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Metabolite Profiles for *Antrodia cinnamomea* Fruiting Bodies Harvested at Different Culture Ages and from Different Wood Substrates

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Supporting Information

ABSTRACT: *Antrodia cinnamomea* is a precious edible fungus endemic to Taiwan that has long been used as a folk remedy for health promotion and for treating various diseases. In this study, an index of 13 representative metabolites from the ethanol extract of *A. cinnamomea* fruiting body was established for use in quality evaluation. Most of the index compounds selected, particularly the ergostane-type triterpenoids and polyacetylenes, possess good anti-inflammation activity. A comparison of the metabolite profiles of different ethanol extracts from *A. cinnamomea* strains showed silmilar metabolites when the strains were grown on the original host wood (*Cinnamomum kanehirai*) and harvested after the same culture time period (9 months). Furthermore, the amounts of typical ergostane-type triterpenoids in *A. cinnamomea* increased with culture age. Culture substrates also influenced metabolite synthesis; with the same culture age, *A. cinnamomea* grown on the original host wood produced a richer array of metabolites than *A. cinnamomea* cultured on other wood species. We conclude that analysis of a fixed group of compounds including triterpenoids, benzolics, and polyacetylenes constitutes a suitable, reliable system to evaluate the quality of ethanol extract from *A. cinnamomea* fruiting bodies. The evaluation system established in this study may provide a platform for analysis of the products of *A. cinnamomea*.

KEYWORDS: *Antrodia cinnamomea*, fruiting body, metabolites profiling, quality control, anti-inflammation activity

INTRODUCTION

Antrodia cinnamomea (Syn. *Antrodia camphorata* and *Taiwanofungus camphorata*) is a precious edible fungus endemic to Taiwan that has long been used as a folk remedy for treating various diseases including liver diseases, hypertension, abdominal pain, and cancer.[1−3] Owing to its perceived efficacy, the selling price of *A. cinnamomea* fruting bodies is above US$10,000 per kilogram in several Asian countries.[4] Over the past decade, numerous bioactivity studies have investigated the physiology and biochemical and pharmacological properties of *A. cinnamomea*,[5−16] and many health-promotion functions of the ingredients of *A. cinnamomea* have been recognized. Although some studies have reported that the health-promotion functions of *A. cinnamomea* are specifically connected to its growth on the aromatic tree *Cinnamomum kanehirai* Hayata,[17−19] the market-driven search for a means to mass produce *A. cinnamomea* mycelium, as well as cultivate its fruiting bodies, has recently led to the successful cultivation of *A. cinnamomea* on other tree species besides *C. kanehirai*.

Quality control, efficacy approbation, and safety are important requirements for the development of ethnomedicines into mainstream international pharmaceutical products. Since the crude material and products of *A. cinnamomea* have a high market value, in addition to investigations into bioactivity and mass-production technique development, establishing a quality control platform has become a pressing issue for *A. cinnamomea* products. Zhao and Leung conducted high-performance liquid chromatography (HPLC) coupled with diode array detection and mass spectrometry to evaluate the quality of *A. cinnamomea* mycelium.[20] However, to the best of our knowledge, a protocol for evaluating the metabolite composition of the fruiting bodies of *A. cinnamomea* has not been established. With regard to the investigation of metabolites, over eighty compounds have been identified in the fruiting body of *A. cinnamomea* to date. Compared with the type of compound between fruiting body with mycelia, the ergostane-type triterpenoid and polyacetylene

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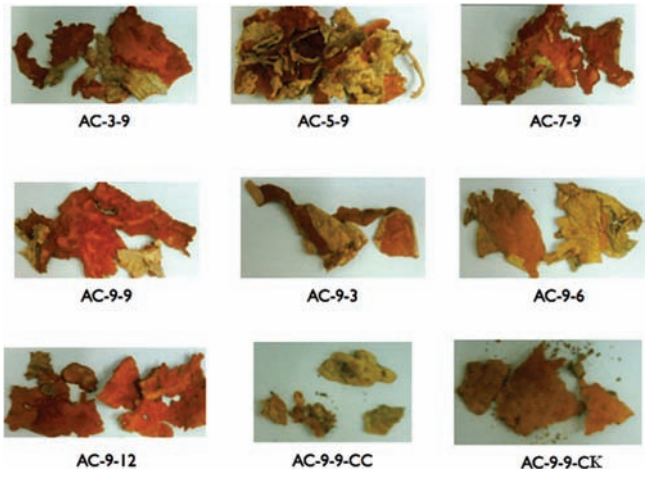

Figure 1. Morphological observations of *A. cinnamomea* fruiting bodies in this study.

are only found in fruiting body and have never been found in mycelia.[2,3] It indicates that ergostane-type triterpenoid and polyacetylene might be used as index compounds for quality control purposes.

Over the past few years, different strains of *A. cinnamomea* have been collected and cultured in our laboratory. Fruiting bodies have been successfully developed and grown on different wood substrates including two broad-leaf trees, *Cinnamomum kanehirai* (the original host) and *C. camphora*; and one conifer, *Cunninghamia konishii*. Here, thirteen compounds representative of *A. cinnamomea* fruting bodies were selected as index compounds to analyze the metabolite composition of *A. cinnamomea* produced at different culture ages and grown on different wood substrates. Analyses of the these index metabolites in various strains of *A. cinnamomea* and grown on various wood substrates for varying periods of time suggest that the profiles of these 13 compounds constitute a suitable, reliable evaluation system of the metabolite composition of *A. cinnamomea* fruiting body extracts.

MATERIALS AND METHODS

***Antrodia cinnamomea* Fruiting Bodies.** The *A. cinnamomea* fruiting bodies analyzed in this study were cultured in the Laboratory of Tree Metabolomics and Natural Medicine, Department of Forestry, National Chung-Hsing University, between June 2008 and December 2009. All the strains of *A. cinnamomea* were provided and identified by Dr. Wen-Wei Hsiao (Experimental Forest, National Taiwan University). Voucher specimens (AC-3, AC-5, AC-7, AC-9) were deposited in the same laboratory. Four different *A. cinnamomea* strains, named AC-3, AC-5, AC-7, and AC-9, were grown on *Cinnamomum kanehirai* wood for nine months, and designated as AC-3-9, AC-5-9, AC-7-9, and AC-9-9. AC-9 was also grown on *Cinnamomum kanehirai* wood for different periods of time. These samples were designated as AC-9-3, AC-9-6, AC-9-9, and AC-9-12, representing culture times of 3, 6, 9, and 12 months, respectively. In addition, two further wood species, *Cinnamomum camphora* (CC) and *Cunninghamia konishii* (CK), were inoculated with AC-9 and the fruiting bodies were also harvested at nine months. All of the woods used in this study were identified by Professor Sheng-Yang Wang (Department of Forestry, NCHU, Taiwan). The harvested ages for the woods of CC and CK are 20 and 35 years, respectively. These two samples were designated AC-9-9-CC and AC-9-9-CK. Figure 1 shows the morphology of all the fruiting bodies obtained.

