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Short Communication

Quantitative determination of maleic and succinic acid derivatives in the mycelium of *Antrodia cinnamomea*

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For the purpose of quantitative determination of novel maleic acid and succinic acid derivatives, antrodins A−E, in the mycelium of *Antrodia cinnamomea*, a high-performance liquid chromatography (HPLC) method was applied to separation of these compounds under the conditions, where the mobile phase was a linear gradient of 1% AcOH/H_2O-CH_3CN, the flow rate was 1.0 ml/min and the detecting wavelength was 235 nm, using an ODS column. The relative standard deviations of this method were less than 4.6% and 4.1% (n=5) for interday and intraday assays, respectively. A good linear correlation was obtained in a range of 0.1 (or 0.2) μg — 1.2 μg. The recoveries of antrodins A, B, C, D and E were 99.8, 102.9, 101.0, 100.5 and 99.9%, respectively.

This method was rapid, accurate and suitable for the quantitative determination of maleic acid and succinic acid derivatives in the mycelium of *Antrodia cinnamomea*.

Key words *Antrodia cinnamomea,* mycelium, antrodin, maleic acid, succinic acid, HPLC.

Abbreviations HPLC, high performance liquid chromatography; ODS, octadesyl silyl; *A. cinnamonea, Antrodia cinnamomea; P. acnes,* Propionibactrium acnes; LPS, lipopolysaccharide; RSD, relative standard deviation; C.V., coefficient of variation.

Introduction

Antrodia cinnamomea Chang TT & WN Chou (Chinese name, niu-chang-chih; synonym *Antrodia camphorata* Wu SH *et al.*) is a medicinal fungus of the family Basidiomycetes, which has been used as a folk medicine in Taiwan. It grows only on the inner heartwood wall of the endemic evergreen *Cinnamomum kanehirai* Hay. (Lauraceae) in Taiwan. It is rare and has not been well cultivated. The fruiting bodies have been used for treating food and drug intoxication, diarrhea, abdominal pain, hypertension, itchy skin, and liver cancer.[1-5] a few chemical and biological studies have been reported hitherto; The pharmacological activities and the chemical constituents in the crude extract of *A. cinnamomea* are quite different from those of *Ganoderma lucidum* (Fr.) Karst.[6,7] Chemical constituents found in the fruiting bodies of *A. cinnamomea* were phenyl compounds, a sesquiterpene lactone, steroids and triterpenoids.[8-17] The triterpenoids have been reported to have anti-cholinergic and anti-serotonergic activities.[13] Hepatoprotective effect was found in a hot water extract of *A. cinnamomea*.[18-20] Recently, we isolated five maleic acid and succinic acid derivatives (antrodins A−E; hepacim®) from the mycelium of *A. camphorata*. Maleimide derivatives (antrodins B and C) showed appreciable cytotoxic activity against LLC cells.[18] Furthermore, we found that antrodin C showed hepatoprotective activity in a hepatic injury model,[21,22] which was induced by a *Propionibacterium acnes (P. acnes)* and lipopolysaccharide (LPS) treatment in ICR mice.

With the successful artificial cultivation of *A. cinnamomea*, this fungus and related products have been widely used as health foods at present. However, quantitative analysis of the constituents in the products of *A. cinnamomea* has rarely been conducted. In this paper, we report the establishment of an HPLC method for the quantitative determination of five maleic acid and succinic acid derivatives. Then using this HPLC method, the fruiting body and four different lots of the mycelia of *A. cinnamomea* were evaluated.

