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# *In vitro* induction of fruiting body in *Antrodia cinnamomea* – a medicinally important fungus

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**ABSTRACT.** The fruiting body of medicinal fungus, *Antrodia cinnamomea*, is a unique traditional medicine originally used by native Taiwanese. *Antrodia cinnamomea* specifically grows inside the rotten trunk of *Cinnamonum kanehirae*, an important native tree species in Taiwan. *In vitro* culture of *A. cinnamomea* on agar plates to induce fruiting body formation has been shown difficult since many of its physiological and developmental processes are unclear. Laboratory culture of *A. cinnamomea* on the *C. kanehirae* trunk showed fruiting body formation occurred on the peripheral and lower sides of trunk, indicating that orientation had played an important role. In addition, humidity and aeration also affected fruiting body formation. Physical wounding of red hyphae was found to induce fruiting body formation on agar plate. Methanol extracts of white, red hyphae, wildly grown and *in vitro* grown fruiting bodies analyzed by HPLC showed a distinct pattern between hyphae and fruiting bodies.

**Keywords:** Abiotic stress; *Antrodia cinnamomea*; HPLC; Secondary metabolite; Wound induction.

## INTRODUCTION

*Antrodia cinnamomea* is a medicinal fungus that grows naturally inside the *Cinnamonum kanehirae* trunk, a native tree species of Taiwan (Chang and Chou, 1995; 2004; Wu et al., 1997). The medicinal use of *A. cinnamomea* was first discovered by native Taiwanese, who used it as an antidote for alcohol intoxication. Recently, many studies indicate that its medicinal applications go far beyond the original usage. It has been reported that many chemical components of *A. cinnamomea* carry functional properties like anti-oxidant (Song and Yen, 2002; Hseu et al., 2002), anti-cancer (Chen and Yang, 1995), anti-virus (Lee et al., 2002), and antibiotic properties (Chen and Yang, 1995). Therefore, demand for *A. cinnamomea* has far exceeded the supply, and it is now considered among the most expensive herbal medicines on the market (more than 5 US dollars per gram of the fresh fungal fruiting body).

The high demand is causing a serious conservation issue since people aggressively harvest the wild *A. cinnamomea* fruiting body by cutting off the *C. kanehirae* trunk and endangering the tree species, which is unique to Taiwan. In an effort to resolve the conservation issue without sacrificing the medicinal benefits, scientists from academia and the pharmaceutical industry have been intensively working to develop *A. cinnamomea* products in the laboratory. One major approach of the laboratory study is to culture the fungus in hyphae forms and optimize the chemical composition, especially production of secondary metabolites, by culturing the hyphae (Song and Yen, 2002; 2003). Several studies have shown this approach might be feasible, and, therefore, many commercial products have been produced. However, no detailed clinical trial has yet been reported, and all commercial products are categorized as health food rather than medicine.

Another approach is to mimic the fungal growth conditions in the laboratory to culture the fungal fruiting body. This approach successfully grows the *A. cinnamomea* fruiting body on the *C. kanehirae* trunk but not on other plant species; hence, it does not resolve the conservation issue. The best solution would be to culture the fungus and grow the fruiting body on agar plates with commercially available nutrient media. No scientific report on this method had been published until very recently when Chang and Wang (2005) reported an *in vitro* fruiting body formation of an *A. cinnamomea* isolate on malt extract agar (MEA) and potato dextrose agar (PDA) media. Here, we present our efforts in another approach to induce the growth of *A. cinnamomea* fruiting body *in vitro* through a novel wounding procedure. The report should stimulate new thinking on the study of *A. cinnamomea* fruiting body formation under *in vitro* conditions.

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## MATERIALS AND METHODS

### Fungal strains and chemicals

The strain ACI3 of *A. cinnamomea* was isolated from a rotten *C. kanehirae* trunk containing *A. cinnamomea* fruiting body and identified as *A. cinnamomea* based on comparison of its morphological features with a standard *A. cinnamomea* strain purchased from the Food Industry Research and Development Institute (Catalog number BCRC35716), Hsinchu, Taiwan. In addition, a molecular comparison of strain ACI3 was performed to confirm the taxonomic identification as mentioned in the following section. All the chemicals used were of analytical grade or higher.