Determination of Optimal *A. cinnamomea* Extraction Time. To evaluate the optimal extraction time for the preparation of the extract for analysis of metabolites, fresh material was lyophilized for 72 h and ground *A. cinnamomea* powder (particle diameter <0.7 mm) was accurately weighed (around 5 g), placed in an Erlenmeyer flask (250 mL) with 100 mL of ethanol (EtOH) and sonicated in an ultrasonicator (Branson 5510, Branson Ultrasonic, Ontario, Canada) for 10, 20, 30, 60, and 120 min. The extracts were then decanted, filtered under vacuum, concentrated in a rotary evaporator and lyophilized. Yield data (w/w) were expressed as mean ± SE (n = 3).

Nitric Oxide Inhibitory Assay. The effect of the compounds isolated from *A. cinnamomea* extracts on NO production was measured indirectly by analysis of nitrite levels using the Greiss reaction.[21,22] Briefly, RAW 264.7 cells grown in a 75 cm^2 culture dish were seeded in 96-well plates at a density of 2×10^5 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_2 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 using the supernatant from the RAW 264.7 cells by the Griess reaction.[23] Results were expressed as a percentage of inhibition relative to the control (cell treated with LPS alone). IC_{50} represents the levels at which 50% of the radicals were scavenged by test samples.

Identification of Index Compounds. Five hundred eighty grams of air-dried fruiting bodies of *A. cinnamomea* (AC-9 strain) was extracted with 95% ethanol (EtOH) at ambient temperature. Total crude EtOH extract was concentrated under vacuum to yield a residue (183.9 g). The crude extract was then redissolved in methanol (10 mg/mL) and passed through an SPE cartridge (Sep-Pak C18, Waters Co., 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(2) column (250 × 10.0 mm; Phenomenex, Torrance CA) was employed with three solvent systems, H_2O (A), MeOH (B) and acetonitrile (C). The gradient elution profile was as follows: 0−5 min, A:B:C = 40:30:30 (isocratic); 5−95 min, A:B:C = 40:30:30 to A:B:C = 10:10:80 (linear gradient); 95−105 min, A:B:C = 10:10:80 to A:B:C = 0:0:100 (linear gradient); 105−115 min, 100% C (isocratic); the flow rate was 0.5 mL/min at 0−95 min; 1.0 mL/min at 95 to 115 min; and the detector wavelength was set at 254 nm. Thirteen compounds in the EtOH extracts were obtained at retention times of 17.9 min (**a**), 20.8 min (**b**), 38.1 min (**c**), 41.2 min (**d**), 42.1 min (**e**), 46.1 min (**f**), 48.7 min (**g**), 51.8 min (**h**), 53.4 min (**i**), 55.0 min (**j**), 67.8 min (**k**), 73.2 min (**l**), and 102.7 min (**m**).

The structures of these compounds were elucidated and confirmed by spectroscopic analysis as follows: compound **a**, (*R*,*S*)-antcin K;[6] compound **b**, 1,4-dimethoxy-2,3-methylenedioxy-5-methylbenzene;[10] compound **c**, (*R*,*S*)-antcin C;[24] compound **d**, antrocamphin A;[10] compound **e**, 2,2′,5,5′-tetramethoxy-3,4,3′,4′-bimethylenedioxy-6,6′-dimethylbiphenyl;[25] compound **f**, (*R*,*S*)-antcin H;[26] compound **g**, dehydrosulfurenic acid;[27] compound **h**, antrocamphin C (new compound); **i**, (*R*,*S*)-antcin B;[24] compound **j**, (*R*,*S*)-antcin G;[28] compound **k**, (*R*,*S*)-antcin A;[24] compound **l**, 15-acetyldehydrosulfurenic acid;[29] compound **m**, aehydroeburicoic acid.[24] UV spectra were recorded on a Jasco V-550 spectrophotometer, and IR spectra were recorded on a Bio-Rad FTS-40 spectrometer. Electrospray ionization-mass spectrometric spectrometry (ESIMS) data was collected with a Finnigan MAT-95S mass spectrometer, and NMR spectra were recorded with Varian Inova 600 and 400 MHz FT-NMR spectrometers, at 600 or 400 MHz (^{1}H) and 150 MHz (^{13}C). *d*-Chloroform ($CDCl_3$) and *d*-methanol (CD_3OD) were used for NMR analysis.

The Metabolite Profiling and Quantification of Ethanol Extract of *A. cinnamomea* Fruiting Bodies. First, the metabolite

Table 1. Influence of Extraction Time on Yield of *A. cinnamomea* Extract

extraction time (min)	yield[a] (%)
10	27.75 ± 2.95 a
20	28.25 ± 1.41 a
30	28.84 ± 1.36 a
60	31.34 ± 2.19 b
120	31.91 ± 4.16 b

[a] Data are given as mean ± SE ($n = 3$). The mean values indicated by the letter "a" were significantly different from the mean values indicated by the letter "b" ($P < 0.05$).

profile of ethanol extract prepared from the AC-9 strain was established by using the 13 index compounds (**a** to **m**) identified above. The standard calibration curves (peak area *vs* concentration) of each index compound were determined at a range of compound concentrations, 10, 25, 50, 100, 250, 500, and 1000 μg/mL. Quantification of the content of each index compound in fruiting bodies from different strains of *A. cinnamomea* was then performed by HPLC analysis. The peak areas of the index compounds in the chromatogram of the EtOH extracts (with known loading concentration) were then defined, and their contents in the extracts were calculated on the basis of the quantity calibrated from the standard calibration curves. The analyses were performed in triplicate, and the results were presented as mean ± SE.

Statistical Analysis. Data are expressed as means ± SE. The significance of differences between group means were analyzed by analysis of variance (ANOVA) using Dunnett's test. Mean values within each column with different labels (a, b, c, d) are significantly different ($p < 0.05$). In the meantime, cluster analysis was performed with MVSP (multivariate statistical package) program[30] to evaluate the similarity of metabolites composition of *A. cinnamomea* fruiting bodies of different AC strains.

RESULTS

More than eighty compounds have been isolated from the fruiting body and mycelium of *A. cinnamomea*. According to a literature survey, benzoquinones, maleic/succinic derivatives, nucleotides and fatty acids as well as triterpenoids and steroids can be found in *A. cinnamomea* mycelium. However, triterpenoids (including steroids), benzolics, and polyacetylenes are the representative compounds.[2,3] In order to establish a means to assess the relative quality of various *A. cinnamomea* extracts here we established a chromatographic profiling of secondary metabolites of *A. cinnamomea*, focusing on triterpenoids (including steroids), benzolics, and polyacetylenes.

Optimum Extraction Time. First, to determine an optimal extraction procedure for analysis of index metabolites in *A. cinnamomea* extracts, we investigated the effect of *A. cinnamomea* extraction time on extraction yield. Table 1 shows the influence of extraction time on yields of *A. cinnamomea* extract. Yields increased with extraction time. As there was no significant difference between the 60 min and the 120 min extraction time, which gave yields of 31.34 ± 2.19% and 31.91 ± 4.16%, respectively, the 60 min extraction time was selected for further assays.

