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Authentication of ten distinctive triterpenoids in Antrodia cinnamomea serves as a crucial aspect for ensuring the quality control of associated nutraceutical products.

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ABSTRACT

Edible mushroom *Antrodia cinnamomea* is distinctive for its use in many health supplement products in relieving of diverse health-related conditions. *A. cinnamomea* is known for its rich array of bioactive secondary metabolites, predominantly terpenoids, that possess anti-inflammatory properties. Despite the abundance of these compounds, only some compounds have demonstrated notable anti-inflammatory activity. Moreover, there is a lack of established quality control methods specifically tailored to the active constituents of these products. Consequently, there is a great need for the development of precise and effective quality control methods for *A. cinnamomea*-based products, targeting their active components to ensure the consistency and reliability of these products in harnessing their anti-inflammatory potential. Herein we report a quantitative HPLC method for better evaluating the quality of *A. cinnamomea* based dietary supplements. Based on their bioactivities, we selected ten benchmark compounds, i. e. antcin K, (25S)-antcin H, (25R)-antcin H, (25R)-antcin C, (25S)-antcin C, (25R)-antcin A, 15α-acetyl-dehydrosulphurenic acid, versisponic acid D, dehydroeburicoic acid, and eburicoic acid and developed and validated a HPLC-UV method for quantification of these compounds simultaneously with high sensitivity, linearity and range, precision, and accuracy. Furthermore, we applied our method to quantify the commercially available *A. cinnamomea* containing supplements and found that the quality of these supplements varies greatly with only one product containing good amount of the active compounds. Our method provides a needed solution to quality control problem of the highly priced *A. cinnamomea* food and nutraceutical products that show great variety and inconsistency.

1. Introduction

Edible fungi with medicinal use have been attracting great attention in the hope to discover new molecules with potential for therapeutic use or for active ingredients for nutraceuticals (Zhang, Wang et al. 2021). *A. cinnamomea* is such a treasured fungus naturally found in Taiwan (Lu et al., 2013) and taken as supplements for treatment of food poisoning, diarrhea (Lu et al., 2022), stomachache (Chen, Thang et al. 2013), hypertension (Lu et al., 2013), itchy skin (Perera, Yang et al. 2018), and liver illness(Peng, Yang et al. 2017), anti-cancer (Lu et al., 2018), and anti-inflammation (Yang, Wang et al. 2022). Successful cultivation of *A. cinnamomea* enables the accessibility of the fungal based health care products derived from both fruiting body and mycelium. The phytochemicals of *A. cinnamomea* have been subjected to extensive characterizations in the hope to establish the chemical principles and scientific evidence for *A. cinnamomea* based natural health care products. From the mycelium, 4-acetylantroquinonol B was found to enhance the immunoprotective function of specific cells against hepatocellular carcinoma stem cells (Li and Chiang 2019). Antrolone has identified the anti-inflammatory activity shown on murine macrophage RAW 264.7

