alter COX-2 expression in the concentration range of 5–20 µg/mL.

**Effect of 4-Acetylanthroquinonol B on ALT, AST, GSH, and MDA in Ethanol-Induced HepG2 Cell Damage.** To evaluate the antioxidant activity of 4-acetylanthroquinonol B in vitro, we induced oxidative damage in HepG2 cells. First, we applied the MTT cell survival assay to evaluate the cytotoxicity of 4-acetylanthroquinonol B in HepG2 cells, and the result showed 4-acetylanthroquinonol B was not cytotoxic to HepG2 cells at doses of 5–20 µg/mL (data not shown); therefore, this dose range was determined to be assayed for the protective effect on ALT, AST, and MDA production in EtOH-induced HepG2 damage. As shown in Figure 5, ALT was increased from 0.98 to 3.25 units/L, AST from 0.75 to 2.44 units/L, and MDA also was increased from 0.38 to 2.96 µg/mg protein after ethanol treatment, indicating that cells were suffering from oxidative damage. However, pretreatment with 4-acetylanthroquinonol B significantly reduced ethanol-induced elevation of ALT and AST in a dose-dependent manner (Figure 5A,B). Of note, ethanol-induced hepatic ALT and AST levels were also decreased by the positive control, silymarin. When cells were pretreated with 4-acetylanthroquinonol B, the ethanol-induced MDA elevation was significantly reduced in a dose-dependent



**Figure 4.** Anti-inflammatory effect of 4-acetylantroquinonol B in vitro. (A) Effects of antroquinonol and 4-acetylantroquinonol B on LPS-induced NO production in RAW 264.7 cells. The nitrite inhibition (%) was calculated by using the following formula: nitrite inhibition (%) =  $[(OD_0 - OD_1)/OD_0] \times 100$ , where  $OD_0$  is the average nitrite level of the LPS-treated negative control and  $OD_1$  is the nitrite level of pretreatment of antroquinonol or 4-acetylantroquinonol B for LPS-treated groups. Values represent the mean  $\pm$  SD of three experiments. (B) Immunoblot result shows LPS-induced inflammatory in macrophage by detection levels of iNOS and COX-2 proteins for whole cell lysates. The indicated doses of 4-acetylantroquinonol B or curcumin were added into culture medium at 1 h prior to 1  $\mu$ g/mL LPS stimulation for an additional 24 h.