Determination of Index Compounds. A comprehensive profile of the ethanol extract of *A. cinnamomea* fruiting body was established as shown in Figure 2. The compounds represented by the thirteen main chromatographic peaks (**a** to **m**, Figure 2) were collected, purified and identified. The structures

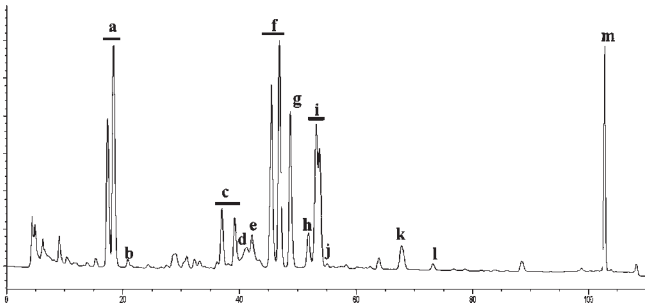

Figure 2. HPLC chemical fingerprint of ethanol extract of *A. cinnamomea* fruiting body (AC-9-9). **a**: (*R*,*S*)-Antcin K. **b**: 1,4-Dimethoxy-2,3-methylenedioxy-5-methylbenzene. **c**: (*R*,*S*)-Antcin C. **d**: Antrocamphin A. **e**: 2,2′,5,5′-Tetramethoxy-3,4,3′,4′-bimethylenedioxy-6,6′-dimethylbiphenyl. **f**: (*R*,*S*)-Antcin H. **g**: Dehydrosulfurenic acid. **h**: Antrocamphin C. **i**: (*R*,*S*)-Antcin B. **j**: (*R*,*S*)-Antcin G. **k**: (*R*,*S*)-Antcin A. **l**: 15-Acetyldehydrosulfurenic acid. **m**: Dehydroeburicoic acid.

of the compounds were confirmed by spectroscopic analysis. The compounds could be divided into three major chemical groups: (1) triterpenoids and steroids [(*R*,*S*)-antcin K (**a**), (*R*,*S*)-antcin C (**c**), (*R*,*S*)-antcin H (**f**), dehydrosulfurenic acid (**g**), (*R*,*S*)-antcin B (**i**), (*R*,*S*)-antcin G (**j**), (*R*,*S*)-antcin A (**k**), 15-acetyldehydrosulfurenic acid (**l**), dehydroeburicoic acid (**m**)]; (2) polyacetylenes [antrocamphin A (**d**) and compound **h**]; and (3) benzenoids [1,4-dimethoxy-2,3-methylenedioxy-5-methylbenzene (**b**), 2,2′,5,5′-tetramethoxy-3,4,3′,4′-bimethylenedioxy-6,6′-dimethylbiphenyl (**e**)]. Besides compound **h**, all of the compounds from the fruiting body of *A. cinnamomea* have been previously identified and our spectral data were in good agreement with the literature (Detailed spectroscopic data for known compounds are listed in the Supporting Information).

The molecular structure of compound **h** was determined to be $C_{15}H_{16}O_4$ by ESI-MS (m/z 260) and HREIMS m/z 260.1042 $[M]^+$ $C_{15}H_{16}O_4$ (calculated 260.1049). Compound **h** showed 15 carbon signals, including six aromatic carbons (δ_C = 139.8, 139.5, 137.1, 136.2, 127.9, 109.8), two acetylenic carbons (δ_C = 97.5, 83.5), two olefinic carbons (δ_C = 127.2, 121.1), two methoxyl groups (δ_C = 60.4, 60.0), two methyl carbons (δ_C = 23.6, 13.9) and one methylene carbon (δ_C = 101.4). In the ^{1}H NMR spectra, compound **h** showed two methoxy groups (δ_H = 3.98, s, 3H; 3.87, s, 3H), one methylenedioxy proton (δ_H = 5.98, s, 2H), two vinyl protons (δ_H = 5.38, br s, 1H; 5.27, br s, 1H), and two methyl protons (δ_H = 2.27, s, 3H; 2.01, s, 3H). The carbon and proton numbers, based on ^{13}C and ^{1}H NMR spectroscopies, were consistent with those of high resolution electron ionization mass spectra (HREIMS). Compound **h** had ^{1}H and ^{13}C NMR data similar to those of antrocamphin A[10] with the exception of methoxyl group number and methylenedioxy. This new isolated compound **h** was named antrocamphin C. The spectra of antrocamphin C are provided in the Supporting Information (Supplement Figure 1 to Supplement Figure 7), and the spectral data are summarized as follows.

Antrocamphin C (h). Yellow liquid, HREIMS m/z 260.1042, $[M]^+$ $C_{15}H_{16}O_4$ (calculated 260.1049). EIMS (70 eV) m/z (rel int): 260 (100) $[M]^+$, 245 (28), 161 (17), 146 (7), 128 (6), 117 (6), 116 (8), 115 (14), 104 (14), 103 (11), 91 (10), 77 (10). ^{1}H NMR δ (600 MHz, $CDCl_3$): 5.94 (2H, s, $COCH_2OC$), 5.38 (1H, br s, H_b-4′), 5.27 (1H, br s, H_a-4′), 3.98 (3H, s, OCH_3-5), 3.87 (3H, s, OCH_3-6), 2.27 (3H, s, CH_3-3), 2.01 (3H, s, CH_3-3′). ^{13}C NMR δ (125 MHz, $CDCl_3$): 139.8 (C-5), 139.5 (C-1),

