Research paper

Differentiation of Mycelia and Basidiomes of *Antrodia cinnamomea* Using Certain Chemical Components

Tun-Tschu Chang,[1,2] Wu-Rong Wang,[1] Cheng-Jen Chou[1]

【Summary】

Using 10 known components in *Antrodia cinnamomea* including 5 ergostanes (antcins C and K, and zhankuic acids A, B, and C), 4 lanostanes (sulphurenic acid, dehydrosulphurenic acid, eburicoic acid, and dehydroeburicoic acid), and 1 monophenyl (4,7-dimethoxy-5-methyl-1,3-benzodioxole) as standards, mycelia and basidiomes of *A. cinnamomea* were differentiated in this study. Natural basidiomes collected from wood of *Cinnamomum kanehirai* in natural forests and cultured basidiomes grown on potato dextrose agar medium contained all 10 test components. However, natural mycelia collected from the wood of *C. kanehirai* in a natural forest and liquid/solid cultured mycelia grown on potato dextrose broth/potato dextrose agar media contained the 4 lanostanes and 4,7-dimethoxy-5-methyl-1,3-benzodioxole but not the 5 ergostanes. These results indicate that the production of ergostanes is related to basidiomatal formation of *A. cinnamomea*, but is not related to the substrate on which the organism is grown.

Key words: *Antrodia cinnamomea*, mycelia, basidiomes, chemical differentiation.

Chang TT, Wang WR, Chou CJ. 2011. Differentiation of mycelia and basidiomes of *Antrodia cinnamomea* using certain chemical components. Taiwan J For Sci 26(2):125-33.

[1] Forest Protection Division, Taiwan Forestry Research Institute, 53 Nanhai Rd., Taipei 10066, Taiwan. 林業試驗所森林保護組，10066台北市南海路53號。

[2] Corresponding author, e-mail:ttchang@tfri.gov.tw 通訊作者。

Received September 2010, Accepted January 2011. 2010年9月送審 2011年1月通過。

研究報告

利用幾種化學成分區別牛樟芝的菌絲體與子實體

張東柱[1,2)] 王武榮[1)] 周正仁[1)]

摘 要

利用10種分離自牛樟芝子實體的化合物，其中包括5種麥角甾烷三萜類(ergostanes: antcins C and K, and zhankuic acids A, B, and C)、4種羊毛甾烷三萜類(lanostanes: sulphurenic acid, dehydrosulphurenic acid, eburicoic acid, and dehydroeburicoic acid)，和1種單苯基類(monophenyl: 4,7-dimethoxy-5-methyl-1,3-benzodioxole)當標準成分，比較天然牛樟芝子實體與菌絲體，和人工培養子實體與菌絲體的成分差異。採自生長於牛樟木材之天然子實體與培養於馬鈴薯葡萄糖洋菜(PDA)培養基產生的子實體，均可檢測出上述10種化合物，然而，採自生長在牛樟木材之天然菌絲體與人工固態與液態培養之菌絲體只能產生上述4種羊毛甾烷三萜類和1種單苯基類化合物，但不能產生5種麥角甾烷三萜類。結果顯示，麥角甾烷三萜類的產生似乎與牛樟芝子實體的形成有相關性，但與子實體生長的基質沒有關係，因為天然子實體生長於牛樟木材，而人工培養子實體則生長於不含任何牛樟木材成分的培養基上。

關鍵詞：牛樟芝、子實體、菌絲體、化學成分差異。

張東柱、王武榮、周正仁。2011。利用幾種化學成分區別牛樟芝的菌絲體與子實體。台灣林業科學 26(2):125-33。

INTRODUCTION

Antrodia cinnamomea TT Chang & WN Chou is a resupinate to effused-reflexed basidiomycete with a porous hymenium. It is known only from Taiwan and is restricted to *Cinnamomum kanehirai* Hayata (Chang and Chou 1995, 2004). The basidiomes produced on infested wood are used as an herbal medicine in Taiwan. Owing to its host specificity and rarity in nature as well as its effectiveness in curing certain illnesses (Shen et al. 2004b), the basidiomes of the fungus are expensive. Artificial cultivation of *A. cinnamomea* basidiomes to satisfy market demand is considered the most effective solution. Chang and Wang (2005, 2008) reported that *A. cinnamomea* produced basidiomes on artificial agar media without containing substrates from wood of *C. kanehirai* indicating that the basidiomatal formation of *A. cinnamomea* was not related to the substrate from wood of *C. kanehirai*. In addition, Lin et al. (2006) reported that physical wounding of *A. cinnamomea* red hyphae induced basidiomatal formation on an MEA (malt-extract agar) plate.