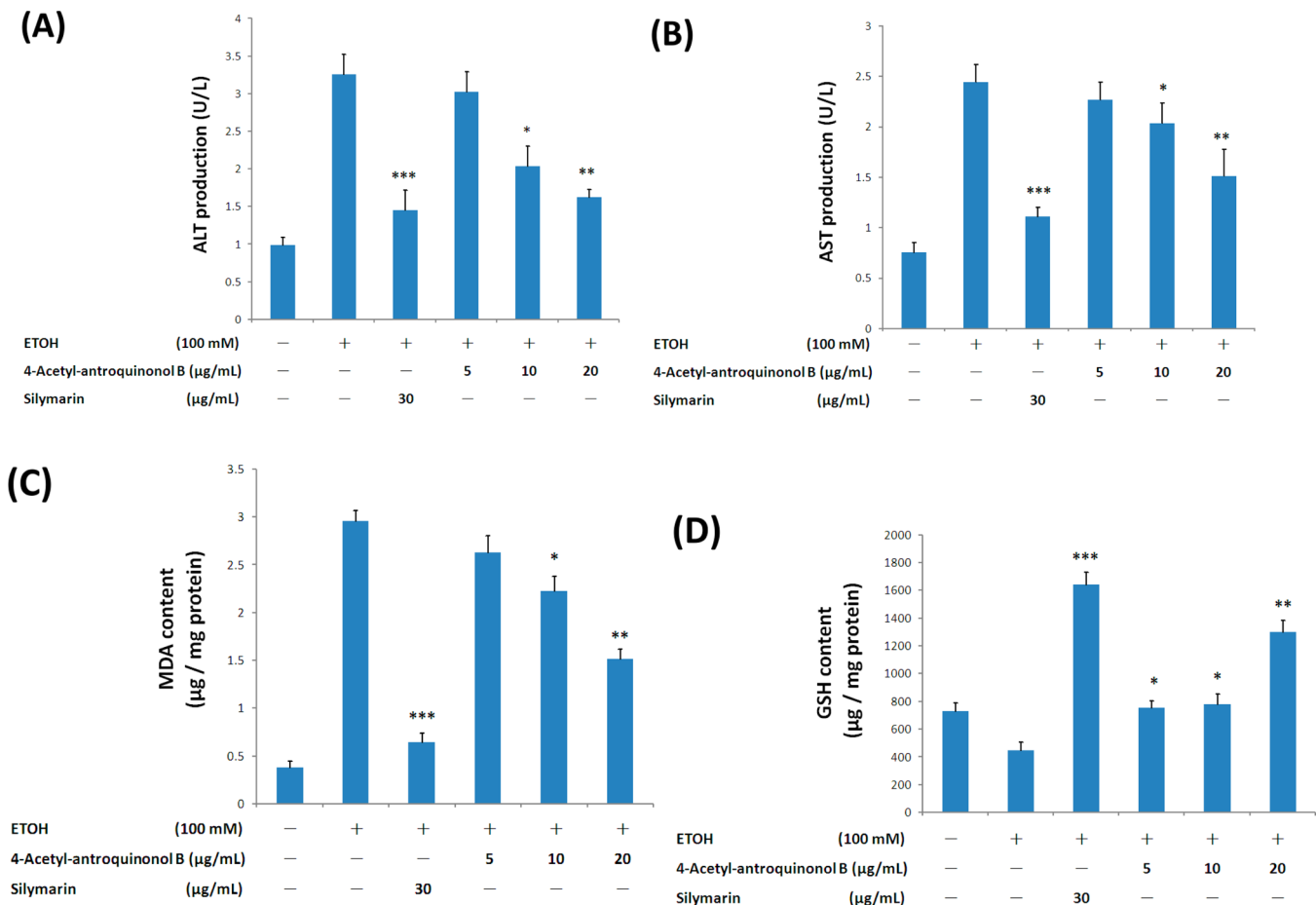
manner (Figure 5C). GSH is the most abundant endogenous antioxidant in hepatocytes. During oxidative stress, increases in GSH consumption result in GSH depletion at the cellular level. We found GSH levels reduced in cultured HepG2 cells treated with ethanol, and pretreatment with 4-acetylantroquinonol B protected against hepatic GSH depletion, as evidenced by the restoration or accumulation of GSH above normal levels (Figure 5D). In comparison with cells exposed to ethanol alone, pretreatment with silymarin also caused highly significant inhibition of MDA and restoration of GSH levels in ethanol-induced HepG2 cells.

**4-Acetylantroquinonol B Increases HO-1 and Nrf-2 Protein Levels in Ethanol-Induced HepG2 cells.** HO-1 is a major antioxidant enzyme that plays an important role in the antioxidant defense system when hepatic cells are damaged by ethanol-induced stress.<sup>26</sup> We hypothesized that 4-acetylantroquinonol B protection on ethanol-induced ALT and AST hepatic enzyme leakage and GSH depression were results from the induction of antioxidant enzymes such as HO-1 and NQO1. As expected, we observed that 5–20  $\mu$ g/mL 4-acetylantroquinonol B significantly increased HO-1 expression levels in ethanol-induced HepG2 cells in a dose-dependent manner (Figure 6A). Silymarin (30  $\mu$ g/mL) also enhanced HO-1 protein expression level. In contrast, antroquinonol did not show a significant effect on protein expression in ethanol-challenged HepG2 cells (data not shown). Furthermore, it has been proved that HO-1 can be activated by Nrf-2, a major transcription factor regulating antioxidant response element