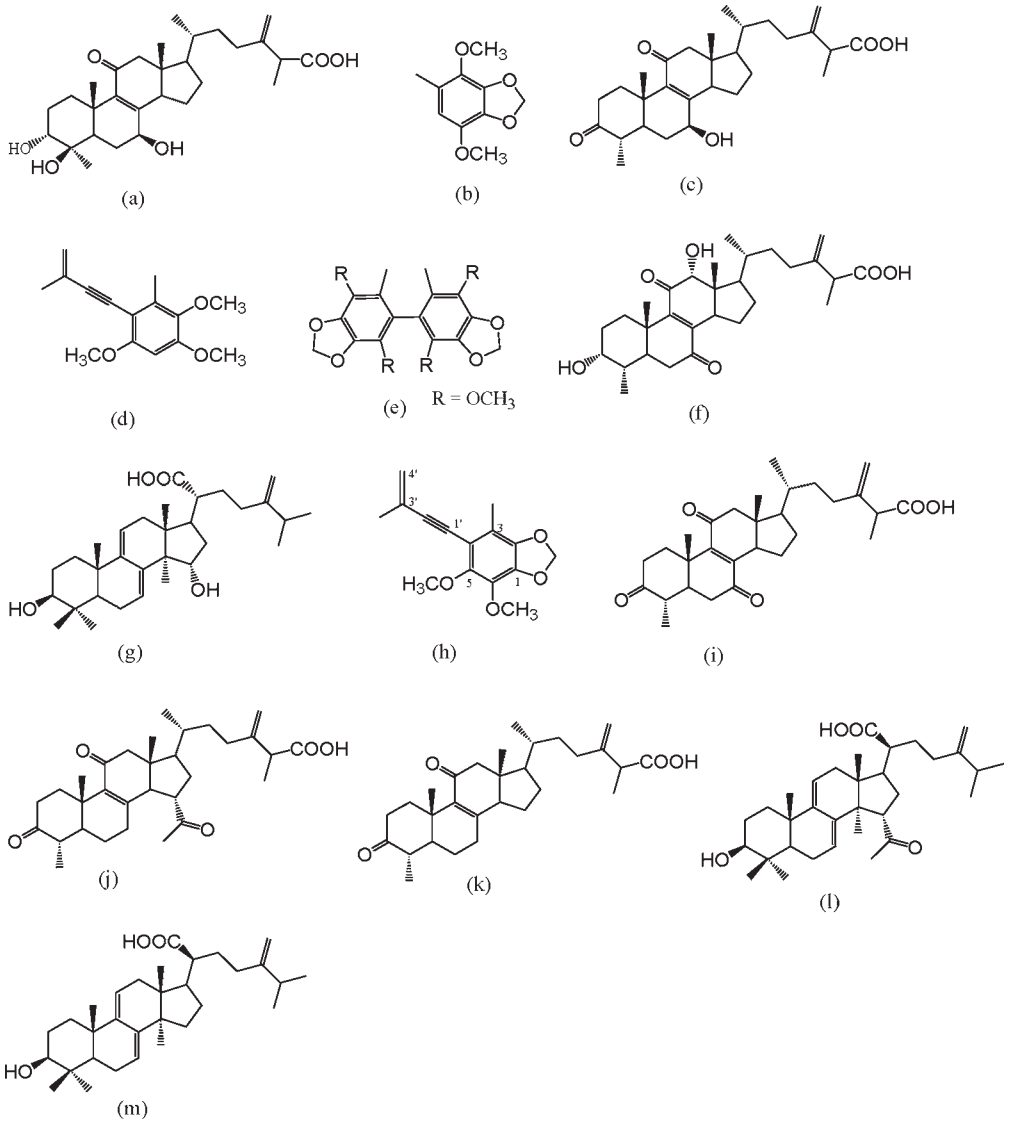

Figure 3. Index compounds of *A. cinnamomea* fruiting body. **a**: (*R*,*S*)-Antcin K. **b**: 1,4-Dimethoxy-2,3-methylenedioxy-5-methylbenzene. **c**: (*R*,*S*)-Antcin C. **d**: Antrocamphin A. **e**: 2,2′,5,5′-Tetramethoxy-3,4,3′,4′-bimethylenedioxy-6,6′-dimethylbiphenyl. **f**: (*R*,*S*)-Antcin H. **g**: Dehydrosulfurenic acid. **h**: Antrocamphin C. **i**: (*R*,*S*)-Antcin B. **j**: (*R*,*S*)-Antcin G. **k**: (*R*,*S*)-Antcin A. **l**: 15-Acetyldehydrosulfurenic acid. **m**: Dehydroeburicoic acid.

137.1 (C-2), 136.2 (C-6), 127.9 (C-3), 127.2 (C-3′), 121.1 (C-4′), 109.8 (C-4), 101.4 ($COCH_2OC$), 97.5 (C-2′), 83.5 (C-1′), 60.4 (OCH_3-5), 60.0 (OCH_3-6), 23.6 (CH_3-3′), 13.9 (CH_3-3).

Figure 3 shows the structures of index compounds identified in *A. cinnamomea*. Among the 13 index compounds obtained in this study, benzenoids **b** and **e** and polyacetylenes **d** and **h** are compounds obtained only in fruiting bodies of *A. cinnamomea*.

Quantification of Index Compounds in *A. cinnamomea* Fruiting Bodies of Various Strains Harvested at Nine Months. Compounds **a** to **m** were then used as index compounds to profile metabolite composition in *A. cinnamomea* extracts from various strains. First, a solution mixed with 13 index compounds (the concentration of each compounds was 1 mg/mL) was profiled by HPLC. The top chromatogram (Standard) in Figure 4 shows profiles of compounds **a** to **m**. Some of the compounds, (*R*,*S*)-antcin K (**a**), (*R*,*S*)-antcin C (**c**), (*R*,*S*)-antcin H (**f**), (*R*,*S*)-antcin B (**i**), and (*R*,*S*)-antcin G (**j**), showed 2 peaks, for *R* and *S* configurations, as these compounds contain a chiral center at the C_{25} position. The absolute configuration for this group of compounds could not be characterized by NMR directly, so further *R*/*S* derivatives were needed to determine their absolute configuration. Thus, both *R*-form and *S*-form triterpenoids were treated as the same compound in this study. To measure the content of the index compounds in different *A. cinnamomea* samples, calibration curves of the index compounds were established using seven dilution standards from 10 to 1000 μg/mL. Supplement Table 1 in the Supporting Information shows the regression parameter and linearity of the proposed HPLC profiling method.

Metabolite profiles for four different *A. cinnamomea* strains (AC-3-9, AC-5-9, AC-7-9, and AC-9-9) harvested at culture age 9 months are shown in Figure 4. Overall, the four strains revealed similar metabolite compositions. The contents of 13 index compounds were determined by the peak area in the HPLC profile and calculated by using the calibration curves of index compounds (purity >99.5%) (Table 2). The ergostane-type

triterpenoid (*R*,*S*)-antcin B was the most aboundant compound in all four different strains, with contents in fruiting bodies ranging from 3.127 mg/g (AC-3-9) to 5.787 mg/g (AC-7-9). Of the lanostane-type triterpenoids, dehydrosulfurenic acid was the most abundant compound, especially in the AC-5-9 strain (content 7.866 mg/g). The AC-5-9 strain contained more antrocamphin A (0.044 mg/g) and antrocamphin C (5.390 mg/g) than the other strains. According to the literature survey and the analysis data obtained in this study, ergostane-type triterpenoids are only found in the *A. cinnamomea* fruiting bodies. By comparison of the contents of ergostane-type triterpenoids, including of antcin A, antcin B, antcin C, antcin G, antcin H, and antcin K, the result of the statistic showed that the components of AC-3-9, AC-7-9, and AC-9-9 are similar (Table 2 and Figure 7). The strain AC-5-9 has different composition compared to the other three strains. It had been reported that the ergostane-type triterpenoids possess various bioactivities; by this analysis protocol we can select the strain which could produce the most ergostane-type triterpenoids in the future.

