Metabolite Profiling and Comparison of Bioactivity in *Antrodia cinnamomea* and *Antrodia salmonea* Fruiting Bodies

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Key words
- *Antrodia cinnamomea*
- *Antrodia salmonea*
- Polyporaceae
- metabolites profiling
- anti-inflammatory activity
- cytotoxicity

Abstract

Antrodia cinnamomea is a precious edible mushroom endemic to Taiwan that has been claimed to have significant health promotion activities. *Antrodia salmonea* is a new species of the genus *Antrodia*. In this study, we compared the metabolites and bioactivity of *A. cinnamomea* and *A. salmonea* fruiting bodies. The volatiles of *A. cinnamomea* and *A. salmonea* were characterized and 3,4,5-trimethoxybenzaldehyde was found to be the most abundant compound in *A. cinnamomea*; the other abundant compounds were δ-guaiene, isolongifolene, 1-octen-3-ol, 4-terpinenol, α-guaiene, and p-cymene. In *A. salmonea*, the main volatiles were α-cedrene, 1-octen-3-ol, D-limonene, cadinadiene, germacrene D, isolongifolene, and α-muurolene. Furthermore, five ergostane-type triterpenoids and two lanostane-type triterpenoids were selected as index compounds characterizing *A. cinnamomea* and *A. salmonea* extracts. The content of each compound varied between the two species. (R,S)-antcin B was the most abundant compound in *A. cinnamomea* fruiting bodies (75.18 ± 0.11 µg/mg). However, (*R,S*)-antcin C (184.85 ± 0.96 µg/mg) was the major triterpenoid in the *A. salmonea* fruiting body. Furthermore, two compounds, antcin M and methyl antcinate K, were only present in the *A. salmonea* fingerprint; therefore, antcin M and methyl antcinate K may be important for distinguishing between *A. cinnamomea* and *A. salmonea* fruiting bodies. Finally, examination of anti-inflammation activity and cytotoxicity showed that *A. salmonea* had more anti-inflammatory activity than *A. cinnamomea*; however, *A. salmonea* was more cytotoxic than *A. cinnamomea*. In conclusion, the composition and bioactivity of the fruiting bodies of *A. cinnamomea* and *A. salmonea* varies. Therefore, it is recommended that further toxicological evaluation and investigation of the biological activity of *A. salmonea* is carried out to ensure its safe and efficacious use as an alternative to *A. cinnamomea*.

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Abbreviations

DMEM:	Dulbecco's modified Eagle medium
FBS:	fetal bovine serum
GC/MS:	gas chromatography/mass spectrometry
HPLC:	high-performance liquid chromatography
LPS:	lipopolysaccharide
MTT:	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide
NMR:	nuclear magnetic resonance
NO:	nitric oxide
SPME:	solid-phase microextraction
UPLC-MS:	ultra-performance liquid chromatography-mass spectrometry

Introduction

Antrodia cinnamomea (syn. *Antrodia camphorata* and *Taiwanofungus camphorate*) is a precious edible mushroom that has long been the most highly valued medicinal fungus in Taiwan. Traditionally, *A. cinnamomea* has been used as a folk remedy for various diseases including cancer, hypertension, abdominal pains, and diarrhea [1]. The extract of *A. cinnamomea* has also been used as a food intoxicant since antiquity [2]. Owing to its perceived efficacy, *A. cinnamomea* dry fruiting bodies are sold at prices exceeding US$15 000 per kilogram in the local market in Taiwan, and the total market value of *A. cinnamomea* products, including raw fruiting bodies and health foods, is estimated to be over US$100 million per year [3]. One of the most important reasons for the high price of

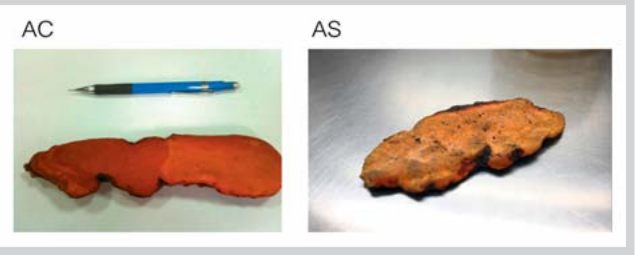

Fig. 1 Samples of *A. cinnamomea* (AC) and *A. salmonea* (AS) fruiting bodies. (Color figure available online only.)