### Molecular identification of *A. cinnamomea* based on 18S rRNA DNA sequence

Genomic DNA of *A. cinnamomea* was purified based on the protocol of QIAamp DNA Mini Kit (Catalog number 51304, QIAGEN Inc., Valencia, CA, USA). Four universal PCR primers were synthesized for 18S rRNA DNA amplification based on the report of White et al. (1990). The primers were NS1 (GTA GTC ATA TGC TTG TCT C), NS3 (GCA AGT CTG GTG CCA GCA GCC), NS4 (CTT CCG TCA ATT CCT TTA AG), and NS8 (TCC GCA GGT TCA CCT ACG GA). The PCR thermo-cycling program was 98ºC for 2 min, followed by 35 cycles of 95ºC for 45 s, 52ºC for 45 s and 72ºC for 2 min, and final step as 72ºC for 10 min. The PCR products were analyzed on 1% TAE agarose gel and purified based on protocol of QIAGEN MiniElute Gel Extraction Kit (Catalog number 28606, QIAGEN Inc., Valencia, CA, USA). The purified DNA was subjected to DNA sequencing analysis. Pairwise alignment was performed based on the software tools of NCBI web site: http://www.ncbi.nlm.nih.gov.

### Growth of *A. cinnamomea*

Growth of *A. cinnamomea* was observed on a 20 ×20×40 cm *C. kanehirae* trunk inoculated with *A. cinnamomea* hyphae. The inoculated trunk was placed in a closed container at room temperature and in a dark environment and with water barely touching the trunk to maintain a high moisture environment. The MEA plates were prepared based on Blakeslee's composition (one liter medium containing 20 g malt extract, 20 g glucose, 1 g peptone and 20 g agar) and were used to culture and maintain the *A. cinnamomea* mycelia. The malt extract broth (MEB) was prepared as MEA without addition of agar for liquid suspension culture. The MEA plates or MEB suspension culture inoculated with *A. cinnamomea* mycelia were kept in an incubator at 28ºC. It took about one month for mycelia to fully cover a 10 cm (diameter) petri dish. These plates were subjected to further experimental treatments for induction of fruiting body. Taking a clue from *A. cinnamomea's* natural habitat, various concentrations of camphor oil produced by *C. kanehirae* were applied to the MEA plates to see whether it would influence the growth of *A. cinnamomea*. The experimental treatments were MEA medium alone as the control, MEA medium containing 1% Tween 20 (v/v) only, MEA medium containing 1% Tween 20 and 0.2% camphor oil, MEA medium containing 1% Tween 20 and 0.4% camphor oil, MEA medium containing 1% Tween 20 and 0.6% camphor oil, and MEA medium containing 1% Tween 20 and 0.8% camphor oil. The 1% Tween 20 was applied to help dissolve the camphor oil in the MEA medium.

### Abiotic factors affecting growth phase of *A. cinnamomea*

***Acetic acid.*** A hole was made in the MEA plate, and different amounts of acetic acid were applied through the hole. After the acetic acid had fully diffused into the agar plates, they were kept upside down on a fully moistened filter paper to have a highly moist environment. The growth of *A. cinnamomea* was observed and compared with the same cultural plates to which no acetic acid was added.

***Air exchange.*** The covers of MEA plates containing three-week-old *A. cinnamomea* hyphae were removed, and the plates were kept face up in an incubator at 28ºC. The continuous growth result of this treatment was compared with the same age hyphae remaining in the sealed MEA plates.

***Wound treatment.*** The MEA plate with fully grown *A. cinnamomea* hyphae was rubbed with cotton swabs to expose the skeletal hyphae. The plates rubbed with cotton swabs were then placed upside down on a fully moist filter paper to maintain a highly moist environment.

### HPLC profile analysis of *A. cinnamomea* methanol extracts

*Antrodia cinnamomea* hyphae and fruiting bodies were placed in an oven at 60ºC for 24 h to measure their dry weight. The samples were then subjected to 100% methanol extraction (Wu and Chiang, 1995; Cherng et al., 1996). The crude methanol extracts were filtered using a 2.2 μm syringe filter and then subjected to HPLC analysis. A 250 × 4.6 mm HyPURITY C18 HPLC column (ThermoHypersil-Keystone, Bellefonte, PA, USA) with a Hitachi L-7100 HPLC pump and 7240 UV detection system (San Jose, CA, USA) were used for the analysis of methanol extracts of *A. cinnamomea* under 254 nm UV detection for secondary metabolite profiles. The mobile phase program of HPLC was 30-100% acetonitrile from 0 - 20 min, 100% acetonitrile for next 20 min, then, linear replacement of acetonitrile with 100% methanol for the next 10 min, and finally 100% methanol for next 10 min.