It is said that wood components of *C. kanehirai* might be related to the bioactive components of *A. cinnamomea*. However, *C. kanehirai* is an endemic and endangered species in Taiwan. Resources of this wood are limited from natural forests; therefore, using *C. kanehirai* wood for cultivation of *A. cinnamomea* is impossible. Whether the basidiomes that are formed on media without *C. kanehirai* substrates contain the bioactive components is worth studying. Methanolic/ethanolic extracts from natural basidiomes of

A. cinnamomea had some bioactive components such as triterpenoids, and monophenyl and biphenyl components (Chiang et al. 1995, Shen et al. 2003a, b, 2004b, 2005). In addition, 2 lanostane-type triterpenoids were identified from cultured mycelia of *A. cinnamomea* and could be involved in its anti-inflammatory actions (Shen et al. 2004a, Chen et al. 2009). We therefore compared the components from the methanolic extract of *A. cinnamomea* among natural basidiomes, cultured basidiomes, natural mycelia, and liquid/agar-cultured mycelia in this study. Based on previous studies from our team (Shen et al. 2003a, b, 2004b, 2005), 9 triterpenoids (antcins C and K, zhankuic acids A, B, and C, sulphurenic acid, dehydrosulphurenic acid, eburicoic acid, and dehydroeburicoic acid) and 1 monophenyl component (4,7-dimethoxy-5-methyl-1,3-benzodioxole) from *A. cinnamomea* were purified and used as standards in a high-performance liquid chromatographic (HPLC) analysis to differentiate mycelia and basidiomes of *A. cinnamomea* in this study.

MATERIALS AND METHODS

Fungal samples

Natural basidiomes: Three basidiomatal samples of *A. cinnamomea* were collected from the host, *C. kanehirai*, in natural forests in Hsinchu, Chiayi, and Kaohsiung Counties. Natural mycelia: A mycelial mat of *A. cinnamomea* growing in decayed wood of *C. kanehirai* in a natural forest in Hsinchu County was collected. This mycelial mat was cultured (isolate TFRI B593) and identified by the first author. Cultured basidiomes: The methods of Chang and Wang (2005) for basidiomatal formation of *A. cinnamomea* were followed. Isolates TFRI B479 and B522 isolated from basidiomes of *A. cinnamomea* respectively collected from Kaohsiung and Taoyuan Counties were used for the basidiomatal production on potato-dextrose-agar (PDA; Himadia, Mumbai, India) medium. One block (3×3 mm) of culture was placed in the center of a Petri dish containing PDA medium. Cultures were incubated at 24℃ in the dark for 4 mo for basidiomatal formation. The basidiomes produced on the PDA medium were collected as cultured basidiomes. In addition, the mycelia near by the basidiomes were also collected for an HPLC analysis. Liquid-cultured mycelia: Isolates TFRI B479 and B86 were used to produce liquid-cultured mycelia. Four blocks (3×3 mm) of cultures were inoculated into each 500-ml flask containing 200 ml potato-dextrose-broth (PDB; Himadia, Mumbai, India) medium. Cultures were incubated at 24℃ in the dark for 1 mo. At the end of incubation, mycelia were rapidly rinsed with 500 ml of NaCl (250 mM) in an aspirator-suction system to remove any contamination from the culture medium. Agar-cultured mycelia: Isolates TFRI B479 and B86 were used to produce agar-cultured mycelia. One block (3×3 mm) of culture was placed in the center of a Petri dish containing PDA medium. Cultures were incubated at 24℃ in the dark for 1 mo. At the end of incubation, the mycelial layer at the top of the culture was removed from the Petri dish. All test samples were dried in an oven at 50℃ until dry before being extracted with methanol.

Preparation of methanolic extracts

Test samples were dried in an oven at 50℃ for 2 d. Five grams of each dry sample was refluxed 4 times with methanol for 6 h; the extract was filtered and evaporated; and 10 mg of each dry extract was diluted with 1 ml methanol immediately before use (Shen et al. 2004b).