A. cinnamomea is that it only grows on the inner surface of the heartwood cavity of the evergreen tree *Cinnamomum kanehirai* Hayata (Lauraceae), which is an endangered species endemic to Taiwan. *Antrodia salmonea* (**Fig. 1**) is a new species of the genus *Antrodia*, which was originally hosted by the indigenous coniferous tree *Cunninghamia konishii* Hayata (Cupressaceae) [4]. This mushroom is similar to *A. cinnamomea*. Both *A. salmonea* and *A. cinnamomea* have a strong bitter taste; however, the color of *A. cinnamomea* is cardinal red whereas *A. salmonea* is salmon-pink, and it is often easy to confuse the two species. Because of the difficulty in producing *A. cinnamomea*, as well as its high market price, *A. salmonea* is often used as a substitute for *A. cinnamomea* in the marketplace. So far, more than 250 scientific papers have been published on *A. cinnamomea* [1,5–7], but literature on *A. salmonea* is rare. To our knowledge, around ten research articles have been published on *A. salmonea* to date. For the development of functional foods or phytomedicines, quality control, efficacy approbation, and safety are the three most important requirements. Since *A. salmonea* now is commonly used as a substitute for *A. cinnamomea*, verification of the chemical ingredients and bioactivity of *A. cinnamomea* and *A. salmonea* is an urgent issue. In the present study, the chemical compositions of *A. salmonea* and *A. cinnamomea* were analyzed by using SPME-GC/MS and HPLC profiling, and the cytotoxicity and anti-inflammation activity of these two mushrooms were also studied.

Results and Discussion

Odor is a significant distinguishing characteristic of the fruiting bodies of *A. cinnamomea* and *A. salmonea*. The volatile compounds of *A. cinnamomea* and *A. salmonea* were collected by using SPME to obtain the volatiles, and analysis was done by GC/MS. **Table 1** shows the compositions of volatile compounds emitted from *A. cinnamomea* and *A. salmonea* fruiting bodies. In the *A. salmonea* fruiting body, 3,4,5-trimethoxybenzaldehyde (30.04%) was the most abundant compound, followed by δ-guaiene (9.88%), isolongifolene (8.00%), 1-octen-3-ol (7.08%), 4-terpinenol (5.99%), α-guaiene (5.99%), and p-cymene (4.96%). In contrast, the main compositions of volatiles of A. salmonea were α-cedrene (14.68%), 1-octen-3-ol (9.31%), D-limonene (9.21%), cadinadiene (7.65%), germacrene D (7.22%), isolongifolene (6.72%), and α-muurolene (5.31%).

To date, more than 80 compounds have been identified from *A. cinnamomea*, including triterpenoids, benzolics, and polyacetylenes [5]. In our previous study, we selected 13 index compounds to establish a comprehensive profile of the ethanol extract

Table 1 Volatile metabolite analysis of *A. cinnamomea* and *A. salmonea* fruiting bodies.

Compound	KI[a]	*A. cinnamomea* (%)	*A. salmonea* (%)	Identification[b]
1-Octen-3-ol	982	7.08	9.31	MS, KI
Octan-3-one	989	2.82	–	MS, KI
Thuj-4(10)-ene	993	–	3.97	MS, KI, ST
3-Octanol	997	0.65	–	MS, KI
α-Phellandrene	1006	–	3.03	MS, KI, ST
1,4-Cineol	1014	1.57	–	MS, KI, ST
p-Cymene	1023	4.96	4.12	MS, KI, ST
D-Limonene	1030	1.12	9.21	MS, KI, ST
γ-Terpinene	1062	0.87	–	MS, KI, ST
Linalool	1100	1.19	–	MS, KI, ST
1-Terpinenol	1146	2.02	–	MS, KI, ST
Camphor	1156	–	3.34	MS, KI, ST
γ-Terpineol	1168	0.67	–	MS, KI, ST
4-Terpinenol	1182	5.99	–	MS, KI, ST
α-Terpineol	1190	0.59	–	MS, KI, ST
Safrole	1295	1.35	–	MS, KI, ST
Isolongifolene	1394	8.00	6.72	MS, KI, ST
α-Copaene	1402	–	1.87	MS, KI
β-Cubebene	1407	0.57	–	MS, KI
α-Guaiene	1432	5.99	–	MS, KI, ST
α-Cedrene	1433	–	14.68	MS, KI, ST
Thujopsene	1440	0.62	1.83	MS, KI
δ-Guaiene	1468	9.88	3.26	MS, KI
Germacrene D	1500	–	7.22	MS, KI, ST
α-Muurolene	1518	–	5.31	MS, KI, ST
Cadinadiene	1542	–	7.65	MS, KI, ST
3,4,5-Trimethoxybenzaldehyde	1549	30.04	–	MS, KI, ST