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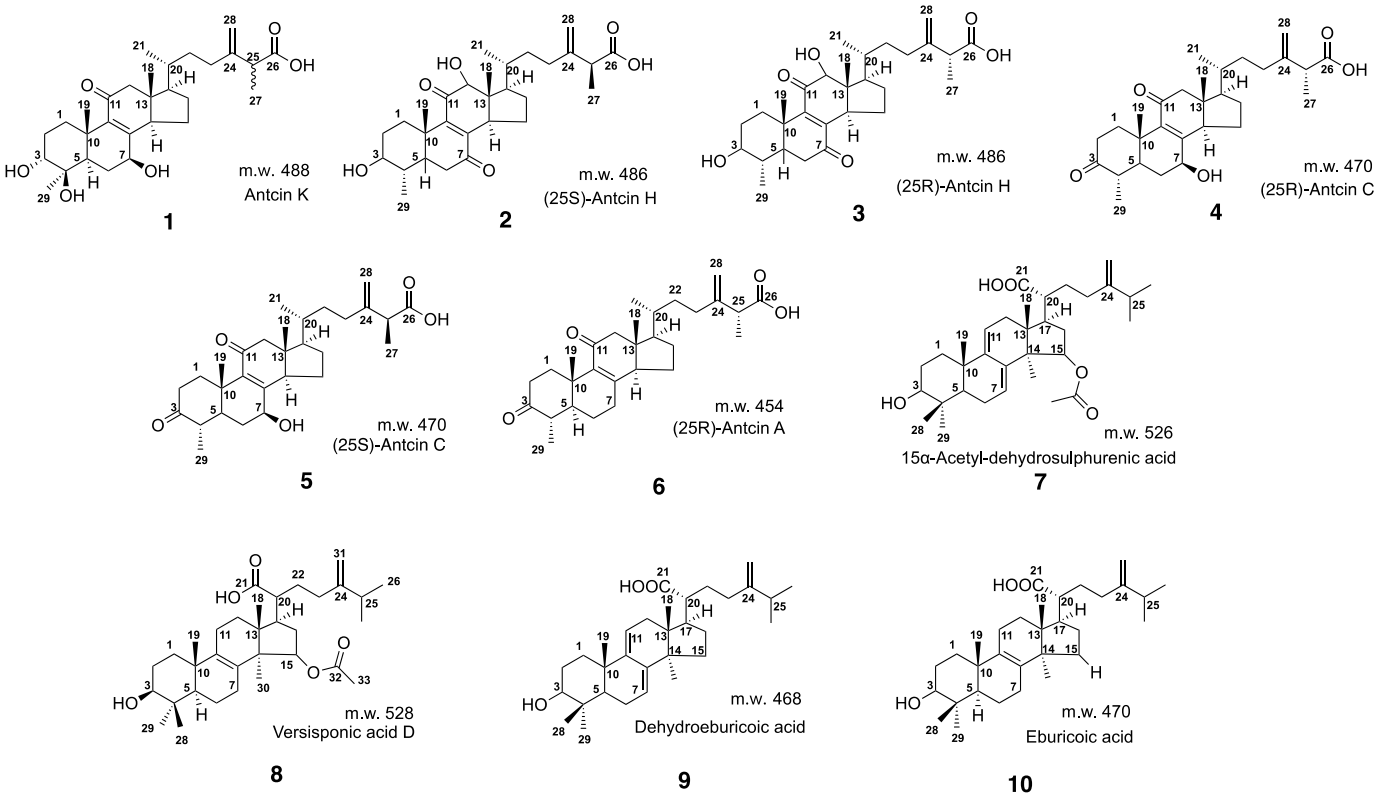

Fig. 1. Structures of selected triterpenoids isolated from *A. cinnamomea*.

cells(Yen, Shi et al. 2018). Terpenoids are the signature phytochemicals found in the fruiting body of *A. cinnamomea,* as over 150 terpenoids with subtle structural variations have been isolated and responsible for their pharmacological effects (Ma et al. 2016).

The intricate nature of the bioactive compounds found in *A. cinnamomea* poses a significant challenge in establishing robust quality control measures for nutraceutical products derived from this source. The complexity of the compounds involved necessitates the development of specialized protocols to accurately assess and ensure the quality of these products. Addressing this challenge is crucial for maintaining the efficacy and reliability of *A. cinnamomea*-based nutraceuticals, as it requires a nuanced approach to capture the diverse array of bioactive components and their potential synergistic effects. The current official method for authentication issued by the Taiwan government only gave a qualitative HPLC fingerprint of the terpenoids, which are mostly not commercially available. Recently, we utilized an activity-guided isolation approach to obtain terpenoids and assess their anti-inflammatory properties using a RAW264.7 cell culture model (Yang, Wang et al. 2022). In the process of our work, we isolated sufficient amounts of pure triterpenoids as standards for quantification of these compounds by HPLC. Our results allow us to rationally select representative and active terpenoids from over 40 different triterpenoids as benchmark compounds for quality indicators for *A. cinnamomea* based nutraceutical products. In this paper, we report our results in the development and validation of an HPLC method for simultaneous quantitation of ten signature triterpenoids (Fig. 1) in *A. cinnamomea* (Wu, Du et al. 2013). Our method provides needed solution to quality control problem of the highly priced *A. cinnamomea* food and nutraceutical products that show great variety and inconsistency.