Materials and Methods

Materials. *A. cinnamomea* mycelium (Ac-S1−4: Lot numbers are #C082002−1, #C092101−1, #C072004−2 and #C042503−3, respectively) and its fruiting body were provided by Simpson Biotech Co. Ltd. (Taiwan). The standard compounds antrodins A−E used in this experiment were isolated from a chloroform ($CHCl_3$) extract of the mycelium of *A. cinnamomea*, as reported previously (Fig. 1).[18] The structures and purity of these compounds were confirmed by spectroscopic and chromatographic methods. The purity of antrodins A, B, C, D and E were 96.8, 97.4, 97.8, 98.7 and 98.8%, respectively. Acetonitrile of analytical HPLC grade was purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan), and acetic acid of analytical reagent grade was from Nacalai Tesque (Kyoto, Japan). Column chromatography was carried out on an ODS Sep-Pak column (Millipore Co. Milford, Mass. USA). Thin-layer chromatography (TLC) was carried out on pre-coated silica gel 60 F-254 plates (0.25 mm, Merck Co., Darmstadt,

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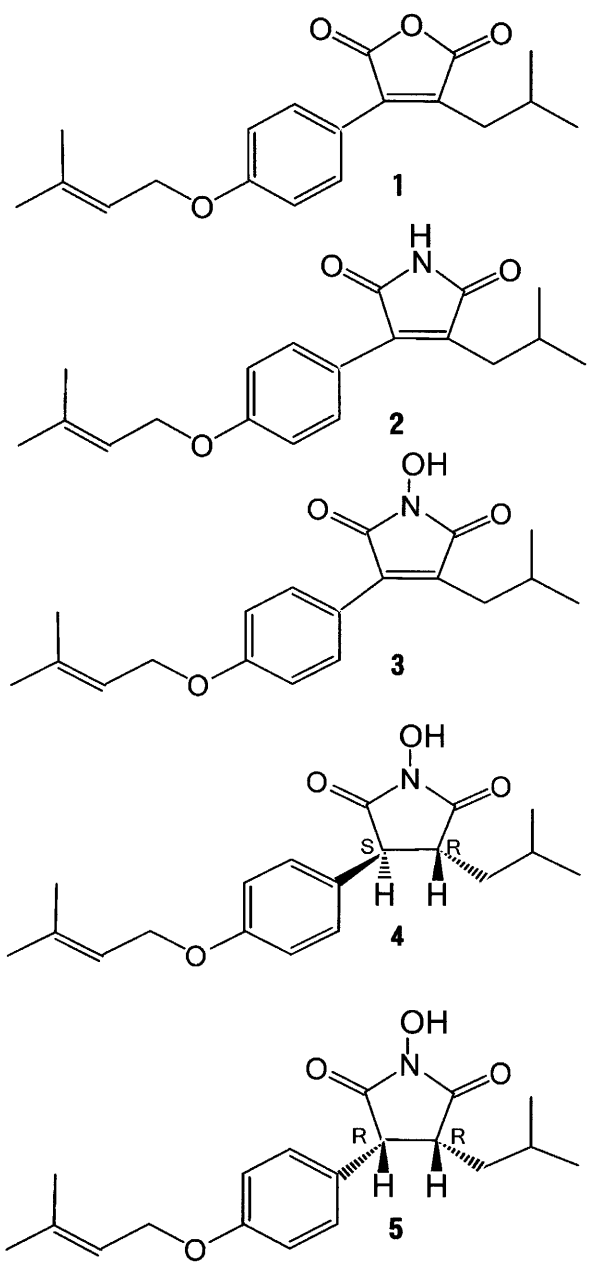

Fig. 1 Structures of antrodins A (**1**), B (**2**), C (**3**), D (**4**) and E (**5**)

Germany), and spots were detected under a UV light and by spraying with 10% H_2SO_4 followed by heating. Millipore syringe filters (Millex-GP, 0.45 μm pore size) were purchased from Nihon Millipore (Tokyo, Japan).

Instruments. Analytical HPLC was carried out on a CCP 8020 system (Tosoh Co., Tokyo, Japan) consisting of a multi-solvent delivery pump (CCPM-II), an auto-injector, and a single wavelength UV detector (UV-8020). The signals from the detector were recorded and analyzed on a C-R8A recorder (Shimadzu Co., Kyoto, Japan).