HPLC system

HPLC was performed on an Agilent 1100 series with DAD(Diode Array Detector) detection. The detection wavelength was set to 210 nm. Separation was obtained with a reversed-phase column (Cosmosil $5C_{18}$- AR-II, 250×4.6 mm, Kyoto, Japan) eluted at a flow rate of 1 ml min^{-1} with a linear solvent gradient elution of A (0.0085% H_3PO_4 in H_2O) and B (acetonitrite), and the column was eluted according to the following profile: 0~65 min, 30~47% B, 65~100 min, 47~47% B, 100~140 min, 47~100% B, 140~170 min, 100~100% B, 170~175 min, 100~30% B, 175~200 min, 30~30% B. The column temperature was set to 30℃. The injection volume was 20 μL. Ten known compounds (with purities of > 99% for each compound) including 5 ergostane triterpenoids (antcins C and K, and zhankuic acids A, B, and C), 4 lanostane triterpenoids (sulphurenic acid, dehydrosulphurenic acid, eburicoic acid, and dehydroeburicoic acid), and 1 monophenyl (4,7-dimethoxy-5-methyl-1,3-benzodioxole) were used as the detected components. Their retention times and patterns of detecting wavelength (210 nm) were based on our previous studies (Shen et al. 2003a, b, 2004b, 2005). Each component and its retention times (in min) in parenthesis were as follows: antcin K (23.1 and 24.3), 4,7-dimethethoxy-5-methyl-1,3-benzodioxole (35.8), antcin C (59.7 and 62.5), zhankuic acid C (62.6 and 64.0), zhankuic acid B (83.4 and 84.5), dehydrosulphurenic acid (83.5), sulphurenic acid (87.5), zhankuic acid A (93.9 and 95.0), dehydroeburicoic acid (140.1), and eburicoic acid (141.4). Because the 5 ergostane triterpenoids have 2 C-25 epimers, they presented 2 close retention times on the HPLC chromatograms.

RESULTS

Comparison of natural and cultured basidiomes

The HPLC chromatogram of 10 standard components is presented in Fig. 1. The HPLC chromatograms of 3 natural basidiomes from

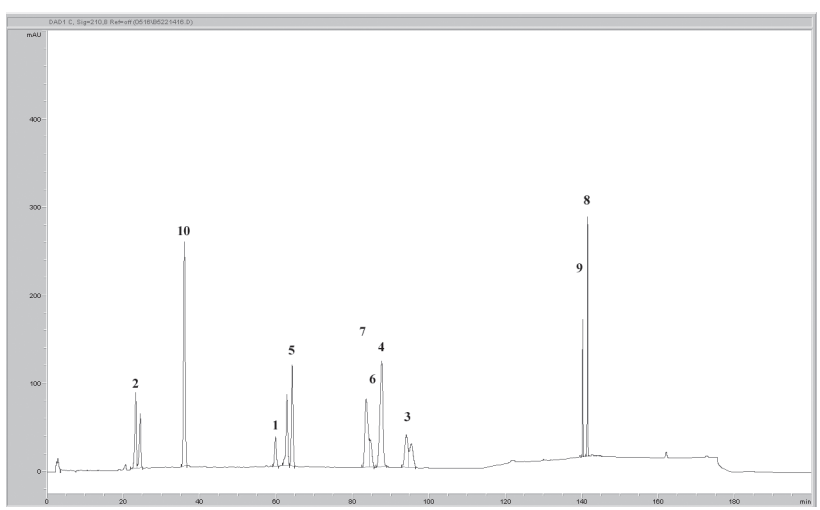

Fig. 1. HPLC chromatogram of 10 known standard components isolated and purified from *Antrodia cinnamomea* fruiting bodies. 1, Antcin C; 2, antcin K; 3, zhankuic acid A; 4, zhankuic acid B; 5, zhankuic acid C; 6, sulphurenic acid; 7, dehydrosulphurenic acid; 8, eburicoic acid; 9, dehydroeburicoic acid; 10, 4,7-dimethoxy-5-methyl-1,3-benzodioxiole.

3 collection sites (Hsinchu, Chiayi and Kaohsiung Counties) were similar, and consequently 1 chromatogram of a sample from Hsinchu County is presented as the standard of natural basidiomes (Fig. 2). For comparison with 10 standard compounds by HPLC, these 3 natural basidiomes contained all 10 test standard compounds. The HPLC chromatograms of 2 cultured basidiomes (isolates B479 and B522) were similar. When compared to the 10 standard compounds of natural basidiomes, these 2 cultured basidiomes also contained all 10 test compounds (Fig. 3).