[a] Kovats index on a DB-5 ms column in reference to *n*-alkanes; [b] MS: NIST and Wiley libraries and literature; KI: Kovats index; ST: authentic standard compounds

of *A. cinnamomea* fruiting body [8]. Triterpenoids in both the mycelium and fruiting body are now considered to be the most biologically active components of *A. cinnamomea* [9]. Thus, five ergostane-type triterpenoids, i.e., (R,S)-antcin K, (R,S)-antcin C, (R,S)-antcin H, (R,S)-antcin B, and (R,S)-antcin A, as well as two lanostane-type triterpenoids, dehydrosulphurenic acid and dehydroeburicoic acid, were used as the index compounds to characterize the *A. cinnamomea* and *A. salmonea* extracts in this study. The structure and MS analysis data are shown in **Fig. 2** and **Table 2**. Compounds **a** to **g** in **Fig. 2** were then used as index compounds to profile metabolites in *A. cinnamomea* and *A. salmonea* extracts. **Fig. 3** shows the HPLC metabolite profiles for *A. cinnamomea* (**Fig. 3A**) and *A. salmonea* extracts (**Fig. 3B**). Some of the compounds, including (R,S)-antcin K, (R,S)-antcin C, (R,S)-antcin H, and (R,S)-antcin B, showed two peaks, for R and S configurations, as these compounds contain a chiral center at the C_{25} position. We were not able to characterize the absolute configuration for this group of compounds directly by NMR; so further R/S derivatives will be 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 *A. cinnamomea* and *A. salmonea* samples, calibration curves of the index compounds were established using five dilution standards from 10 to 1000 µg/mL. The contents of eight 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 3**). The ergo-

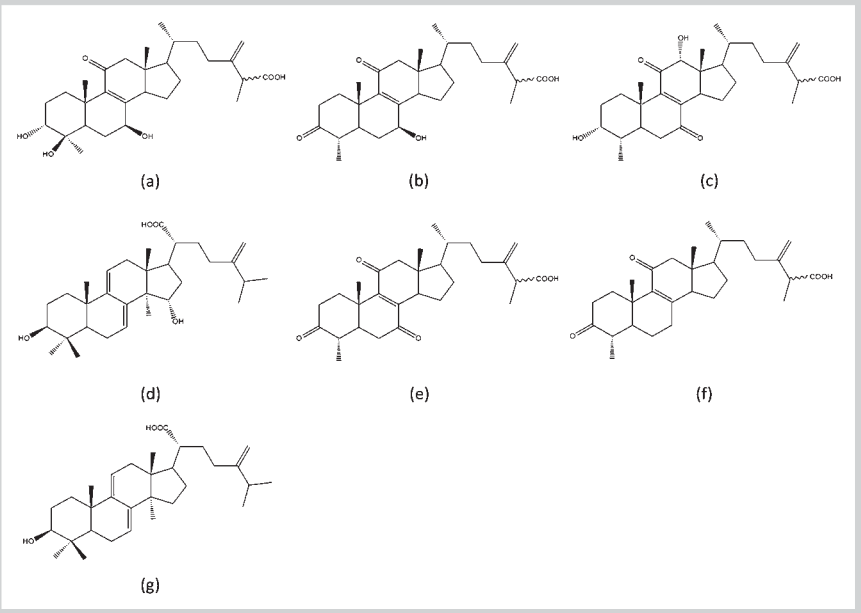

Fig. 2 Index compounds of *A. cinnamomea* and *A. salmonea* fruiting bodies. **a** (R,S)-Antcin K, **b** (R,S)-Antcin C, **c** (R,S)-Antcin H, **d** Dehydrosulphurenic acid, **e** (R,S)-Antcin B, **f** (R,S)-Antcin A, and **g** Dehydroeburicoic acid.