2. Materials and methods

2.1. Chemical reagents

HPLC-grade methanol was purchased from Sigma-Aldrich. Chromatographic grade formic acid and all other chemical reagents have a purity of over 98% and used without further purification unless specified. The fruiting body and commercial dietary supplements were purchased from the local markets in Taipei, Taiwan, China, and provided by AgriGADA Biotech Pte. Ltd (Singapore).The terpenoids standards were obtained from separating the fruiting body of *A. cinnamomea* provided by AgriGADA Biotech Pte. Ltd based on our previous report (Yang, Wang et al. 2022). They are antcin K (1), (25S)-antcin H (2), (25R)-antcin H (**3**), (25R)-antcin C (**4**), (25S)-antcin C (**5**), (25R)-antcin A (**6**), 15α-acetyl-dehydrosulphurenic acid (**7**), Versisponic acid D (**8**), dehydroeburicoic acid (**9**), and eburicoic acid (**10**). The purities of these ten compounds were more than 98% based on ^{1}H NMR spectral analysis.

2.2. HPLC system

A Waters high-performance liquid chromatograph (HPLC) system was used which contained by an autosampler, a gradient pump with degas option and gradient mixer, a 2998 Photodiode Array (PDA) Detector (Waters Corporation, Milford, MA, USA) and a multi-solvent delivery system. Full-wavelength scanning was applied in HPLC analytical system, all injection volumes of samples and standard solutions were 10 μL. The operating temperature of HPLC system was maintained at 37 °C.

2.3. Sample extraction procedure

Three hundred grams of dried, chipped, and milled *A. cinnamomea* fruiting bodies were finely ground into powder and subsequently extracted with 95% ethanol at approximately 37 °C. This procedure was repeated in triplicate for each sample. The slurry was filtered under

Table 1
Parameters of qualification for ten selected triterpenoids found in *A. cinnamomea*.

Compound	Wavelength (nm	calibration curves	Linear Range(mM)	R^2	LOD* (μM)	LOQ* (μM)
1	254	$y = 3 \times 10^6x+112285$	0.16–5.128	0.9998	0.084	0.253
2	254	$y = 2 \times 10^6x+21578$	0.058–1.855	0.9998	0.852	2.581
3	254	$y = 2 \times 10^6x-4685.9$	0.031–1.005	0.995	0.628	1.902
4	254	$y = 5 \times 10^6x+96607$	0.019–0.599	0.9992	0.103	0.313
5	254	$y = 3 \times 10^6x+10584$	0.019–0.519	0.9998	0.194	0.589
6	254.5	$y = 10^6x+12600$	0.033–0.522	0.996	0.237	0.717
7	254	$y = 2.78 \times 10^5x+1307.6$	0.0372–1.19	0.999	0.337	1.021
8	200.2	$y = 4 \times 10^6x-51943$	0.063–1.004	0.9983	0.106	0.32
9	242.6	$y = 10^6x+45976$	0.053–1.712	0.9986	4.050	12.27
10	254.5	$y = 5.80 \times 10^4x+564.32$	0.09–1.437	0.9944	3.36	10.18

Note: *LOD: limit of determination, LOQ: limit of quantitation.

reduce pressure and the filtrate was evaporated using a rotary evaporator to yield crude extract residue (58.4 g). The residue was reconstituted in water and subjected to sequential extractions using varying polarities of solvents: diethyl ether (DE), hexane (HE), ethyl acetate (EA), and n-butanol (BuOH). Each extraction was performed three times to ensure thorough extraction of target compounds. The respective solution was collected for analysis.

Separation of the fractions with targeted bioactive compounds were further well carried out via semi-prep HPLC equipped with a 2998 PDA detector. Moreover, a binary solvent mixture was employed with H_2O with 0.1% formic acid (A) and MeOH (B) were applied for obtaining the optimal chromatographic separation.

The terpenoids were isolated and the purity determined by the HPLC profiles and ^{1}H NMR data reported previously (Yang, Wang et al. 2022).