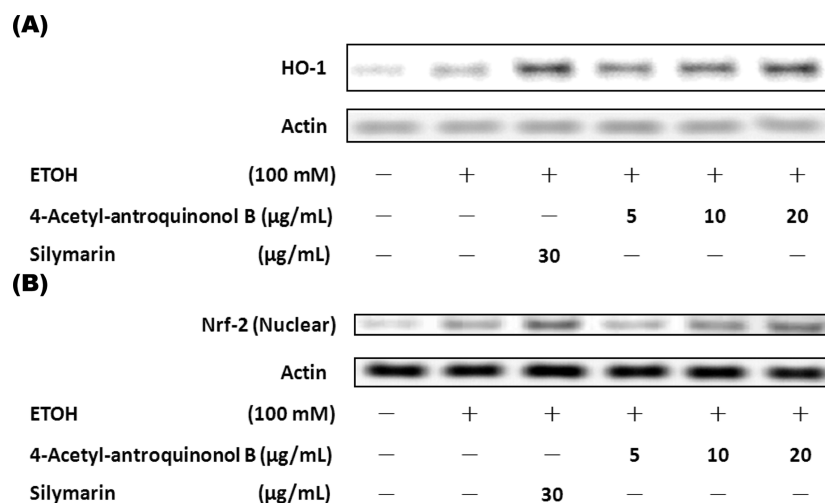
(ARE)-driven phase-II gene expression.<sup>29</sup> We therefore attempted to determine whether 4-acetylantroquinonol B up-regulates HO-1 expression in association with Nrf-2 activation. It is well-known that activated Nrf-2 disassociates from Keap-1 and translocates into the nucleus, where it binds to the ARE of targeted gene promoters. Activation of Nrf-2 was determined by Western blot using nuclear extracts from cultured HepG2 cells. As illustrated in Figure 6B, 4-acetylantroquinonol B treatment increased Nrf-2 accumulation in the nuclear fraction. These results support that 4-acetylantroquinonol B against ethanol-induced hepatic oxidative stress at least through activation of Nrf-2 and induction of HO-1 expression.

## DISCUSSION

*A. cinnamomea* is a unique and precious medicinal fungus indigenous to Taiwan. Many studies have reported evidence of the various biological activities of its fruiting bodies and mycelium. However, wild-growing fruiting bodies of *A. cinnamomea* are very expensive and difficult to obtain; thus, mycelium fermented in either liquid- or solid-state cultures are becoming important commercial products. In our previous study,<sup>21</sup> we demonstrated the hepatoprotective effects of mass-produced mycelium products of *A. cinnamomea* and suggested that a potent bioactive compound, antroquinonol, may responsible for the hepatoprotective activity. However, the complete metabolite fingerprint of this government-certified health food has not been ascertained. Quality control, efficacy approbation, and safety are important requirements for the



**Figure 5.** Antioxidant effect of 4-acetylanthroquinol B in vitro. Effects of 4-acetylanthroquinol B on ethanol-induced ALT, AST, MDA, and GSH depletion in HepG2 liver cells. Cells were preincubated with indicated concentrations of 4-acetylanthroquinol B and silymarin for 1 h and stimulated by ethanol for 12 h. Hepatic ALT (A) and AST (B) were measured in the culture supernatant. Hepatocellular MDA (C) and GSH depletion (D) were assessed from HepG2 cell lysates. Values represent the mean  $\pm$  SD of three experiments. (\*)  $P < 0.05$ , (\*\*)  $P < 0.01$ , and (\*\*\*)  $P < 0.001$  were considered significant for EtOH alone.



**Figure 6.** 4-Acetylanthroquinol B enhances antioxidative protein expression. Immunoblot result shows antioxidative capacity in HepG2 liver cells by detection level of HO-1 protein for whole cell lysates (A) and Nrf-2 protein in nuclear fraction (B). The indicated doses of 4-acetylanthroquinol B or silymarin were added into culture medium at 1 h prior to 100 mM alcohol for an additional 12 h.

development of natural products into health foods and/or pharmaceutical products.<sup>17</sup> Because the crude material and products of *A. cinnamomea* have a high economic value, establishing a quality control platform is a pressing issue for *A.*

*cinnamomea* products. In this study, the metabolite profile of *A. cinnamomea* mycelium was established. Using an HPLC technique, 14 compounds (Figure 1) were selected as index compounds. Among them, anthroquinol (1.78 μg/mg) was

the most abundant compound in the extract of mycelium, followed by inosine (1.61  $\mu\text{g}/\text{mg}$ ), 4-acetylanthroquinol B (1.48  $\mu\text{g}/\text{mg}$ ), and 3-isobutyl-4-[4-(3-methyl-2-butenyloxy)-phenyl]furan-2,5-dione (1.31  $\mu\text{g}/\text{mg}$ ). The contents of two lanostane-type triterpenoids, dehydroeburicoic acid and dehydrosulfurenic acid, were 0.93 and 0.91  $\mu\text{g}/\text{mg}$ , respectively (Table 1 and Figure 2). The evaluation system established in this study may provide a platform for analysis of the solid-state culture mycelium products of *A. cinnamomea*.