Compound	(M ± H)	Product ions *m/z* at 35 eV (RI%)
(R,S)-Antcin K	489 $[M + H]^+$	435 (100), 453 (67), 443 (38), 407 (33), 417 (31)
(R,S)-Antcin C	469 $[M - H]^-$	425 (100), 426 (31), 427 (5), 341 (4), 407 (3)
(R,S)-Antcin H	485 $[M - H]^-$	441 (100), 413 (69), 457 (41), 442 (32), 414 (23)
Dehydrosulphurenic acid	485 $[M + H]^+$	425 (100), 423 (62), 424 (17), 257 (16)
(R,S)-Antcin B	467 $[M - H]^-$	423 (100), 424 (32), 425 (6), 408 (2)
(R,S)-Antcin A	483 $[M - H]^-$	409 (100), 410 (30), 411 (3)
Dehydroeburicoic acid	467 $[M - H]^-$	337 (100), 371 (94), 339 (87), 373 (47), 338 (28)

Table 2 Molecular ions [M ± H], product ions (35 eV), and percentage of relative intensities (RI%) of triterpenoids for characterization of the fruiting bodies of *A. cinnamomea* and *A. salmonea*.

stane-type triterpenoid (R,S)-antcin B was the most abundant compound in the *A. cinnamomea* fruiting body (75.18 ± 0.11 µg/mg) followed by (R,S)-antcin H (48.77 ± 0.31 µg/mg), (R,S)-antcin C (43.36 ± 0.76 µg/mg), and (R,S)-antcin A (19.86 ± 0.12 µg/mg). However, (R,S)-antcin C was the dominant triterpenoid in the *A. salmonea* fruiting body with the content of (R,S)-antcin C in the extract being up to 184.85 ± 0.96 µg/mg. (R,S)-antcin A was the second most abundant triterpenoid in *A. salmonea* with the content being 57.85 ± 0.11 µg/mg, followed by (R,S)-antcin H (19.86 ± 0.28 µg/mg) and (R,S)-antcin K (18.61 ± 0.33 µg/mg). A comparison of the quantity of the lanostane-type triterpenoids with ergostane-type-triterpenoids (dehydrosulphurenic acid and dehydroeburicoic acid) showed that the amounts of lanostane-type triterpenoids in *A. cinnamomea* and *A. salmonea* were lower than ergostane-type triterpenoids.

The results of the triterpenoid analysis for *A. cinnamomea* and *A. salmonea* showed that all the index compounds selected in this study could be detected in both *A. cinnamomea* and *A. salmonea* fruiting bodies, but the content of each triterpenoid varied in the two mushrooms. A comparison of the metabolite profiles of *A. cinnamomea* and *A. salmonea* (**Fig. 3**) showed that there were two more peaks (X and Y) present in *A. salmonea* at the retention time of 60 to 62 min (**Fig. 3B**). The MS, ^{1}HNMR, and ^{13}CNMR spectral data were in good agreement with antcin M (X) and methyl antcinate K (Y), which were reported previously [10]. Antcin M and methyl antcinate K (**Fig. 4**) might be important constituents for distinguishing *A. cinnamomea* and *A. salmonea* fruiting bodies.