2.4. HPLC signal-to-noise ratios

Chromatographic separation of all analytes was successfully attained utilizing a C18 column (Phenomenex, Luna 5μ C18, 250 × 4.6 mm) coupled with a guard column, both of which were maintained at ambient temperature. A binary mobile phase system comprised of water with 0.1% formic acid (A) and methanol (B). The system mobile phase consisted of solvent A (HPLC grade water + 0.1% formic acid) and solvent B (HPLC grade methanol) with a total flow rate of 0.8 mL per minute into the detector. At the time of injection (time zero), the mobile phase composition consisted of 100% mobile phase A (water) for a duration of 15 min before gradient program as follows: 0–35% A at 0–35 min; linear gradient 30% A at 35.01–50 min; linear gradient 30–0% A at 50–80 min; linear gradient 15% A at 80.01–100 min, injection volume is ten μL. Detection was performed using a 2998 Photodiode Array (PDA) Detector configured to operate at a wavelength of 245 nm.

2.5. Preparation of standards

The standard solutions of the target compounds (**1** to **10**) were dissolved in methanol to make stock solution with concentrations almost four hundreds mM for storage at 4 °C until HPLC analysis. During experimentation, a series of standard solutions were meticulously diluted with methanol to achieve desired concentrations, facilitating the construction of calibration curves. Subsequently, these solutions were filtered through a polyvinylidene fluoride (PVDF) membrane filter with a pore size of 0.45 μm before injection.

2.6. Construction of calibration curves

Determine the linearity of the method by injecting the standard solution into HPLC chromatographic system and analyzing via the HPLC method as described before. All samples repeated triplicate and completed within one day (Wang and Yang 2007). The linear least squares regression equations (Y = a+bX) for the ten compounds were obtained by plotting the peak areas from the chromatogram versus the corresponding concentrations of ten compounds (Wang and Yang 2007). The high-correlation coefficient values (r2 ≥ 0.99) of all the analytes showed high-related linearity within test concentration ranges (Figs. S1–S10).

Table 2
Precision of the HPLC analytic method for triterpenoids from *A. cinnamomea*.

Compounds	Theoretical concentration(mM)	CV	
		Intra-day precision (n = 6)	Inter-day precision (n = 18)
1	2.01	0.008	1.889
2	0.53	3.745	2.27
3	0.603	3.386	5.557
4	0.113	1.345	2.424
5	0.208	1.089	1.636
6	0.113	1.345	2.424
7	0.396	1.294	1.929
8	0.402	3.351	0.897
9	0.685	1.163	0.8
10	0.575	0.463	4.025

Note: CV: coefficient of variation = (S.D/mean) x 100%.

2.7. Method determination of LOD and LOQ

In accordance with previous studies, the limit of detection (LOD) and limit of quantification (LOQ) were investigated based on signal-to-noise ratios (S:N) of 3:1 and 10:1, respectively (Ling, Liu et al. 2011). A proper dilution method utilizing methanol was employed in preparing the working solutions of the ten analytes, resulting in six appropriate concentrations for each analyte. The LODs and LOQs of the 10 labeled marker standards were experimentally determined by conducting 3 repeated injections of each compound at the known concentrations, respectively. The related values were calculated according to the following Equations (Wang and Yang 2007):

$$LOD = \frac{3.3\sigma}{S}; LOQ = \frac{10\sigma}{S}$$

where σ and S are the mean standard deviation of three repeated injections results and the slope of the standard curves equation, respectively (Wang and Yang 2007).The results are shown in Table 1.

2.8. Precision, accuracy

Determination of precision and accuracy were carried out in accordance with International Conference on Harmonization (ICH) guidelines (Branch 2005). For precision validation, different concentrations of antcin K (**1**), (25S)-antcin H (**2**), (25R)-antcin H (**3**), (25R)-antcin C (**4**), (25S)-antcin C (**5**), (25R)-antcin A (**6**), 15α-acetyl-dehydrosulphurenic acid (**7**), versisponic acid D (**8**), dehydroeburicoic acid (**9**), eburicoic

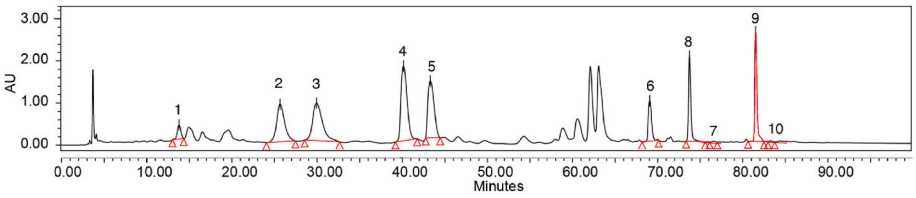

Fig. 2. HPLC profile of standard compounds detector wavelength is set at 254 nm. The column was equilibrated with 100% mobile phase A (water) for 10 min, and then gradient program changes to 35% A for 35 min, 30% A for following 20 min, and then 30–0% A from 50 to 80 min, 0% A from 80.01 to 90 min at a flow rate of 0.8 mL/min.