Comparison of natural and cultured mycelia

The HPLC chromatograms showed that the methanolic extracts of natural mycelia contained the 4 detected lanostane triterpenoids (sulphurenic acid, dehydrosulphurenic acid, eburicoic acid, and dehydroeburicoic acid) and 4,7-dimethoxy-5-methyl-1,3-benzodioxole (Fig. 4). The natural mycelia did not contain the 5 detected ergostane triterpenoids (antcins C and K, and zhankuic acids A, B, and C). The HPLC chromatograms of 2 liquid-cultured mycelia and agar-cultured mycelia (isolates B479 and B86) also contained the 4 lanostanes and 1 monophenyl compound as did natural mycelia (Fig. 5). In addition, the mycelia near by the basidiomes from the nutrient agar medium of isolates TFRI B479 and B522 also only contained the 4 lanostanes and this monophenyl compound as did natural mycelia, but not the 5 detected ergostane triterpenoids produced by the cultured basidiomes on the same plates.

DISCUSSION

These results indicate that production of ergostane triterpenoids such as antcins C and K, and zhankuic acids A, B and C is associated with the basidiomatal formation of *A*.

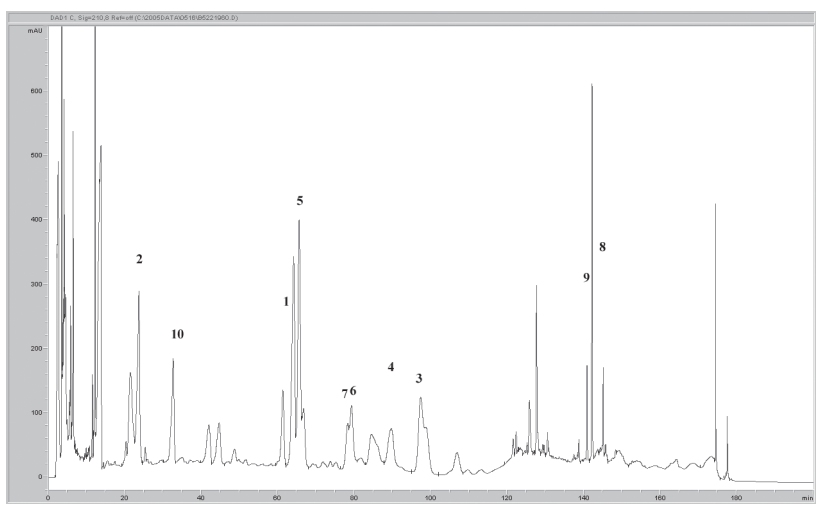

Fig. 2. HPLC chromatogram of the methanolic extract from natural fruiting bodies of *Antrodia cinnamomea*. 1, Antcin C; 2, antcin K; 3, zhankuic acid A; 4, zhankuic acid B; 5, zhankuic acid C; 6, sulphurenic acid; 7, dehydrosulphurenic acid; 8, eburicoic acid; 9, dehydroeburicoic acid; 10, 4,7-dimethoxy-5-methyl-1,3-benzodioxiole. The purity of these standards was higher than 98%. One milligram of each standard compound was dissolved in 10 ml methanol as the injected sample. The injection volume was 20 µl.

cinnamomea, since both natural basidiomes grown on wood of *C. kanehirai* and cultured basidiomes produced on PDA medium contained the 5 detected ergostane triterpenoids, but these were found in neither the natural nor cultured mycelia (Table 1). The 4 detected lanostane triterpenoids (sulphurenic acid, dehydrosuphurenic acid, eburicoic acid, and dehydroeburicoic acid) and 1 monophenyl (4,7-dimethoxy-5-methyl-1,3-benzodioxole)