To evaluate the anti-inflammation activity of the extracts from *A. cinnamomea* and *A. salmonea* fruiting bodies, an LPS-stimulated murine macrophage assay system was used. **Table 4** shows the inhibitory effects of the extracts of *A. cinnamomea* and *A. salmonea* fruiting bodies. IC_{50} values (50% inhibitory concentration) of *A. cinnamomea* and *A. salmonea* were 73.89 µg/mg and 66.3 µg/mg, respectively. Test cells were healthy and viable at doses ranging from 10 to 80 µg/mL, as determined by the MTT colorimetric assay (data not shown). In addition to anti-inflammation activity, the cytotoxicity was evaluated for *A. cinnamomea* and *A. salmonea* extracts. As shown in the results presented in **Table 4**, the *A. cinnamomea* extract possessed stronger cytotoxicity than *A. salmonea*. The IC_{50} value against MCF-7 cells was 59.18 µg/mL for *A. cinnamomea* and 91.45 µg/mL for *A. salmonea*. *A. cinnamomea* grown on its original host, *C. kanehirai*, has the highest market value [4]. However, excessive felling of *C. kanehirai* is prohibited by the government of Taiwan. *A. salmonea* is a similar mushroom to *A. cinnamomea*, and because the cultivation of wood for *A. salmonea* is easy to obtain, *A. salmonea* has become a common substitute for *A. cinnamomea* in the marketplace. Numerous studies have discussed the metabolites of *A. cinnamomea* and its bioactivities; however, to date, investigations of *A. salmonea* are rare.

Although some studies have analyzed the volatile compounds emitted from the mycelium of *A. cinnamomea*, no volatiles have been characterized for *A. cinnamomea* and *A. salmonea* fruiting bodies until now [11]. In the current study, first we characterized the volatiles of *A. cinnamomea* and *A. salmonea* fruiting bodies. According to our analysis, 3,4,5-trimethoxybenzaldehyde was

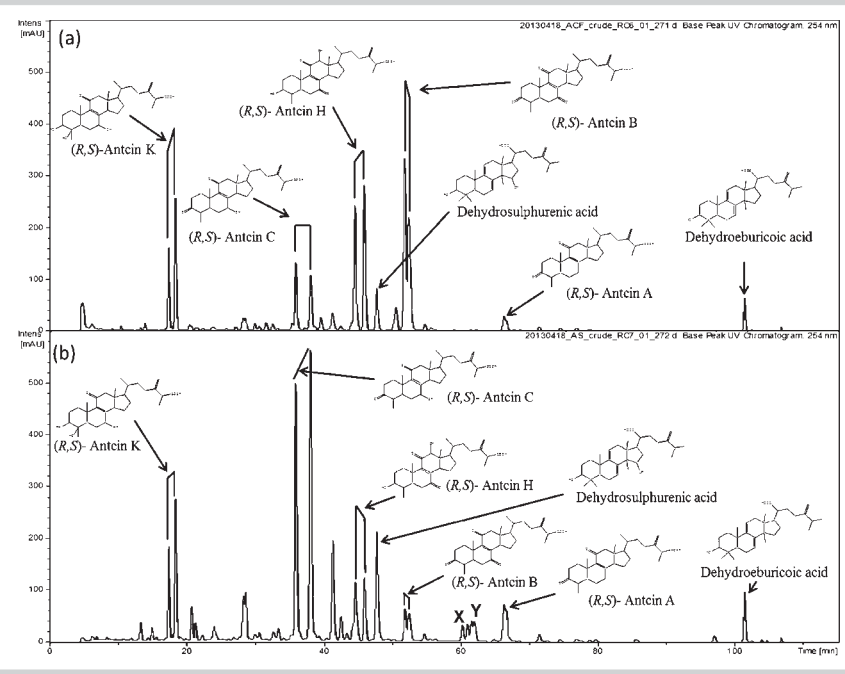

Fig. 3 HPLC profiling of metabolites of *A. cinnamomea* (**a**) and *A. salmonea* (**b**) fruiting bodies.

Table 3 Quantification of triterpenoid contents in extracts of fruiting bodies of *A. cinnamomea* and *A. salmonea*.

Triterpenoids	Contents (μg/mg dried fruiting bodies)	
	A. cinnamomea	*A. salmonea*
(R,S)-Antcin K	17.30 ± 0.29	18.61 ± 0.33
(R,S)-Antcin C	43.36 ± 0.76	184.85 ± 0.96
(R,S)-Antcin H	48.77 ± 0.31	19.86 ± 0.28
Dehydrosulphurenic acid	4.73 ± 0.04	12.85 ± 0.16
(R,S)-Antcin B	75.18 ± 0.11	15.47 ± 0.10
(R,S)-Antcin A	19.86 ± 0.12	57.85 ± 0.11
Dehydroeburicoic acid	3.74 ± 0.05	5.98 ± 0.01