Quantification of Index Compounds of *A. cinnamomea* Fruiting Bodies at Different Ages and Grown on Different Wood Substrates. When the AC-9 strain was grown on the original host, *Cinnamomum kanehirai*, for varying periods of time (3, 6, 9, or 12 months), various metabolite patterns were seen (Figure 5). In the extract of the AC-9 strain fruiting body harvested at age 3 months (AC-9-3), there were only two significant peaks, compound **g** (dehydrosulfurenic acid) and

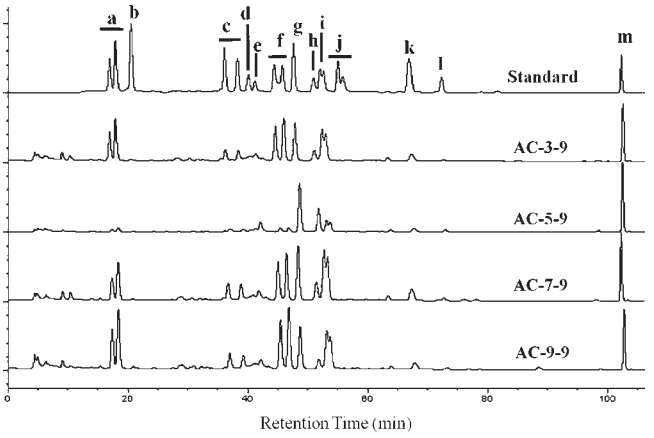

Figure 4. HPLC profiling of index compounds of *A. cinnamomea* fruiting body and ethanol extracts of four different *A. cinnamomea* strains (AC-3, AC-5, AC-7, and AC-9) grown on *Cinnamomum kanehirai* wood for nine months. Peaks: **a**, (*R*,*S*)-antcin K; **b**, 1,4-dimethoxy-2,3-methylenedioxy-5-methylbenzene; **c**, (*R*,*S*)-antcin C; **d**, antrocamphin A; **e**, 2,2′,5,5′-tetramethoxy-3,4,3′,4′-bimethylenedioxy-6,6′-dimethylbiphenyl; **f**, (*R*,*S*)-antcin H; **g**, dehydrosulfurenic acid; **h**, antrocamphin C; **i**, (*R*,*S*)-antcin B; **j**, (*R*,*S*)-antcin G; **k**, (*R*,*S*)-antcin A; **l**, 15-acetyldehydrosulfurenic acid; **m**, dehydroeburicoic acid.

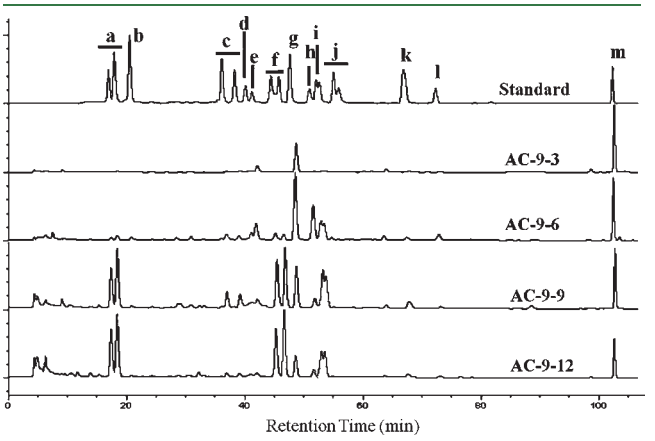

Figure 5. HPLC profiling of index compounds of *A. cinnamomea* fruiting body and ethanol extracts of the same *A. cinnamomea* strains (AC-9) grown on *Cinnamomum kanehirai* wood for different months (3, 6, 9 and 12). Peaks: **a**, (*R*,*S*)-antcin K; **b**, 1,4-dimethoxy-2,3-methylenedioxy-5-methylbenzene; **c**, (*R*,*S*)-antcin C; **d**, antrocamphin A; **e**, 2,2′,5,5′-tetramethoxy-3,4,3′,4′-bimethylenedioxy-6,6′-dimethylbiphenyl; **f**, (*R*,*S*)-antcin H; **g**, dehydrosulfurenic acid; **h**, antrocamphin C; **i**, (*R*,*S*)-antcin B; **j**, (*R*,*S*)-antcin G; **k**, (*R*,*S*)-antcin A; **l**, 15-acetyldehydrosulfurenic acid; **m**, dehydroeburicoic acid.

Table 2. Quantification of Index Compound Contents from 4 Different Strains of Fruiting Body of *A. cinnamomea* Grown on *Cinnamomum kanehirai* Wood for 9 Months

compound	content (mg/g dried fruiting bodies)[a]			
	AC-3-9	AC-5-9	AC-7-9	AC-9-9
(*R*,*S*)-antcin K (**a**)	1.956 ± 0.154 b	0.191 ± 0.013 a	3.250 ± 0.110 c	4.319 ± 0.587 d
1,4-dimethoxy-2,3-methyl-enedioxy-5-methylbenzene (**b**)	<0.001 a	<0.001 a	<0.001 a	0.001 ± 0.000 b
(*R*,*S*)-antcin C (**c**)	1.265 ± 0.104 a	0.820 ± 0.063 a	0.917 ± 0.015 a	1.537 ± 1.020 a
antrocamphin A (**d**)	<0.001 a	0.044 ± 0.005 b	0.083 ± 0.002 c	0.046 ± 0.005 d
2,2′,5,5′-tetramethoxy-3,4,3′,4′-bimethylenedioxy-6,6′-dimethylbiphenyl (**e**)	0.013 ± 0.000 a	0.238 ± 0.001 d	0.216 ± 0.001 c	0.081 ± 0.007 b
(*R*,*S*)-antcin H (**f**)	3.319 ± 0.304 b	0.072 ± 0.005 a	3.760 ± 0.273 c	3.921 ± 0.162 c
dehydrosulfurenic acid (**g**)	1.226 ± 0.032 a	7.866 ± 0.224 c	2.816 ± 0.298 b	1.389 ± 0.077 a
antrocamphin C (**h**)	0.722 ± 0.002 c	5.390 ± 0.003 d	0.286 ± 0.002 a	0.426 ± 0.040 b
(*R*,*S*)-antcin B (**i**)	3.127 ± 0.408 a	2.889 ± 0.364 a	5.787 ± 0.501 b	3.381 ± 0.364 a
(*R*,*S*)-antcin G (**j**)	<0.001 a	0.002 ± 0.000 b	0.010 ± 0.002 d	0.006 ± 0.000 c
(*R*,*S*)-antcin A (**k**)	0.635 ± 0.051 a	0.767 ± 0.002 b	1.153 ± 0.089 d	0.983 ± 0.059 c
15-acetyldehydrosulfurenic acid (**l**)	0.008 ± 0.010 a	0.232 ± 0.021 c	0.225 ± 0.026 c	0.073 ± 0.020 b
dehydroeburicoic acid (**m**)	2.402 ± 0.191 c	6.284 ± 0.492 d	1.921 ± 0.220 ab	1.683 ± 0.187 a

[a] All contents are calculated as the standard linear equation, where *y* is the peak area and *x* is the concentration of the analyzed material. Mean values within each column with different labels (a, b, c, d) are significantly different ($p < 0.05$).