HPLC analysis. A TSK gel ODS-80 Ts column (4.6 x 150 mm i.d., Tosoh Co., Japan) was used for analysis. The mobile phase was composed of 1% AcOH/H_2O-CH_3CN (0 min, 60:40; 40 min, 30:70). The flow rate was 1.0 ml/min, and the detecting wavelength was set at 235 nm. The operating temperature was maintained at 25℃.

Standard curve preparation. Every standard compound was accurately weighed and dissolved in methanol to prepare a concentration of 1 mg/ml. Each standard solution (200 μl) was taken and diluted with methanol to give a 1 ml solution. One to six micro liter was injected to an HPLC column. The linearity was determined with three injections for each concentration and linear regression of the peak-area ratio *versus* the injected quantity of each standard compound. The contents of these constituents in the test sample were calculated using the regression parameters obtained from the standard curve.

Precision of peak area. To verify the precision of this method, we analyzed a standard mixture of 3 μl (0.2 μg/μl) with five repetitions on the same day and five consecutive days. The relative standard deviations (RSD) of peak areas of the intraday and interday assays were analyzed.

Accuracy of standard curves. To verify the accuracy of the standard curves of antrodins A－E, we analyzed the respective standard mixtures of 0.4 μg (0.2 μg/μl × 2 μl), 0.6 μg (0.2 μg/μl × 3 μl), and 0.8 μg (0.2 μg/μl × 4 μl). According to each standard curve of antrodins A－E, the quantity of each compound was determined, and coefficient of variation (C.V.) was calculated.

Sample preparations for HPLC. One gram of the mycelium of *A. cinnamomea* was accurately weighted and extracted three times with 20 ml of $CHCl_3$ by refluxing in a boiling water bath for 3 h. After filtration, the combined solutions were evaporated to dryness *in vacuo*. These $CHCl_3$ extracts were made to a 1 mg/ml solution with methanol, then 3 ml of the solution was chromatographed on a ODS Sep-Pak column eluted with 12 ml of water, followed by 3 ml of methanol. The methanol eluate was evaporated in vacuo to give a residue, which was dissolved in 3 ml of methanol. Then 5 μl of the methanol solution was analyzed by HPLC.

Reproducibility of the extracting method. To verify the suitability of an extracting method, five portions of the mycelium of *A. cinnamomea* (1 g) were accurately weighted and extracted with $CHCl_3$ as mentioned above for preparation of analytical samples. A 5 μl portion of the methanol solution was analyzed using HPLC, and the RSD of peak areas were calculated.

Recovery tests. Three portions of the mycelium of *A. cinnamomea* (1 g) were accurately weighted and extracted three times with 20 ml of $CHCl_3$ by refluxing in a boiling water bath for 3 h. After filtration, combined solutions were evaporated to dryness *in vacuo*. The $CHCl_3$ extract was made to the respective 1 mg/ml solutions with methanol, and two portions (1 ml each) of the solution were taken. One was control, and 20, 40 and 80 μg of antrodins A－E were added to the other portions. Then the solutions were applied to ODS Sep-Pak columns, which were eluted with 4 ml of water and 1 ml of methanol. The methanol solution was evaporated *in vacuo* to give a residue, which was dissolved in 1 ml of methanol. Then all sample solutions were

filtered and a 5 μl portion was subjected to HPLC analysis to calculate the recovery rates.

Quantitative determination of antrodins A－E in the mycelium of *A. cinnamomea*. The mycelium of *A. cinnamomea* (1 g) was accurately weighted, extracted and treated as described above. The methanol solutions were filtered through a 0.45 μm Millipore filter unit. Then 5 μl of each sample solution was analyzed by HPLC. The contents of constituents in the test samples were calculated using regression parameters obtained from standard curves.