the most abundant compound in the *A. cinnamomea* fruiting body. The other abundant compounds were δ-guaiene, isolongifolene, 1-octen-3-ol, 4-terpinenol, α-guaiene, and p-cymene. In the case of *A. salmonea*, the most abundant volatiles were α-cedrene, 1-octen-3-ol, D-limonene, cadinadiene, germacrene D, isolongifolene, and α-muurolene. The odor of the *A. cinnamomea* and *A. salmonea* fruiting bodies is quite different. *A. cinnamomea* has a relatively rich woody odor, while *A. salmonea* has a lighter odor. δ-guaiene, isolongifolene, 4-terpinenol, and α-guaiene have distinguished woody odors and, therefore, the volatile compound composition might determine the different odors of *A. cinnamomea* and *A. salmonea*.

In addition to a comparison of the chemicals of *A. cinnamomea* and *A. salmonea*, the metabolite fingerprints of *A. cinnamomea* and *A. salmonea* were established by using HPLC. We purified the major triterpenoids, including five ergostane-type triterpenoids and two lanostane-type triterpenoids, the compound structures of which were confirmed by UPLC-MS and NMR analysis. These index compounds were used as the index compounds to identify and quantify the triterpenoids in the *A. cinnamomea* and *A. salmonea* metabolite fingerprints. Overall, the ergostane-type and lanostane-type triterpenoids were detected both in the fingerprints of *A. cinnamomea* and *A. salmonea*. The *A. cinnamomea* fingerprint was similar to the metabolite profiling, which we established previously [8]. All of the index compounds shown in the *A. salmonea* fingerprint were observed in *A. cinnamomea*; however, the content of each compound was different in the two mushrooms. For the *A. cinnamomea* fruiting body, (R,S)-antcin B, (R,S)-antcin H, and (R,S)-antcin C were abundant compounds at 75.18 ± 0.11, 48.77 ± 0.31, and 43.36 ± 0.76 μg/mg, respectively. Interestingly, (R,S)-antcin C was the dominant compound in *A. salmonea* with levels of up to 184.85 ± 0.96 μg/mg. The second most abundant triterpenoid in *A. salmonea* was (R,S)-antcin A (57.85 ± 0.11 μg/mg) followed by (R,S)-antcin H (19.86 ± 0.28 μg/mg) and (R,S)-antcin K (18.61 ± 0.33 μg/mg). In addition, we found a further two compounds in *A. salmonea* that were absent in *A. cinnamomea*. According to the spectral analysis, the compounds were antcin M and methyl antcinate K. These two compounds might be important for distinguishing *A. cinnamomea* and *A. salmonea* fruiting bodies.

Obviously, the compositions of *A. cinnamomea* and *A. salmonea* were dissimilar. We speculate that these different compositions might affect the bioactivity of *A. cinnamomea* and *A. salmonea*. In our previous study, the ethanolic extracts of *A. cinnamomea* exhibited potent anti-inflammatory activity *in vitro* and *in vivo* [2]. To evaluate the anti-inflammatory activity of *A. cinnamomea* and *A. salmonea* extracts, an LPS-stimulated murine macrophage assay system was used. The anti-inflammatory activity of *A. salmonea* extract (IC_{50} = 66.3 μg/mg) was slightly higher than that of *A. cinnamomea* (IC_{50} = 73.89 μg/mg). (R,S)-antcin C is the strongest anti-inflammatory triterpenoid in the *A. cinnamomea* fruiting body with an IC_{50} = 5.48 μg/mL [8]. However, *A. salmonea* contained a higher amount of (R,S)-antcin C than *A. cinnamomea*. This might be the reason why *A. salmonea* possessed higher anti-inflammatory activity than *A. cinnamomea*. In addition to evaluating the anti-inflammatory activity, we examined the cytotoxicity of *A. cinnamomea* and *A. salmonea* against MCF-7 cell lines. The results showed that *A. cinnamomea* (IC_{50} = 59.18 μg/mg) was more cytotoxic than *A. salmonea* (IC_{50} = 91.45 μg/mg). According to the results of a cytotoxicity assay by Du and his coworkers, (R,