Table 3. Quantification of Index Compound Contents from the Fruiting Bodies of *A. cinnamomea* Strains Grown on *Cinnamomum kanehirai* Wood for 3, 6, 9, and 12 Months

compound	content (mg/g dried fruiting bodies)[a]			
	AC-9-3	AC-9-6	AC-9-9	AC-9-12
(*R*,*S*)-antcin K (**a**)	<0.001 a	0.322 ± 0.004 a	4.319 ± 0.587 b	4.329 ± 0.207 b
1,4-dimethoxy-2,3-methyl-enedioxy-5-methylbenzene (**b**)	<0.001 a	0.005 ± 0.001 c	0.001 ± 0.000 b	0.001 ± 0.000 b
(*R*,*S*)-antcin C (**c**)	<0.001 a	2.059 ± 0.080 b	1.537 ± 1.020 b	0.362 ± 0.071 a
antrocamphin A (**d**)	<0.001 a	0.166 ± 0.004 c	0.046 ± 0.005 b	0.003 ± 0.002 a
2,2′,5,5′-tetramethoxy-3,4,3′,4′-bimethylenedioxy-6,6′-dimethylbiphenyl (**e**)	0.841 ± 0.001 d	0.530 ± 0.004 c	0.081 ± 0.007 b	0.025 ± 0.001 a
(*R*,*S*)-antcin H (**f**)	<0.001 a	0.911 ± 0.020 b	3.921 ± 0.162 c	4.807 ± 0.226 d
dehydrosulfurenic acid (**g**)	10.312 ± 0.511 c	6.234 ± 0.302 b	1.389 ± 0.077 a	1.135 ± 0.025 a
antrocamphin C (**h**)	<0.001 a	1.874 ± 0.016 d	0.426 ± 0.040 b	0.660 ± 0.001 c
(*R*,*S*)-antcin B (**i**)	<0.001 a	3.109 ± 0.792 b	3.381 ± 0.364 b	2.598 ± 0.497 b
(*R*,*S*)-antcin G (**j**)	0.011 ± 0.001 b	0.121 ± 0.001 d	0.006 ± 0.000 a	0.014 ± 0.000 c
(*R*,*S*)-antcin A (**k**)	0.103 ± 0.002 a	0.451 ± 0.000 b	0.983 ± 0.059 c	0.068 ± 0.022 a
15-acetyldehydrosulfurenic acid (**l**)	0.032 ± 0.042 a	1.041 ± 0.106 b	0.073 ± 0.020 a	0.058 ± 0.010 a
dehydroeburicoic acid (**m**)	18.058 ± 1.351 c	5.821 ± 0.740 b	1.683 ± 0.187 a	0.887 ± 0.128 a

[a] All contents are calculated as the standard linear equation, where y is the peak area and x is the concentration of the analyzed material. Mean values within each column with different labels (a, b, c, d) are significantly different ($p < 0.05$).

compound **m** (dehydroeburicoic acid) (Figure 5). Both of these two compounds are lanostane-type triterpenoids. When the growth period was increased to 6 months (AC-9-6 in Figure 5), ergostane-type triterpenoids started to be synthesized. Results for all four culture ages are summarized in Table 3. The amount of ergostane-type triterpenoids increased significantly when the growth on wood was over six months; whereas the amounts of lanostane-type triterpenoids decreased with increase in culture age.

AC-9 was also cultured on different wood substrates, *Cinnamomum kanehirai* (AC-9-9), *Cinnamomum camphora* (AC-9-9-CC), and *Cunninghamia konishii* (AC-9-9-CK), for nine months. The metabolite profiles of *A. cinnamomea* grown on different wood substrates are shown in Figure 6. The metabolite profiles for *A. cinnamomea* that was grown on nonoriginal substrate woods, *C. camphora* (AC-9-9-CC), and *C. konishii* (AC-9-9-CK), with the same culture time, were considerably different from that of *A. cinnamomea* cultured on *C. kanehirai* (AC-9-9). In AC-9-9-CC and AC-9-9-CK the lanostane-type triterpenoids, dehydrosulfurenic acid (**g**) and dehydroeburicoic acid (**m**), were the main constituents. AC-9-9-CC had 8.717 mg/g and 11.895 mg/g of **g** and **m**, respectively. Similar results were obtained for AC-9-9-CK which had 5.895 mg/g and 3.914 mg/g of **g** and **m**, respectively. In addition, there was considerably less of the ergostane-type triterpenoids, polyacetylenes, and benzenoids in AC-9-9-CC and AC-9-9-CK than in AC-9-9 (Table 4). From the result of similarity analysis (Figure 7), the composition of AC-9 cultured on *C. kanehirai* is significantly different from AC-9 cultured on the other wood (AC-9-9-CC and AC-9-9-CK) in the same period, nine months. However, the compositions are similar in the AC-7-9, AC-9-9, and AC-3-9 strains, rather than AC-5-9. From these results it may be concluded that ergostane-type triterpenoids were only produced from *A. cinnamomea* which were cultured on the original substrate *C. kanehirai*.

Antiinflammation Activity of *A. cinnamomea* Fruiting Body Index Compounds. To evaluate the antiinflammation activity of index compounds from *A. cinnamomea* fruiting bodies, an LPS-stimulated murine macrophage assay system was used. Figure 8 shows the inhibitory effects of the 13 index compounds.

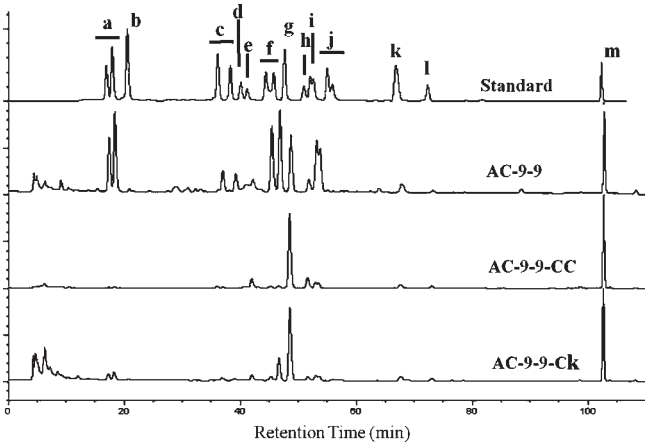

Figure 6. HPLC profiling of index compounds of *A. cinnamomea* fruiting body and ethanol extracts of the same *A. cinnamomea* strains (AC-9) grown on *Cinnamomum kanehirai* wood, *Cinnamomum camphora* wood and *Cunninghamia konishii* wood for nine months. Peaks: **a**, (*R*,*S*)-antcin K; **b**, 1,4-dimethoxy-2,3-methylenedioxy-5-methylbenzene; **c**, (*R*,*S*)-antcin C; **d**, antrocamphin A; **e**, 2,2′,5,5′-tetramethoxy-3,4,3′,4′-bimethylenedioxy-6,6′-dimethylbiphenyl; **f**, (*R*,*S*)-antcin H; **g**, dehydrosulfurenic acid; **h**, antrocamphin C; **i**, (*R*,*S*)-antcin B; **j**, (*R*,*S*)-antcin G; **k**, (*R*,*S*)-antcin A; **l**, 15-acetyldehydrosulfurenic acid; **m**, dehydroeburicoic acid.