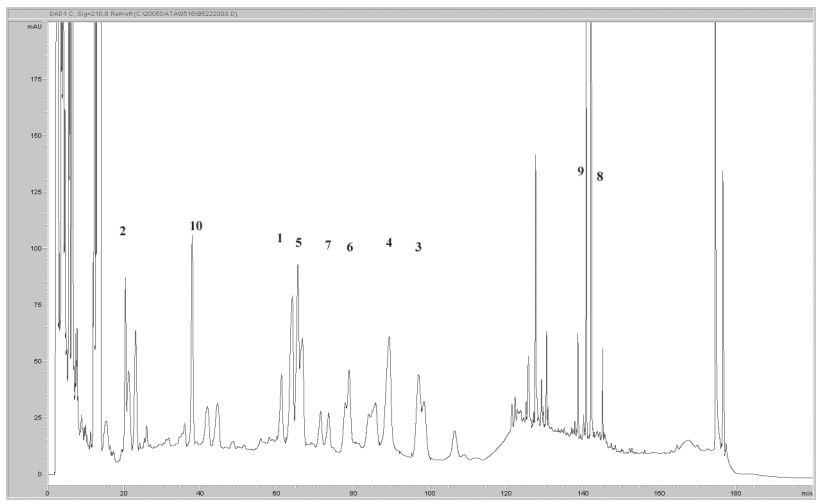

Fig. 3. HPLC chromatogram of the methanolic extract from cultured fruiting bodies of *Antrodia cinnamomea* (isolate TFRI B479). 1, Antcin C; 2, antcin K; 3, zhankuic acid A; 4, zhankuic acid B; 5, zhankuic acid C; 6, sulphurenic acid; 7, dehydrosulphurenic acid; 8, eburicoic acid; 9, dehydroeburicoic acid; 10, 4,7-dimethoxy-5-methyl-1,3-benzodioxiole.

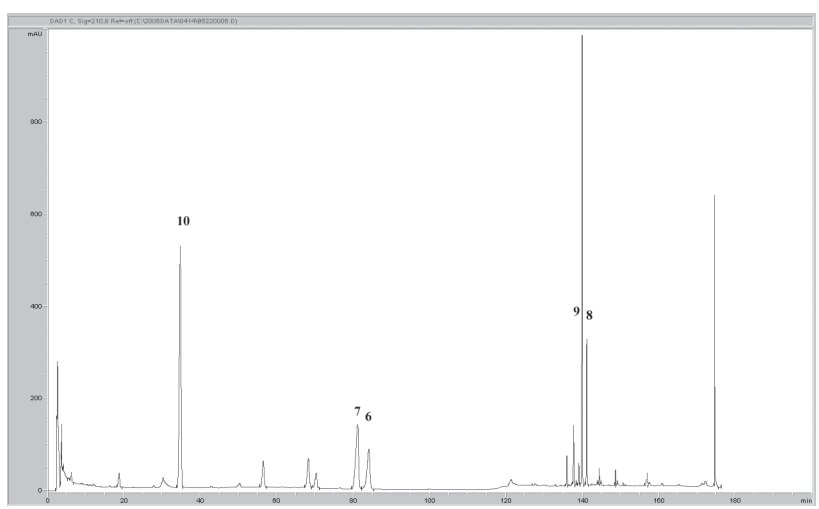

Fig. 4. HPLC chromatogram of the methanolic extract from natural mycelia of *Antrodia cinnamomea*. 6, Sulphurenic acid; 7, dehydrosulphurenic acid; 8, eburicoic acid; 9, dehydroeburicoic acid; 10, 4,7-dimethoxy-5-methyl-1,3-benzodioxole.