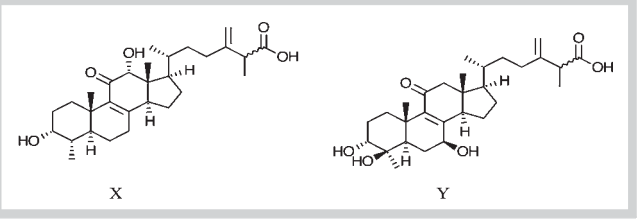

Fig. 4 Compounds distinguishing the difference between *A. cinnamomea* and *A. salmonea* fruiting bodies; antcin M (**X**) and methyl antcinate K (**Y**).

S)-antcin H and (R,S)-antcin B exhibited stronger cytotoxicity against human leukemia cell lines in comparison with other ergostane-type triterpenoids in the *A. cinnamomea* fruiting body [9]. (R,S)-antcin H and (R,S)-antcin B were abundant in *A. cinnamomea*. This might be why *A. cinnamomea* possessed stronger cytotoxicity than *A. salmonea*.

According to this study, we can distinguish between *A. cinnamomea* and *A. salmonea* by fingerprints, especially since two compounds, antcin M and methyl antcinate K, were only present in the *A. salmonea* fingerprint. *A. salmonea* had more anti-inflammatory activity than *A. cinnamomea*, however, *A. salmonea* was more cytotoxic than *A. cinnamomea*. Nowadays, *A. salmonea* is often used as a substitute for *A. cinnamomea*. However, the composition and levels of bioactivity are different in these two mushrooms. Therefore, for safety reasons, we recommend a toxicological evaluation and biological activity investigation of *A. salmonea* for use as an alternative to *A. cinnamomea*.

Materials and Methods

Antrodia cinnamomea and *Antrodia salmonea* fruiting bodies

The *A. cinnamomea* and *A. salmonea* fruiting bodies were collected by Mr. Wen-Wei Hsiao who is an assistant research fellow in Experimental Forest, National Taiwan University. *A. cinnamomea* was collected from the remained wood of *C. kanehirai* distributed at the secondary growth of forest, which is located at Suili countryside, Nantou County, Taiwan. *A. salmonea* was also collected from its host wood, *C. konishii*. The remained wood of *C. konishii* was located at Sun-Link-Sea region in Nantou County, Taiwan. The samples were identified by Dr. Sheng-Yang Wang (Professor of Department of Forestry, Nation Chung-Hsing University). The voucher specimens (TCFAC0001 and TCFAS0001) were deposited in the herbarium of the same university. **Fig. 1** shows the morphology of the fruiting bodies analyzed in this study.

Volatile metabolite analysis of *Antrodia salmonea* and *Antrodia cinnamomea* fruiting bodies by using solid-phase microextraction combined with gas chromatography coupled to mass spectrometry

An SPME holder and carboxen-polydimethylsiloxane-coated fibers (75 mm) were purchased from Supelco (Bellefonte). The sample bottle was placed in a water bath (40 ± 2 °C) and conditioned (15 min, without fiber). Before use, SPME fibers were conditioned by heating in a hot injection port of a GC at 200 °C for 15 min to remove contaminants. After the equilibration time, the fiber was introduced into the sample bottle and exposed to the gases in the headspace of the *A. salmonea* and *A. cinnamomea* powder for 15 min. After 15 min, the SPME fiber was inserted into the injection port of the GC using an SPME liner for desorption at 120 °C for a 5-s splitless period. The analysis of all samples was performed using a Thermo Scientific ITQ 900 GC/MS equipped with a DB-5 column (30 m × 0.25 mm × 0.25 µm, Agilent J&W Scientific). The GC oven temperature was programmed from 40 °C, held 1 min, raised to 100 °C at 4 °C/min, then raised to 250 °C at 15 °C/min and held for 3 min. The sample injection was in the splitless mode. The flow rate of the carrier gas, helium, was at 1.0 mL/min. The Kovats indices were calculated for all volatile constituents using a homologous series of n-alkanes C_9-C_{24}. The major components were identified by coinjection with standards (wherever possible), confirmed with Kovats indices using the Wiley (Ver. 8.0) and National Institute of Standards and Technology (NIST) Ver. 2.0 GC/MS libraries.