According to the definition provided by the U.S. National Cancer Institute (NCI), chemoprevention is the use of drugs, vitamins, or other agents to try to reduce the risk of, or delay the development or recurrence, of cancer. In carcinogenesis, oxidative stress and inflammatory injury are closely related. More and more evidence is accumulating that suggests that dietary phytochemicals and/or functional foods (food supplements) can reduce oxidative and inflammatory conditions in the body and therefore reduce the risk of human malignancies. EMAC showed significant anti-inflammation and antioxidation activities in vivo. Expressions of both LPS-induced iNOS and COX-2 in mouse liver tissue were suppressed after treatment with EMAC (Figure 3B). Meanwhile, the antioxidative activity of mice was also enhanced by uptake of EMAC (Figure 3C). Worthy of note, we did not find particular phenolic compounds in EMAC; thus, the increase in antioxidant activity might be due to EMAC enhancing antioxidative enzymes in mice.

We performed LPS-induced NO production assay as a bioactivity-guided fractionation assay platform and obtained two active compounds, anthroquinol and 4-acetylanthroquinol B. In our previous study, anthroquinol was demonstrated to have liver protective activity by protecting HepG2 cells against ethanol-induced hepatic enzyme leakage, cellular lipid peroxidation, and sustained GSH depletion.<sup>21</sup> Lin and Chiang had demonstrated that 4-acetylanthroquinol B inhibits proliferation of HepG2 cells via affecting p53, p21, and p27 proteins and can be considered as a potential cancer drug.<sup>30</sup> In this study, 4-acetylanthroquinol B was newly found to possess better anti-inflammation activity than anthroquinol (Figure 4A). The anti-inflammatory activities of EMAC and 4-acetylanthroquinol B were separately evidenced by suppression of LPS-induced iNOS and COX-2 expression in vitro and in vivo (Figures 3B and 4B). Like anthroquinol, we found 4-acetylanthroquinol B also has strong antioxidant activity. According to our observation, pretreatment of HepG2 cells with 4-acetylanthroquinol B significantly inhibited ethanol-induced ALT and AST release in a dose-dependent manner (Figure 5A,B). Moreover, the increases in MDA level were significantly attenuated by 4-acetylanthroquinol B pretreatment (Figure 5C). Our results also demonstrated that 4-acetylanthroquinol B pretreatment significantly prevents ethanol-induced GSH depletion and lipid peroxidation in HepG2 cells (Figure 5D). Furthermore, the transcription activator Nrf-2 binds to ARE in the upstream promoter region of many antioxidant genes including HO-1. Our results showed that 4-acetylanthroquinol B treatment significantly increased Nrf-2 activity by means of its nuclear translocation and might further induce HO-1 expression (Figure 6). We suggest that 4-acetylanthroquinol B and anthroquinol are important indices for quality control of *A. cinnamomea* mycelium extract.

Both reactive oxygen species (ROS) and reactive nitrogen species (RNS) participate in normal physiology; however, overproduction of ROS and RNS leads to several pathological processes including carcinogenesis, aging, and inflammation.<sup>31</sup>

These basic research studies thoroughly changed people's health concept and behavior. Along with the increasing demand for health supplements, most phenolic compound containing foods were successfully developed to scavenge harmful reactive radicals. In the present study, we established the metabolite profiling for a solid-state growth mycelium product of *A. cinnamomea*, which has been certified as a Health Food by the Department of Health in Taiwan, and the majority of constituents are not phenolic, significantly different from other products. Our in vivo and in vitro study results demonstrated that this *A. cinnamomea* product possessed significant anti-inflammatory and antioxidative activities through alteration of gene expressions. In this way, it might have potential for use as a chemoprevention supplement.

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### Notes

The authors declare no competing financial interest.

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