Results and Discussion

The standard compounds, antrodins A－E, used in this experiment were isolated from a chloroform extract of the mycelium of *A. cinnamomea*,[17] and the purity of these compounds were more than 96.8% as estimated by a chromatographic method. As shown in Table 1, the retention times of the respective standard compounds were apparently distinguishable. In calibration curves obtained, where Y is the peak area and X is the quantity of the respective compounds, the regression of these standard compounds showed good linear relationships between the peak-area and the quantity. The RSD of the peak area of antrodins A－E were 0.52－2.13% for standard compounds (Table 2). The RSD of the interday and intraday assays were 0.52－4.14% and 2.23－4.63% for standard compounds (Table 3). The Accuracy of standard curves of antrodins A－E were 99.6 (C. V., 1.00), 100.6 (C. V., 1.21), 99.6 (C. V., 1.47), 97.7 (C. V., 2.10) and 98.3% (C. V., 1.60), respectively (Table 4). These results showed that the method and the regression parameters obtained from the standard curves were suitable for quantitative determination of antrodins A－E in the samples.

Table 1. Retention time reproducibilities, regression equations, correlation coefficients (γ), and linearity ranges of antrodins A—E

Compound	Retention time (min, n=3)	Regression equation	γ	Linearity range (μg)
Antrodin A (**1**)	37.07±0.016	y = 219.74 x - 2.58	0.9998	0.20-1.20
Antrodin B (**2**)	27.24±0.021	y = 305.74 x - 5.65	0.9995	0.10-1.20
Antrodin C (**3**)	22.62±0.019	y = 354.82 x - 5.78	0.9997	0.10-1.20
Antrodin D (**4**)	16.61±0.024	y = 163.99 x - 4.91	0.9985	0.10-1.20
Antrodin E (**5**)	15.97±0.026	y = 132.29 x - 2.48	0.9991	0.20-1.20

Table 2. Precision of peak area of antrodins A—E

Compound	Injucted (μL)	Peak Area (Mean±S.D.)	RSD (%)
Antrodin A (**1**)	1	42.75±0.30	0.70
	3	125.47±1.01	0.81
	6	269.41±5.73	2.13
Antrodin B (**2**)	1	59.99±0.61	1.01
	3	176.02±0.92	0.52
	6	372.41±7.28	1.95
Antrodin C (**3**)	1	69.00±0.38	0.55
	3	203.38±2.44	1.20
	6	431.77±7.75	1.80
Antrodin D (**4**)	1	30.90±0.30	0.99
	3	89.15±1.50	1.60
	6	194.78±2.70	1.39
Antrodin E (**5**)	1	24.90±0.33	1.33
	3	74.77±0.74	0.99
	6	157.56±2.86	1.80

Table 3. Intraday and interday relative standard deviations of antrodins A—E

Compound	Intraday (%)	Interday (%)
Antrodin A (**1**)	1.51	3.61
Antrodin B (**2**)	0.52	2.46
Antrodin C (**3**)	1.20	2.64
Antrodin D (**4**)	4.14	2.23
Antrodin E (**5**)	0.99	4.63

In the reproducibility of an extracting method, RSD values of antrodins A－E were 3.42, 4.39, 1.91, 2.62 and 1.58%, respectively. The recovery rates of antrodins A－E were 99.8 (RSD, 3.34), 102.9 (RSD, 3.82), 101.0 (RSD, 3.56), 100.5 (RSD, 4.56) and 99.9% (RSD, 3.92), respectively, suggesting that the extracting method was suitable.

These findings indicated that the method was satisfactory in terms of linearity, accuracy, and reproducibility. It was suitable for quantitative determination of antrodins A－E in the mycelium of *A. cinnamomea* in a wide range of concentrations.