## RESULTS

### Growth pattern of *A. cinnamomea* on *C. kanehirae*

Identification of strain ACI3 based on 18S rRNA gene sequencing analysis showed 98% homology when

compared with the sequences of *A. cinnamomea* strain BCRC 35398 (1015/1027 with NS1/NS4 as the PCR primers, and 822/832 with NS3/NS8 as the PCR primers), indicating that the strain ACI3 was *A. cinnamomea*. This conclusion was further confirmed by morphological observation of its fruiting body, including the resupinate growth over the *C. kanehirae* trunk, bright orange color, strong bitter taste, and porous surface (Chang and Chou, 1995). It took two months after inoculation of *A. cinnamomea* on *C. kanehirae* to form a fruiting body. The fruiting bodies grown on the peripheral and bottom sides of trunk showed a healthy orange to brown porous surface (Figure 1A, B). However, the fruiting body structure degraded and became white when the fruiting body's face was turned upward (Figure 1C).

## Camphor effects on *A. cinnamomea* growth

To see whether *A. cinnamomea* growth is affected under camphor oil environment, we performed a series of experiments to see the effects of camphor oil on *A. cinnamomea* growth. As shown in Figure 2, the *A. cinnamomea* could tolerate camphor oil concentration up to 400 µl per 100 ml (shown as 0.4% in Figure 2) MEA medium without any noticeable decrease in growth. It was observed that 200 µl of camphor oil in 100 ml (shown as 0.2% in Figure 2) MEA medium increased the growth by 20%. This result indicates that *A. cinnamomea* grows better under a *C. kanehirae* environment.

## Physical induction of *A. cinnamomea* fruiting body

Acetic acid was tested since the natural growth environment of *A. cinnamomea* is acidic. Application of a few drops of acetic acid to MEA plates caused fungal hyphae to stop extension and withdraw from the spots of acetic acid. In addition, the red color of the hyphae

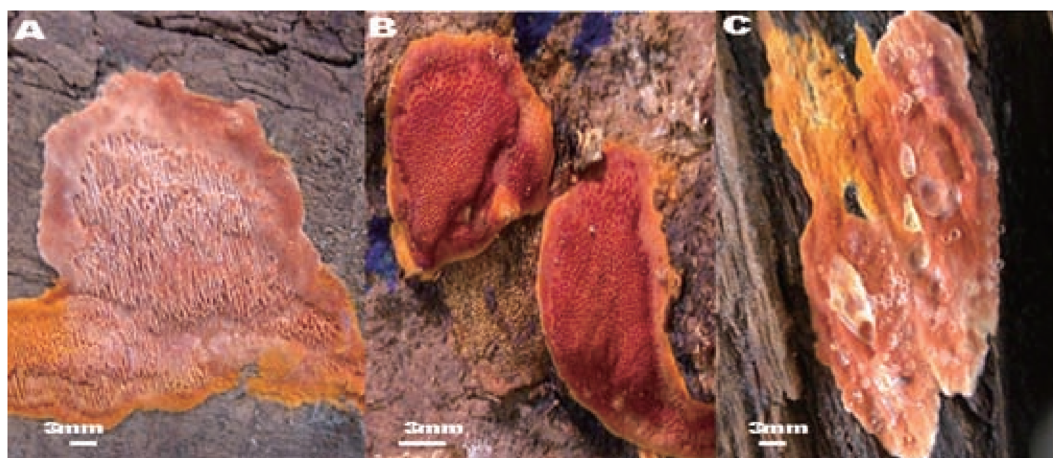


**Figure 1.** Fruiting body of *A. cinnamomea* on the *C. kanehirae* trunk. (A) The fruiting body growing on the peripheral side of trunk; (B) The fruiting body growing on the lower side of trunk; (C) The fruiting body growing on the higher side of trunk.