acid (**10**) were prepared and ten μL was replicate analysis of each compound with HPLC system. The intra-day precisions were determined by injecting the known concentrations of the 10 analytes 6 times injected into HPLC column during a single day and inter-day precisions were examined by duplicating the same procedures over 3 consecutive days at room temperature, overall 18 injections were conducted and analyzed (Ling, Liu et al. 2011).The variations of results were expressed by relative standard deviations (RSD). As shown in Table 2, the coefficient of variation (CV) was taken as a measure of precision and repeatability.

Accuracy was assessed by spike and recovery study, adding three level concentrations of the specific standard solutions to known amounts of selected commercial (AgriGADA Biotech Pte. Ltd) sample solution with the same volume, respectively. Moreover, 10 μL of the mixed solution was injected into HPLC column for triplicate times and compare the peak areas obtained from multiple analyses. To assess the validity and accuracy of the method, the average sample recovery of triterpenoids in the sample solution was evaluated by calculating the ratio of the amount detected to the addition of a known amount of each compound to the mixed solution, the following equation was used:

$$Recovery\ (\%) = \left(1 - \frac{F_f - C_f}{F_f}\right) \times 100\%$$

Where $F_f =$ theoretical concentration, $C_f =$ measured concentration.

where F_f is the theoretical concentration in the spiked sample, C_f is the amount of measured concentration observed in the spiked sample, and recovery means the percentage of the spiked samples was detected in the spiked sample. Intra- and inter-day imprecision (coefficient of variation (CV%)) and accuracy taken as measure were evaluated by the standard deviation (S.D.) to the mean value. The results are shown in Table 2.

2.9. Statistical analysis

Descriptive statistical analyses were performed using Origin 8.0 to calculate the means and the standard error of the mean. Results were expressed as the mean ± standard deviation (SD).

3. Results and discussion

3.1. Selection of standard compounds as markers of quality control

Terpenoids are the major phytochemicals in *A. cinnamomea* and were credited as the active compounds responsible for the health promotion property of *A. cinnamomea* based product. However, there are more than a hundred terpenoids isolated from the mushroom and they have wide structural diversity. Anti-inflammation activity is suggested to be the major underlying principle for the health promotion activity of *A. cinnamomea.* Recently, we found that only a few terpenoids shown anti-inflammation property on murine macrophages RAW 264.7 cells using compounds at concentrations of 50 μM and 100 μM. Among these, (25R)-antcin A (**6**), 15α-acetyl-dehydrosulphurenic acid (**7**), versisponic acid D (**8**), dehydroeburicoic acid (**9**), and eburicoic acid (**10**) showed promising anti-inflammatory bioactivity compared to blank groups, and these compounds did not exhibit any toxic effects at which the concentrations their activity was tested. Some major and unique terpenoids found in *A. cinnamomea* such as antcin K (**1**), (25S)-antcin H (**2**), (25R)-antcin H (**3**), (25R)-antcin C (**4**), and (25S)-antcin C (**5**) were included to the list of active compounds. Compound **6** and **8** (10 μM) have the highest activity in suppressing the inflammation reaction on RAW264.7 cell stimulated by lipopolysaccharides (LPS) with half-inhibitory concentration (IC_{50}) of 19.61 ± 0.8 μM and 17.16 ± 1.0 μM respectively, these values were significantly lower than the rest compounds and thus compound **6** and **8** may be used as reference standard for evaluation of anti-inflammatory activity of products.