Table 4 Anti-inflammation activity and cytotoxicity against MCF-7 cells of *A. cinnamomea* and *A. salmonea*.

	IC_{50} (µg/mg)			
	A.cinnamomea	***A. salmonea***	**Plumgagin**	**Curcumin**
NO inhibition activity	73.89	66.30	–	4.50
Cytotoxicity	59.18	91.45	4.54	–

Metabolite profiling and quantification of ethanol extract of *A. cinnamomea* and *A. salmonea* fruiting bodies

The protocol for the preparation of the ethanol (EtOH) extract of *A. salmonea* and *A. salmonea* fruiting bodies was according to our previous method [8]. Briefly, fresh material was lypholized for 72 h and *A. cinnamomea* and *A. salmonea* were ground to a powder (particle diameter < 0.7 mm), accurately weighed (around 5 g), placed in an Erlenmeyer flask (250 mL) with 100 mL EtOH, and sonicated in an ultrasonicator (Branson 5510, Branson Ultrasonic) for 60 min. The extracts were then decanted, filtered under vacuum, concentrated in a rotary evaporator, and lyophilized. The metabolite profile of the ethanol extract prepared from the *A. cinnamomea* and *A. salmonea* were established by using seven index compounds, namely (R,S)-antcin K, (R,S)-antcin C, (R,S)-antcin H, dehydrosulphurenic acid, (R,S)-antcin B, (R,S)-antcin A, and dehydroeburicoic acid, which were identified by us previously [8] and the structure was reconfirmed by using UPLC-MS analysis. An amaZon speed ion trap (Bruker) was set to an ion source temperature 250 °C with N_2 as dry gas at 9.0 L min-1 with the capillary voltage at 4500 V, the end plate offset at 500 V, and a scan range of 70 to 600 *m/z* in the positive and negative ionization modes. The unknown peaks presented in the metabolite profiling of *A. salmonea* were collected and identified by spectra analysis. 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 data were collected with a Finnigan MAT-95 S mass spectrometer, and NMR spectra were recorded with Bruker Avance 400 MHz FT-NMR spectrometers at 400 MHz (^{1}H) and 100 MHz (^{13}C). d-Chloroform ($CDCl_3$) was used for NMR analysis. All spectroscopic analysis data were in good agreement with the literature. The standard calibration curves (peak area vs. concentration) of each index compound were determined at the range of compound concentrations of 10, 25, 50, 100, 250, 500, and

1000 µg/mL. Quantification of the content of each index compound in fruiting bodies from *A. cinnamomea* and *A. salmonea* 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 are presented as mean ± SE.

Nitric oxide inhibitory assay

The effect of *A. cinnamomea* and *A. salmonea* extracts on NO production was measured indirectly by analysis of nitrite levels using the Greiss reaction [12, 13]. Briefly, RAW 264.7 cells grown in a 75-cm^2 culture dish and 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* and *A. salmonea* extracts. The nitrite concentration (as an estimate of NO production) was measured using the supernatant from the RAW 264.7 cells by the Griess reaction [14]. Curcumin (> 95%, Sigma Co.) was used as a positive control.

Cytotoxicity analysis

MCF-7 (human breast adenocarcinoma, BCRC 60436) was purchased from the Bioresource Collection and Research Center (BCRC), Food Industry Research, and Development Institute, Taiwan. MCF-7 cells were cultured in DMEM supplemented with 10% FBS, 1% penicillin–streptomycin, and 1 mM sodium pyruvate, and were maintained at 37 °C and 5% CO_2. All cells (1×10^3 per well) were seeded in 96-well plates and incubated for 24 h, and different dosages of extracts of *A. cinnamomea* and *A. salmonea* extracts were added to each well in triplicate for 24 h. The cell viability was determined by the MTT assay [15]. Plumbagin (> 95%, Sigma Co.) was used as a positive control.

Statistical analysis

Data are expressed as means ± SE. The significance of the differences between group means was determined by analysis of variance (ANOVA) using Dunnett's test. Mean values within each column with different labels (a, b, c, d) are significantly different ($p < 0.05$).

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Conflict of Interest

The authors declare that they have no competing interests.

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