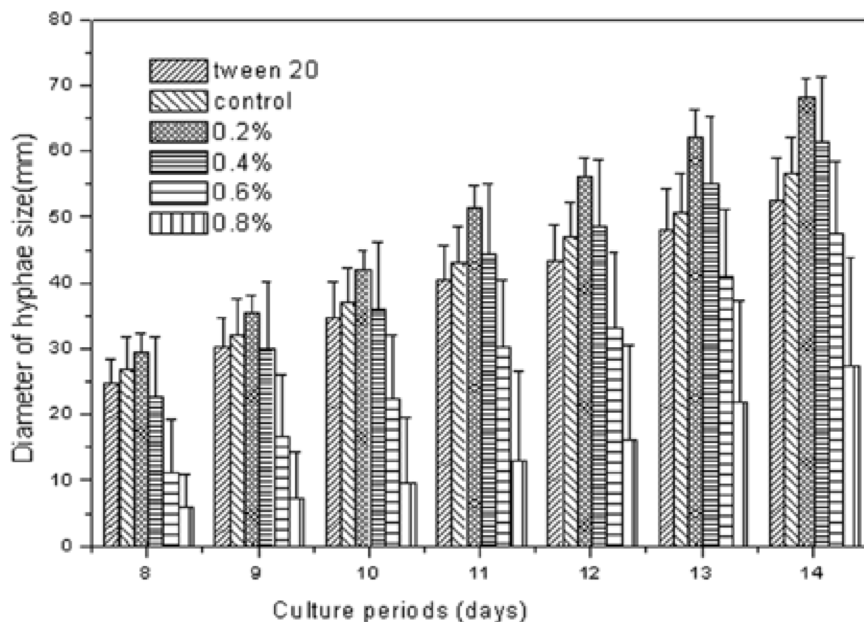


**Figure 2.** Growth of *A. cinnamomea* hyphae in MEA plates with various concentrations of camphor oil of *C. kanehirae*; "control" as MEA medium alone, "tween 20" as MEA medium containing 1% Tween 20 (v/v) only, "0.2%" as MEA medium containing 1% Tween 20 and 0.2% camphor oil, "0.4%" as MEA medium containing 1% Tween 20 and 0.4% camphor oil, "0.6%" as MEA medium containing 1% Tween 20 and 0.6% camphor oil, and "0.8%" as MEA medium containing 1% Tween 20 and 0.8% camphor oil. The 1% Tween 20 is applied to help dissolving camphor oil in MEA medium. Data are the average ± SD of five experiments.

turned pale and even white. Thus, it appears that acetic acid regulated the growth rate of hyphae and changed the growth phase.

In the air exchange experiment we found, instead of paling, an increase in the red color hyphae in well aerated petri dishes compared to the sealed petri dishes. This indicates that aeration may be an important factor in the growth of hyphae, especially in change white hyphae into red hyphae.

Wounding treatment with cotton swabs on MEA plates with fully grown hyphae was found to induce fruiting bodies in agar plates (Figure 3). Fruiting bodies from MEA plates subjected to HPLC analysis showed patterns identical to fruiting bodies obtained from *C. kanehirae* (Figure 4).

### HPLC analysis of *A. cinnamomea* methanol extracts

Figure 4 shows the HPLC profiles of methanol extracts from different growth stages of *A. cinnamomea*. In general, hyphal and fruiting body tissues were significantly different. Hyphal tissues contained a higher proportion of polar compounds than fruiting body tissues, which contained more non-polar compounds. In addition, the high degree of similarity between the fruiting bodies grown from *C. kanehirae* trunks and those from MEA plates with wounding treatment indicating that fruiting body production by wounding treatment can potentially replace wildly grown fruiting bodies for medicinal purposes.

## DISCUSSION

Medicinally important fungi play an important role in traditional Chinese medical practice. *Antrodia cinnamomea* has been proved effective in several reports at treating liver diseases and tumors (Chen and Yang, 1995; Hseu et al., 2002; Song and Yen, 2002). Further characterization of this fungal species may therefore yield medicinal benefits. However, its slow growth rate and exclusive host requirement have made its large-scale production for medicinal purposes difficult. This report presents a successful effort to induce *A. cinnamomea* fruiting bodies from a non-*C. kanehirae* environment.