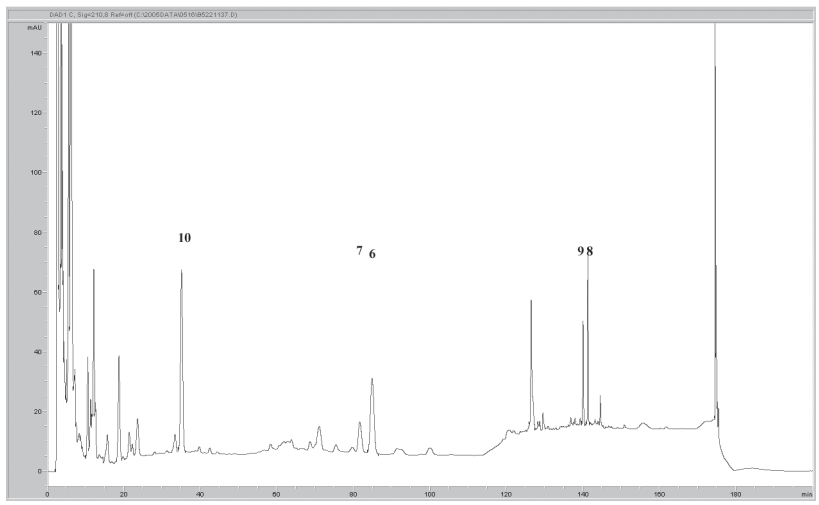

Fig. 5. HPLC chromatogram of the methanolic extract from agar-cultured mycelia of *Antrodia cinnamomea* (isolate TFRI B479). 6, Sulphurenic acid; 7, dehydrosulphurenic acid; 8, eburicoic acid; 9, dehydroeburicoic acid; 10, 4,7-dimethoxy-5-methyl-1,3-benzodioxole.

Table 1. Comparison of 10 chemical components between mycelia and basidiomes of *Antrodia cinnamomea*

Form of *Antrodia cinnamomea*	Chemical component[a)]									
	1	2	3	4	5	6	7	8	9	10
Basidiomes from nature										
Hsinchu County	+	+	+	+	+	+	+	+	+	+
Chiayi County	+	+	+	+	+	+	+	+	+	+
Kaohsiung County	+	+	+	+	+	+	+	+	+	+
Basidiomes from culture										
TFRI B479	+	+	+	+	+	+	+	+	+	+
TFRI B522	+	+	+	+	+	+	+	+	+	+
Mycelia from nature	-	-	-	-	-	+	+	+	+	+
Mycelia from culture										
Liquid culture TFRI B479	-	-	-	-	-	+	+	+	+	+
TFRI B86	-	-	-	-	-	+	+	+	+	+
Agar culture TFRI B479	-	-	-	-	-	+	+	+	+	+
TFRI B86	-	-	-	-	-	+	+	+	+	+

[a)] 1, antcin C; 2, antcin K; 3, zhankuic acid A; 4, zhankuic acid B; 5, zhankuic acid C; 6, sulphurenic acid; 7, dehydrosulphurenic acid; 8, eburicoic acid, 9, dehydroeburicoic acid; 10, 4,7-dimethoxy-5-methyl-1,3-benzodioxole.

were produced by basidiomes and mycelia of *A. cinnamomea* regardless of the living substrate. Because prices of basidiomatal products of *A. cinnamomea* are much higher than those of mycelia, it is said that some manufacturers have used mycelia to substitute for basidiomes. It is almost impossible to identify whether the commercial products of *A.*

cinnamomea are from mycelia or basidiomes when the products are a dry powder or capsulated/tableted. However, in this study, only the basidiomes of *A. cinnamomea* produced ergostane triterpenoids and not the mycelia, so these results can be applied to commercial quality checks of the fungal products.

In addition to *A. cinnamomea*, basidiomes of *A. salmonea*, a congeneric species of *A. cinnamomea*, also contained the 5 ergostane triterpenoids that have not been found in any other fungi (Chen et al. 1995, Shen et al. 2007). The monophenyl compound (4,7-dimethoxy-5-methyl-1,3-benzodioxole) was only found in *A. cinnamomea*, indicating that it could be as an indicator component of *A. cinnamomea*. However, the 4 lanostane triterpenoids were found in other fungi except for dehydrosulphurenic acid that was only found in *A. cinnamomea* and *A. salmomea* (Tai et al. 1993, Yoshikawa et al. 2000). But it is believed that dehydrosulphurenic acid should be present in other fungi because its main structure is the same as sulphurenic acid.

In this study, the production of the 10 detected components was not related to the wood of *C. kanehirai* because they were present in cultured mycelia and basidiomes grown on substrates that contained no wood of *C. kanehirai*. Five ergostanes (antcins C and K, and zhanknic acids A, B, and C) isolated from basidiomes exhibited anti-inflammatory activities in isolated peripheral human neutrophils (Shen et al. 2004b). The ergostane antcin C, and 3 lanostanes (sulphurenic acid, eburicoic acid, and dehydroeburicoic acid) isolated from basidiomes of *A. cinnamomea* contributed to the immunomodulatory activity (Shen et al. 2003). The ability of cultured basidiomes and mycelia without the wood of *C. kanehirai* to produce these bioactive compounds indicates that artificial cultures of *A. cinnamomea* possess some effective compounds.

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