Typical chromatograms of these samples are shown in Fig. 2. A sample Fb derived the fruiting body of *A. cinnamomea* showed a quite different elution profile when compared to those of samples of the mycelium of *A. cinnamomea*, Ac-S1, Ac-S2, Ac-S3 and Ac-S4, except a peak A. Though the retention time of peak A was close to that of the peak of antrodin C, both showed different chromatographic behavior on a thin layer chromatography. These findings indicated that antrodins A－E were characteristic compounds present in the mycelium, but not present in the fruiting body of *A. cinnamomea*.

The quantity of antrodins A－E varied from lot to lot (Table 5). It is suggested that artificially cultivating conditions may be important for production of novel maleic acid and succinic acid derivatives.

In summary, HPLC was demonstrated to be an accurate, precise and reliable analytical method for determination of the maleic acid and succinic acid derivatives in the

Table 4. Accuracy of standard curves of antrodins A—E (n=3)

Compound	Injucted (μg)	Measured (μg)	Accuracy (%)	Mean±S.D.	C.V. (%)
Antrodin A (**1**)	2μL (0.4)	0.3999±0.0007	99.993 ±0.1996	99.643±1.004	1.00
	3μL (0.6)	0.5827±0.0046	97.121 ±0.7720		
	4μL (0.8)	0.8145±0.0163	101.814 ±2.040		
Antrodin B (**2**)	2μL (0.4)	0.4074±0.0029	101.856 ±0.7191	100.593±1.22	1.21
	3μL (0.6)	0.5942±0.0030	99.031 ±0.5028		
	4μL (0.8)	0.8071±0.0194	100.893 ±2.429		
Antrodin C (**3**)	2μL (0.4)	0.4024±0.0026	100.602 ±0.6479	99.576±1.4588	1.47
	3μL (0.6)	0.5895±0.0068	98.249 ±1.1473		
	4μL (0.8)	0.799 ±0.0206	99.877 ±2.5813		
Antrodin D (**4**)	2μL (0.4)	0.4025±0.0102	100.637 ±2.5669	97.662±2.0550	2.10
	3μL (0.6)	0.5735±0.0091	95.5873±1.5203		
	4μL (0.8)	0.7741±0.0166	96.763 ±2.0770		
Antrodin E (**5**)	2μL (0.4)	0.3954±0.0074	98.862 ±1.8502	98.324±1.5732	1.60
	3μL (0.6)	0.5839±0.0056	97.320 ±0.9371		
	4μL (0.8)	0.7903±0.0155	98.324 ±1.9324		