Our observations of *A. cinnamomea* growth patterns on the *C. kanehirae* trunk agree with the report of Bulter and Wood (1988) on the fruiting body formation of *Polyporaceae* species, which indicated that the growth orientation affects fruiting body formation and may be related to its sexual reproduction processes. Further study of this aspect may help us understand the mechanism of *A. cinnamomea* fruiting body formation.

*Cinnamonum kanehirae* is a unique host to *A. cinnamomea* in the natural environment. One possible reason is that the camphor oil produced by *C. kanehirae* deters possible competitors of *A. cinnamomea*. Our observation showed that camphor oil indeed accelerated the growth of *A. cinnamomea* indicating a positive influence of the oil on its growth.

Generally, fungal fruiting body formation is considered part of the aging process, which can be caused by nutrient exhaustion, mechanical injury, chemical stimulation, or other environmental changes (Thomas and Stanley, 1968; Sawao et al., 1984; Hideo et al., 1985; Yoshiiyuki et al.,

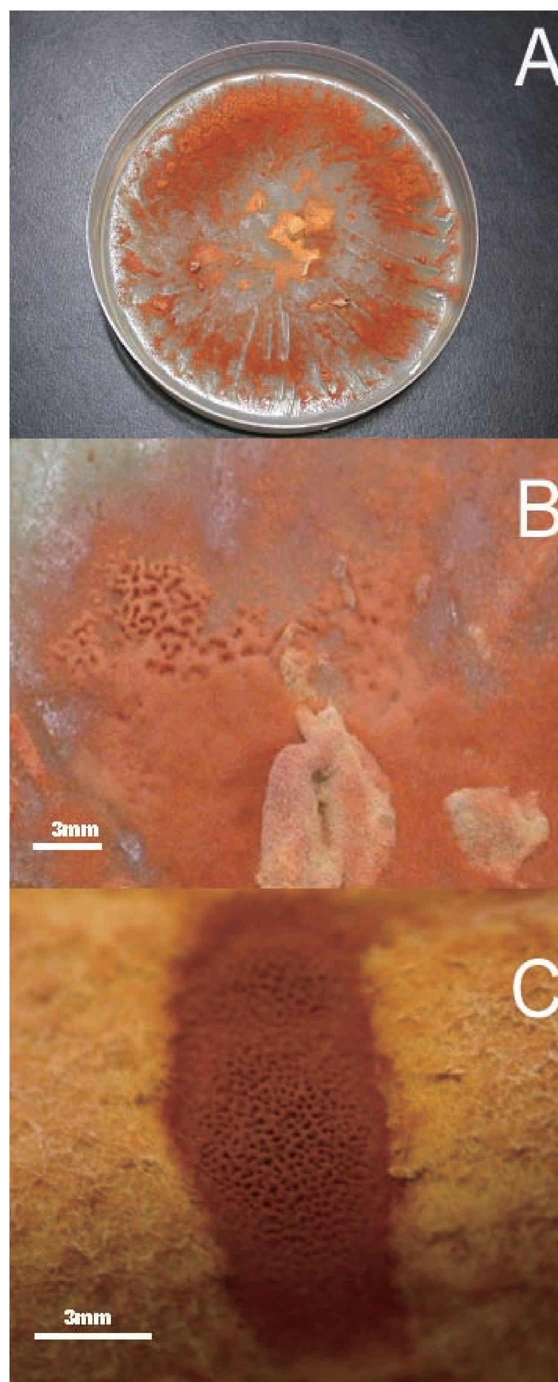


**Figure 3.** In vitro fruiting body formation in *A. cinnamomea* in MEA plates after cotton swab rubbing. (A)-(C) indicate different magnification images. The porous structure of fruiting body was clearly visible on surface of the fruiting bodies.