The selected terpenoids showcase diverse chromophores, contributing to their distinct absorption maxima. Identifying the optimal detection wavelengths via photodiode array necessitated pinpointing their individual absorbance maxima. The UV-VIS spectra of these compounds are illustrated in Figs. S10–S22. Furthermore, these terpenoids demonstrate significant anti-inflammatory activity and are recognized as key active compounds, contributing to its biological effects. For instance, antcin K (Huo, Win et al. 2017), eburicoic acid, and dehydroeburicoic acid (Deng, Chen et al. 2009; Du et al., 2012) have shown protective effects against CCl4-induced acute hepatic damage in mice through antioxidant and anti-inflammatory mechanisms. Antcin H (Chen, Chang et al. 2019) has demonstrated anti-tumor activity in vivo, while the liver protective effects of antcin C (Gokila Vani, Kumar et al. 2013) have been reported, with antcin C confirmed to protect against free radical and alcohol-induced acute liver injury *in vivo*. Moreover, based on our previous research, versisponic acid D and Antcin A exhibited significant anti-inflammatory activity compared to LPS-treated cells, suggesting it may be major anti-inflammatory compounds in *A. cinnamomea* (Yang, Wang et al. 2022).

3.2. Construction of calibration curves

For linearity validation, standard stock solutions of the analytes of antcin K (**0.16–5.128 mM**), (25S)-antcin H (**0.058–1.855 mM**), (25R)-antcin H (**0.031–1.005 mM**), (25R)-antcin C (**0.019–0.599 mM**), (25S)-antcin C (**0.019–0.519 mM**), (25R)-antcin A (**0.033–0.522 mM**), 15α-acetyl-dehydrosulphurenic acid (**0.0372–1.19 mM**), versisponic acid D (**0.063–1.004 mM**), dehydroeburicoic acid (**0.053–1.712 mM**), eburicoic acid (**0.09–1.437 mM**) were prepared before injecting into HPLC column. For plotting the standard curves of the 10 analytes, at least choosing six concentrations of solutions and triplicate analyses are performed to obtain the linear least-squares regression equation for each analyte (Ling, Liu et al. 2011). The linear equations related results were showed in Table 1.

3.3. Analytical specificity

The HPLC grade solvent (methanol in this case) serves as the blank solution used in control groups. Under the conditions of the optimized method, standard mixtures of the 10 labeled compounds were injected into the HPLC column for analysis. The specificity in the samples was determined by comparing the retention times and wavelengths of the UV

Table 3
Accuracy for quantitative analysis of triterpenoids in *A cinnamomea*.

Compound	initial amount (mM)	spiked level (mM)	Recovery[a](%)			Mean(%)	CV[b](%)
			1	2	3		
1	0.616	0.933	91.09	89.44	86.55	89.03 ± 2.30	2.58
		1.866	94.22	89.91	95.35	93.16 ± 2.87	3.09
		3.732	100.98	93.02	93.05	95.68 ± 1.53	1.60
2	0.000	0.308	99.54	99.40	100.22	99.72 ± 0.437	0.44
		0.616	98.43	98.64	101.97	99.68 ± 1.988	1.99
		1.232	99.63	99.63	99.52	99.59 ± 0.065	0.06
3	0.066	0.201	87.20	90.42	84.21	87.27 ± 3.11	3.56
		0.401	95.99	95.99	94.32	95.43 ± 0.97	1.02
		0.802	99.78	96.95	96.95	97.89 ± 1.64	1.67
4	0	0.12	98.82	98.75	99.47	99.014 ± 0.397	0.44
		0.24	100.10	99.84	99.02	99.025 ± 0.564	1.99
		0.479	99.90	96.08	97.75	97.913 ± 1.913	0.06
5	0.028	0.104	98.81	97.73	100.44	98.99 ± 1.37	3.56
		0.207	91.87	99.56	95.66	95.70 ± 3.85	1.02
		0.415	99.3	98.34	100.73	99.45 ± 1.20	1.67
6	0.061	0.061	97.01	97.35	95.39	96.58 ± 1.05	1.09
		0.121	101.69	101.77	103.28	102.25 ± 0.89	0.88
		0.242	96.21	97.47	105.89	99.86 ± 4.34	4.35
7	0.117	0.256	97.46	94.79	97.59	96.61 ± 1.58	1.64
		0.511	99.25	102.37	102.69	101.439 ± 1.90	1.87
		1.02	102.35	103.08	102.44	102.62 ± 0.33	0.32
8	0	0.251	99.13	99.18	98.63	98.98 ± 0.305	0.308
		0.502	99.04	99.59	101.00	99.88 ± 1.014	1.016
		1.003	98.96	99.21	100.68	99.62 ± 0.928	0.931
9	0	0.281	96.97	94.49	97.65	96.37 ± 1.67	1.73
		0.561	97.59	97.15	97.39	97.37 ± 0.22	0.23
		1.122	93.77	93.62	93.27	93.55 ± 0.26	0.28
10	0	0.251	98.94	96.96	99.20	98.37 ± 1.23	1.25
		0.503	99.84	99.81	100.62	100.09 ± 0.46	0.46
		1.01	99.68	99.77	99.80	99.75 ± 0.06	0.06