Most of the compounds were able to inhibit nitric oxide (NO) production, with IC_{50} values (50% inhibitory concentration) of less than 20 μg/mL. The IC_{50} values of two lanostane triterpenoids, compounds **e** and **m**, as well as two benzolics, compounds **b** and **e**, were between 20 to 35 μg/mL (Figure 8, IC_{50} higher than 20 μg/mL). Overall, the ergostane-type triterpenoids and polyacetylenes showed greater inhibition of NO production than the benzolics and lanostane-type triterpenoids. Compound **c**, antcin C (IC_{50} = 5.48 μg/mL), and compound **d**, antrocmaphin A (6.24 μg/mL), showed the strongest NO inhibitory activity. Test cells were healthy and viable at doses ranging from 5 to

Table 4. Quantification of Index Compound Contents from the Fruiting Bodies of *A. cinnamomea* Strains Grown on *Cinnamomum kanehirai*, *Cinnamomum camphora* (CC) and *Cunninghamia konishii* (CK) Wood for 9 Months

compound	content (mg/g dried fruiting bodies)[a]		
	AC-9-9-CC	AC-9-9-CK	AC-9-9
(*R*,*S*)-antcin K (**a**)	<0.001 a	0.937 ± 0.012 b	4.319 ± 0.587 c
1,4-dimethoxy-2,3-methylenedioxy-5-methylbenzene (**b**)	<0.001 a	0.003 ± 0.001 c	0.001 ± 0.000 b
(*R*,*S*)-antcin C (**c**)	0.051 ± 0.020 a	0.599 ± 0.008 ab	1.537 ± 1.020 b
antrocamphin A (**d**)	<0.001 a	<0.001 a	0.046 ± 0.005 b
2,2′,5,5′-tetramethoxy-3,4,3′,4′-bimethylenedioxy-6,6′-dimethylbiphenyl (**e**)	0.272 ± 0.004 c	0.126 ± 0.003 b	0.081 ± 0.007 a
(*R*,*S*)-antcin H (**f**)	<0.001 a	1.455 ± 0.002 b	3.921 ± 0.162 c
dehydrosulfurenic acid (**g**)	8.717 ± 0.463 c	5.895 ± 0.284 b	1.389 ± 0.077 a
antrocamphin C (**h**)	1.259 ± 0.004 c	0.728 ± 0.001 b	0.426 ± 0.040 a
(*R*,*S*)-antcin B (**i**)	1.336 ± 0.004 b	0.899 ± 0.022 a	3.381 ± 0.364 c
(*R*,*S*)-antcin G (**j**)	<0.001 b	0.043 ± 0.005 a	0.006 ± 0.000 c
(*R*,*S*)-antcin A (**k**)	1.164 ± 0.045 b	0.724 ± 0.025 a	0.983 ± 0.059 c
15-acetyldehydrosulfurenic acid (**l**)	0.424 ± 0.027 c	0.338 ± 0.019 b	0.073 ± 0.020 a
dehydroeburicoic acid (**m**)	11.895 ± 0.499 c	3.914 ± 0.336 b	1.683 ± 0.187 a

[a] All contents are calculated as the standard linear equation, where *y* is the peak area and *x* is the concentration of the analyzed material. Mean values within each column with different labels (a, b, c) are significantly different ($p < 0.05$).

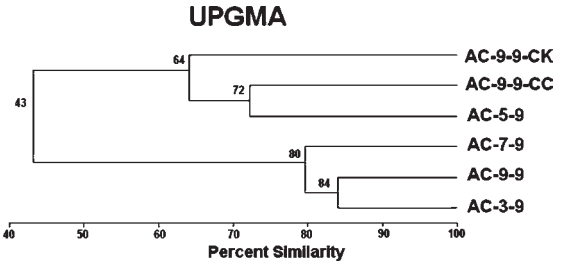

Figure 7. Similarity analysis of metabolites composition of *A. cinnamomea* fruiting bodies of different AC strains. UPGMA means unweighted pair group method with arithmetic mean.

40 μg/mL, as determined by MTT colorimetric assay (data not shown).

DISCUSSION

A. cinnamomea has traditionally been used in Taiwan by aborigines for detoxifiaction, and many people in Taiwan believe that *A. cinnamomea* is an effective remedy for liver diseases, diarrhea, hypertension, and cancer. Owing to the high demand for *A. cinnamomea*, in recent years techniques to mass-produce the fruiting body and mycelia have been developed by several biotech companies. There is, thus, an urgent need to address issues of quality control for this fungus. Here, we used HPLC to establish a metabolic profiling for *A. cinnamomea*. According to the literature survey and isolation results obtained by us, thirteen represented compounds, including triterpenoids, benzolics, and polyacetylenes, were selected as index compounds to analyze the secondary metabolites in *A. cinnamomea* fruiting bodies produced with different culture ages and on different wood substrates. The most important reasons for these compounds selected as index compounds are that they frequently are obtained in fruiting bodies reported by different research groups, and all of them are dominant ingredients in fruiting bodies compared with other compounds. Different strains of *A. cinnamomea* grown on the original host wood (*Cinnamomum kanehirai*) for 9 months resulted in similar metabolite profiles, demonstrating that this combination of 13 compounds is suitable for use as an index to evaluate the metabolites in *A. cinnamomea* fruiting bodies. *A. cinnamomea* grown on *C. kanehirai* wood for over 6 months synthesized a rich array of metabolites, especially ergostane-type triterpenoids and benzolics.

The ergostane-type triterpenoids only found in the fruiting body may have an important role to the activities of *A. cinnamomea*.[1−3] The amount of ergostane-type triterpenoids increased whereas the amounts of lanostane-type triterpenoids decreased with increase in culture age (Figure 5). In our previous study,[31] we demonstrated that the expression of sterol 14α-demethylase (CYP51), which is one of the key enzymes for sterol biosynthesis in fungi,[32] is higher in natural fruiting bodies than in other cell types. It might indicate that the lanostane-type triterpenoids, which also are produced in the mycelium of *A. cinnamomea*, would be transfer to ergostane-type triterpenoids by CYP51 in the matured fruiting bodies. The putative biosynthesis pathway of lanostane-type and ergostane-type triterpenoids from the fruiting bodies of *A. cinnamomea* was proposed as shown in Figure 9. Lanostane-type triterpenoids can be oxidized to compound **g** and derivatives (**a**, **l** and **m**). Under the representation of CYP51, ergostane-type triterpenoids were synthesized from lanostane-type triterpenoids and were further oxidized to compound **c** and derivatives (**f**, **i**, **j** and **k**). Both lanostane-type and ergostane-type triterpenoids were discovered clearly in the fruiting bodies at 9 months, but only lanostane-type triterpenoids were found at 3 months (Figure 5). It might be caused by the different maturation levels of fruiting bodies. The contents of ergostane-type triterpenoids increase with culture time. Moreover, the wood substrate seems to be an important factor for metabolite production. According to our results, when *A. cinnamomea* is cultivated in nonoriginal host wood, it does not generate the same metabolites as when it is grown in the orginal *C. kanehirai* host wood. Both lanostane-type and ergostane-type triterpenoids were found in fruiting bodies grown on *C. kanehirai* (AC-9-9); however, ergostane type triterpenoids were not obtained from *C. camphora* (AC-9-9-CC) nor