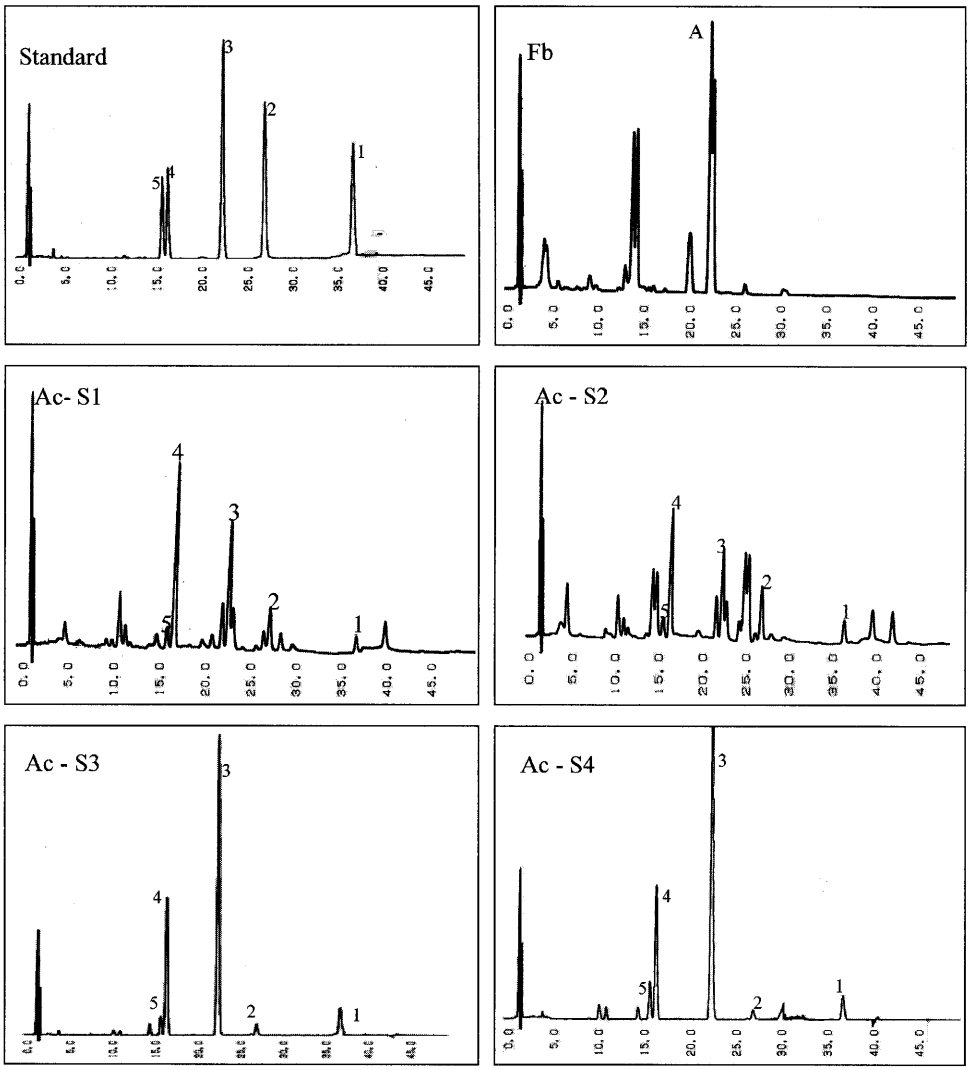

Fig. 2 HPLC chromatograms of various samples of the mycelium of *Antrodia cinnamomea*
Fb: the fruiting body of *A. cinnamomea*.; Ac-S1－4: the mycelium of *A. cinnamomea*; Lot numbers are #C082002－1, #C092101－1, #C072004－2 and #C042503－3, respectively.

Table 5. Contents of antrodins A—E in the mycelium of *Antrodia cinnamomea*

Sample	Antrodin				
	A	B	C	D	E
Ac-S1	—[a)]	0.81[b)]	1.69	24.95	3.53
Ac-S2	—	2.15	3.22	24.96	4.05
Ac-S3	9.23	4.13	51.96	45.96	7.39
Ac-S4	7.80	2.97	52.90	44.05	14.12

a): Not detected; b): mg/g (dry weight of sample)

mycelium of *A. cinnamomea*, and this method may provide useful means of chemical evaluation, not only for scientific purposes but also for industrial applications.

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Japanese abstract

樟芝菌糸体中の新規マレイン酸及び琥珀酸誘導体（antrodin A－E）の定量分析法を確立する目的で，高速液体クロマトグラフィー（HPLC）による分析条件を検討した。Antrodin A－Eは，移動相：1％ AcOH/H_2O-CH_3CN (0 min, 60 : 40 ; 40 min, 30 : 70)；流速：1.0 ml/min；測定波長：235 nm；カラム温度：室温の条件下，ODSカラムを用い定量を行った。この方法による日内と日間の相対的な標準偏差は4.6%及び4.1% (n=5) であり，0.1 (or 0.2) μg から1.2 μg までの濃度範囲で良好な直線性が得られた。Antrodin A－Eの回収率はそれぞれ，99.8, 102.9, 101.0, 100.5及び99.9%であった。本測定法は迅速且つ正確であり，樟芝菌糸体中の新規マレイン酸及び琥珀酸誘導体の定量に適していると考えられる。

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