Note: All values are mean S.D. obtained by triplicate analysis. CV (coefficient of variation) = (S.D./mean) x 100%.

spectra of the samples with those of the standard compounds (Figs. 1 and 2). Based on HPLC fingerprint, it was found that for all analytes, there was no interference in the retention time region of interest and well-dissolved peaks indicated reasonable analytical specificity and the ability to distinguish analyte peaks. The ten triterpenoids present in the *A. cinnamomea* samples were separated by the HPLC method with PDA detectors and the quantification were done by measuring the main absorption wavelengths of these markers are 242 and 254 nm to get good sensitivity and detection of these chemical markers. The selected compounds were well separated with retention times of 14.0, 25.78, 30.06, 40.26, 43.40, 66.35, 73.80, 74.24, 81.55 and 81.97 min respectively (Fig. 2). The total run time per injection was controlled within 90 min, which is a bit long, but the polarity difference of the compounds requires longer time to elute them all. It was conducive to the HPLC simultaneous quantitative analysis of all compounds in a single operation is much simpler and more accurate than using previous procedures.

3.4. Linearity ranges, LOD and LOQ

Quantification parameters for the ten terpenoids using the above-described analytical HPLC method were examined. Standard stock solutions containing the ten analytes were prepared and diluted to appropriate concentrations with methanol to yield a series of appropriate concentrations for plotting the calibration curves. Multi-point calibration equations of the standard curves of each component were constructed by plotting the peak areas versus the concentration of each analyte, 100 percent methanol solvent as blank control and these procedures were analyzed in triplicate. The calculated standard curves results were given in Table 1. The markers were linear in the ranges of 0.033–0.522, 0.16–5.128, 0.053–1.712, 0.019–0.599, 0.019–0.519, 0.031–1.005, 0.058–1.855, 0.0372–1.19, 0.063–1, 0.09–1.437 mM, respectively. Good regression coefficients (r^2 0.9944–0.9998) were found all compounds.

The limits of detection (LOD) and the limits of quantification (LOQ) under the present chromatographic conditions were established at signal-to-noise ratios of approximately 3 and 10, respectively. In which low concentrations levels of marker triterpenoids can be reliably detected (LOD) and quantified (LOQ) in the following analysis.

3.5. Precision

The analytical method precision levels for ten markers were evaluated by both the intra-day and inter-day tests by six analyses per day over a successive 3-day period. The accuracy of the method was verified by calculating the %CV of six replicate HPLC analysis to determine the variation in retention time and concentration values for each component. The values for the markers ranging from 0.008% to 3.7% and 0.8%–5.6%, respectively. All the measures we used here were lower than 6%. The corresponding results indicated that the analytical method for quantification of the ten bioactive markers deriving from *A. cinnamomea* revealed good precision (Table 2).

3.6. Accuracy

Accuracy was determined by a spike and recovery experiment (Betz, Brown et al. 2011) by accurately spiked a known amount of the corresponding compound at three concentration levels to a sample of *A. cinnamomea*. Following the spiking of the ten compounds into the *A. cinnamomea* sample solution, recoveries for this mixture was analyzed in triplicate to assess the recovery study. Different acceptance criteria, with acceptable recoveries ranging from at least 80–120% for the concentration levels in our study (Commission 2002; Guideline 2005). As shown in Table 3, the spike experimental measurements were within the acceptable range, which ranged from 89.03 ± 2.30 to 102.62 ± 0.33% with variation coefficients ranging from 0.06 to 3.56%. The values of percentage recovery spiked with the mixture of standards at different developmental stages indicating that the analytical method for these ten triterpenoids from *A. cinnamomea* samples exhibited quite good

Table 4
Nutraceutical products containing *A. cinnomomea* from the marketplace in Taiwan, China.