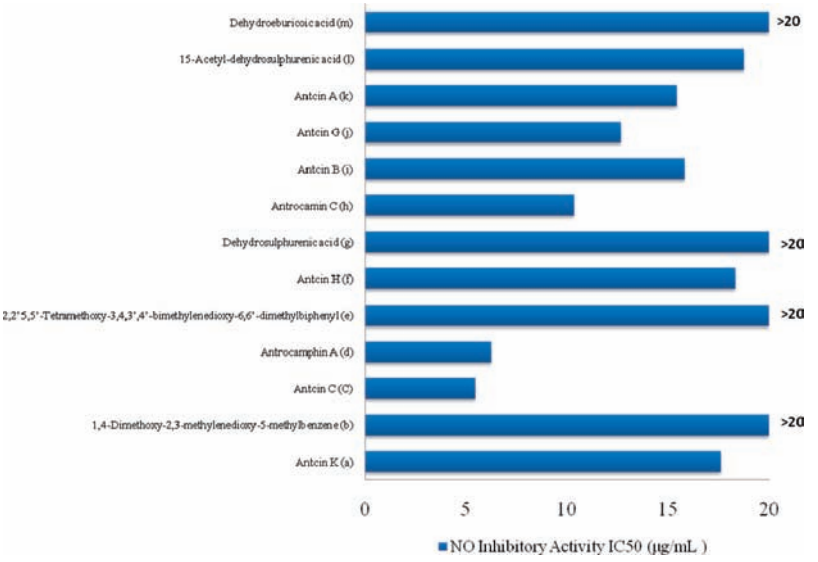

Figure 8. Effects of *A. cinnamomea* fruiting body index compounds on nitric oxide production in LPS-challenged RAW 264.7 cells.

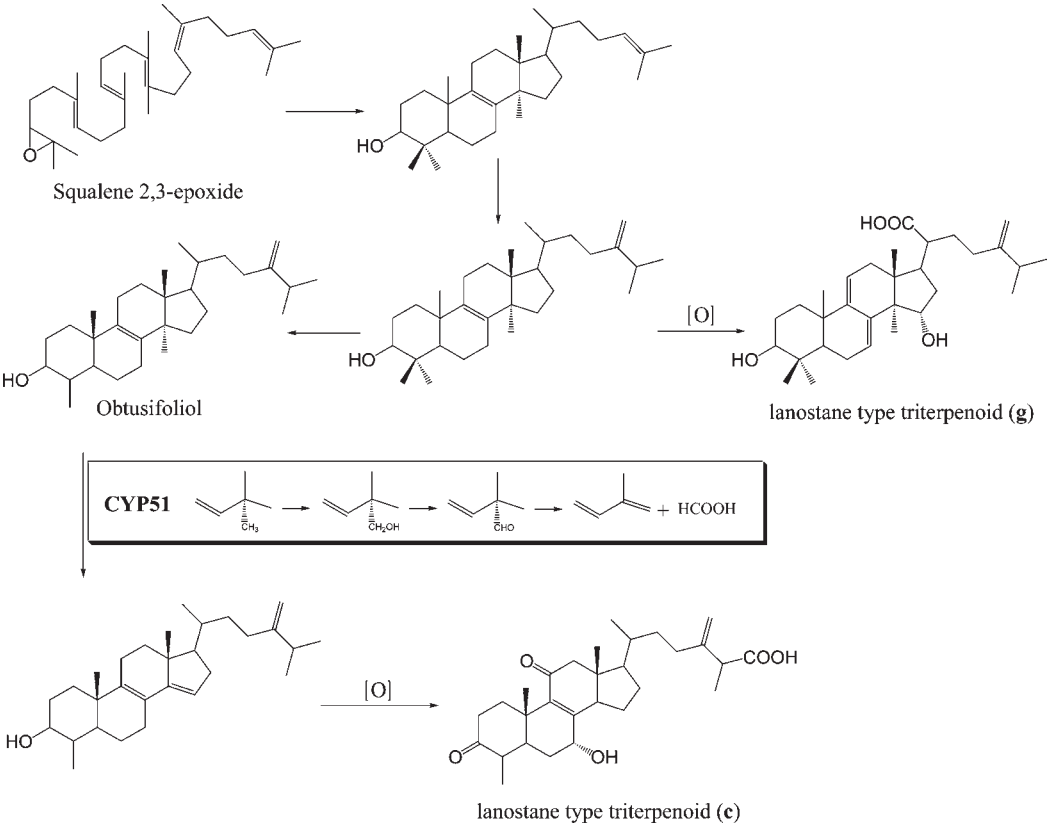

Figure 9. Putative biosynthesis pathway of lanostane-type and ergostane-type triterpenoids in the fruiting body of *A. cinnamomea*.

C. konishii (AC-9-9-CK) (Figure 6). This finding indicates that the composition of *C. kanehirai* wood influences the synthesis of *A. cinnamomea* metabolites. We thus suggest that some metabolites in the *C. kanehirai* (AC-9-9) substrate are correlated with the maturation of fruiting bodies. Further studies are needed to investigate this important topic.

Finally, in our previous report,[1] we demonstrated that the ethanolic extracts of *A. cinnamomea* exhibited potent antiinflammatory activity *in vitro* and *in vivo*. Moreover, we also elucidated the antiinflammatory mechanism of antrocamphin A, which is the antiinflammation principal that can be isolated from ethanolic extracts using a bioactivity guided fractionation protocol.[1] In this study we further obtained the results of antiinflammation activity for other key metabolites contained in *A. cinnamomea*, including lanostane and ergostane triterpenoids, benzolics, and polyacetylenes. Of note, the lanostane-type

triterpenoids and polyacetylenes also possess significant anti-inflammation activity (Figure 8). The IC_{50} value of antcin C was 5.48 μg/mL, even higher than that of antrocamphin A. We conclude that reliable evaluation of *A. cinnamomea* fruiting bodies can be achieved by a metabolite profile, which comprises lanostane- and ergostane-type triterpenoids, polyacetylenes and benzolics.

■ ASSOCIATED CONTENT

Supporting Information. The spectroscopic dada for he known compounds and Figures S1 to S7 (spectra of antrocamphin C). This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

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