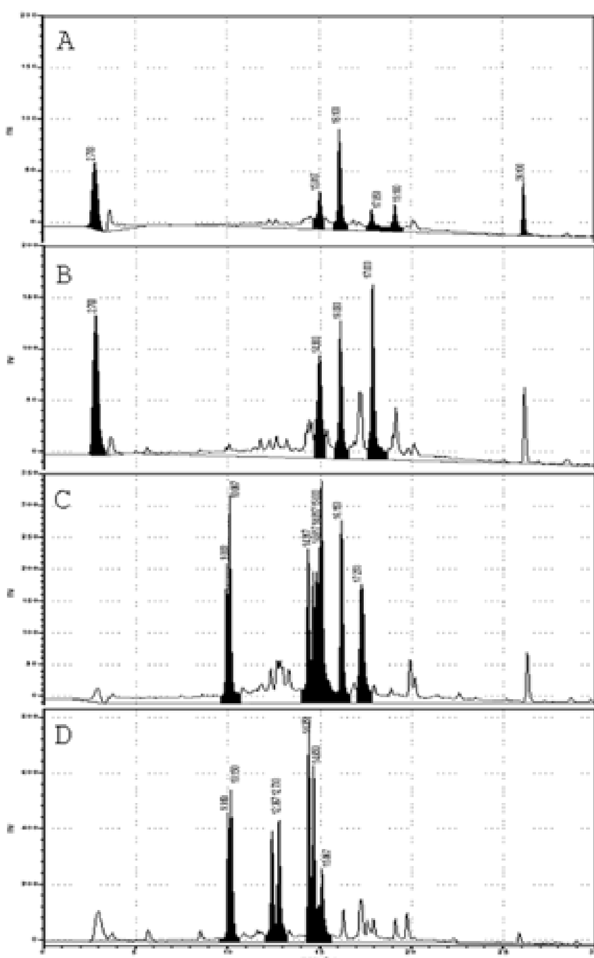


**Figure 4.** HPLC chromatograms of methanol extracts from *A. cinnamomea* white mycelia (A), red mycelia (B), fruiting body grown on *C. kanehirae* (C), and fruiting body grown *in vitro* in MEA plates with cotton swab rubbing treatment (D). Several key peaks have been shaded to enhance visual comparison.

1998). For commercial mushroom production, factors like low temperature, increased aeration, increased humidity, suitable light radiation, and wounding treatments are commonly used. We adapted a similar treatment for *A. cinnamomea* and found three factors, i.e. addition of acetic acid, air exchange, and wound treatments, had unique effects on growth. The wounding treatment was found especially important for the formation of fruiting bodies. This report opens further avenues for induction of *A. cinnamomea* fruiting body *in vitro* and a case for study of this phase change. The MEA plates with *A. cinnamomea* hyphae before wounding treatment were one-month-old. It is possible that nutrient levels were depleted in the MEA plates, and this triggered phase change.

The methanol extraction of *A. cinnamomea* has been used to study its secondary metabolite composition (Wu and Chiang, 1995; Cherng et al., 1996). Since the composition of secondary metabolites is an important factor to influence the medicinal activities of herbal medicines, we were interested to learn whether secondary product profiles at different stages of *A. cinnamomea* growth showed any changes. We found that hyphal tissues contain a higher proportion of polar compounds than the fruiting body tissues, which contained more non-polar compounds. This is important information for the pharmaceutical companies interested in the medicinal application of *A. cinnamomea*. The present study may also aid the conservation of *C. kanehirae* since cutting off the tree to collect *A. cinnamomea* fruiting bodies will no longer be necessary.

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# 人工環境誘導藥用真菌牛樟芝之子實體

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藥用真菌牛樟芝的子實體為起始於台灣原住民的傳統草藥，但只寄生於台灣國寶級樹種牛樟的中空腐朽的樹幹內，由於牛樟芝的生理與發生過程尚不清楚，因此希望大量地以人工環境栽培牛樟芝子實體仍舊非常困難。我們觀察牛樟芝在牛樟樹幹上的生長情形發現牛樟芝子實體只能發生於樹幹四周及向下的一面，向上的一面則無法長出牛樟芝的子實體，因此牛樟芝在樹幹上的生長方位對子實體的形成非常重要。另外，牛樟芝子實體的形成也受到溼度與空氣品質的影響。我們也發現物理性的傷害可誘導洋菜膠培養基上的牛樟芝菌絲產生子實體，此子實體的甲醇萃取物經 HPLC 圖譜分析證明，非常類似野生的牛樟芝子實體 HPLC 圖譜，且不同於菌絲體的 HPLC 圖譜。

**關鍵詞：**非生物性逆境；牛樟芝；高效能液相層析法；二次代謝物；創傷誘導。