Sample No.	Mean Contents of the terpenoids[a]									
	1[b]	2	3	4	5	6	7	8	9	10
A[c]	3.493	ND	ND	ND	ND	ND	ND	ND	ND	ND
B	0.815	ND	ND	ND	ND	ND	ND	ND	0.477	ND
C	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
D	0.224	ND	ND	ND	ND	ND	ND	ND	4.343	ND
E	0.385	ND	ND	ND	ND	ND	ND	ND	0.718	ND
F	2.10	ND	ND	ND	ND	ND	ND	0.17	1	ND
G	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
H	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
I	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
J	ND	ND	ND	ND	0.059	2.8	ND	ND	ND	71.187
K	ND	ND	4.2	0.1	3.9	0.3	ND	0.15	0.07	128.12
L	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
M	ND	1.233	ND	ND	ND	ND	ND	ND	ND	ND

Note: [a] Compound numbers correspond to structures shown in Fig. 1.
[b] Values based on crude extracts.
[c] The product information can be found in supporting information Table S1. A-M mg/g sample. ND: not detectable.

accuracy.

3.7. Validation of sample extraction procedure

To obtain satisfactory extraction efficiency for all analytes, one-way experiments were compared to assess the extraction efficiency due to extraction conditions such as extraction method (sonication and reflux), extraction solvent (95% ethanol and 100% methanol) and extraction method. The method developed herein was successfully applied to the quantitative determination of 10 triterpenoids from the commercial products of *A. cinnamomea*. methanol was chosen as the extraction solvent since the most of bioactive compounds could be efficiently extracted and well separated from background by three replicate measurements at a concentration of 80 mg/mL for each capsule of commercial samples. The optimal extraction efficiency was indicated by shaker extraction with 100% methanol at room temperature for 60 min.

Our results showed that abundance and profile of triterpenoids have great variations among different nutraceutical products claiming to contain *A. cinnamomea*. This may be due to the difference in raw material grade, manufacturing process, and the different parts of the fungus biomass used. The constituents of products in capsule and pill form were different (Table 4) (products A–I, L-M vs. J and K). Among these products, dripping pills (product J and K) contained more bioactive compounds than other samples and eburicoic acid (**10**) was only identified from dripping pills J and K.

It is worth noting that Product K contains relatively high amounts and diverse type of triterpenoids compared to other products. And the content of eburicoic acid (**10**) in product K was approximately 2 times larger than in the product J which is another brand's drip pill product.

Two bioactive triterpenoids detected in the commercial products B, D and E, and the categories of triterpenoids among them were comparable, although the contents of bioactive compounds not vary significantly except dehydroeburicoic acid (**9**) (products B vs. D vs. E). The content of dehydroeburicoic acid (**9**) in product D was nearly 5 times higher than that in products B and E. It was also found that five products (C, G, H, I, L) contain only trace amount of terpenoids while compound **1** and **9** is the most commonly found compounds.

4. Conclusions

In summary, the simultaneous quantitation method of ten characteristic triterpenoids in *A. cinnamomea* was developed using a reversed-phase high-performance liquid chromatography and the method features high sensitivity, precision and accuracy for quality control of the commercial samples claiming containing the precious *A. cinnamomea*. It would be important to correlate the purported bioactivity of these products and the contents of these terpenoids to further establish the chemical principles of these products and differentiate the quality of the products and safe guard the consumer well-being and promote evidence based nutraceutical products. Further multiple lab validation is needed to establish our method as an industrially recognized method for *A. cinnomomea* industry.

CRediT authorship contribution statement

Chunyuhang Xu: Experimental, Data curation, Writing manuscript. **Xin Yang:** Investigation, Formal analysis, Data curation, Project administration. **Linzhi Jing:** Investigation. **Xiang Wang:** data processing. **Zhuoyu Zhou:** Improving the manuscript and addressing the reviewers' comments. **Yujia Cao:** Investigation. **Hongling Zheng:** Investigation. **Chien-Liang Kuo:** Conceptualization. **Dejian Huang:** Supervision, Conceptualization, Writing – review & editing, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.crfs.2024.100721.

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