

Effect of Chinese Herb Extract on the Formation of Triterpenoids in Shake-Flask Cultures of *Antrodia cinnamomea*

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Abstract

Antrodia cinnamomea is a well-known medicinal mushroom producing potent bioactive triterpenoids; it only grows in Taiwan. Its anti-tumor activity remains the focus of current research on triterpenoids. However, a main drawback of producing mycelia by submerged cultures is the rather low levels of triterpenoid produced. In this research, different kinds of Chinese herb water extracts were added into the media to study their effects on the formation of triterpenoids of *A. cinnamomea* in the thin stillage submerged cultures. Astragalus water extract was the most effective for enhancing triterpenoids production. With an addition of Astragalus water extract of 2% (v/v), the content and concentration of triterpenoids were 10.36 mg/g DW and 85.28 mg/L, respectively, which were 3-fold and 5-fold higher than the control, respectively, on the 21th day. The results reveal that although the addition of Chinese herb water extract lengthens the exponential phase and reduces the specific growth rate, the production rate of biomass, intracellular polysaccharide (IPS) and triterpenoids were still significantly enhanced. Moreover, this study also demonstrates the feasibility of reusing thin stillage for the mycelia culture of *A. cinnamomea*.

Keywords: *Antrodia cinnamomea*; Triterpenoids; Chinese herb; Thin stillage; Submerged cultures

Introduction

Antrodia cinnamomea is a parasitic fungus found exclusively on the inner wall of the endemic species *Cinnamomum kanehirai* Hay. It is commonly used as a traditional Chinese remedy for alcohol and drug intoxication, hypertension, abdominal pain and cancer [1,2] and recently becomes the most expensive medicinal mushroom in Taiwan. Many researchers have revealed that *A. cinnamomea* possesses antioxidant, antitumor and immunomodulating activities. Various types of bioactive compounds from the fruiting bodies of *A. cinnamomea* have been identified, including triterpenoids, polysaccharide, steroids and sesquiterpene lactone [1,3-5]. Apart from polysaccharide, triterpenoids were well recognized as potential anticancer agents due to their activity against growing tumors in the fruiting bodies of *A. cinnamomea* [1,3,5-8]. Cultivated mycelium has been reported to exhibit similar anticancer effects with wild fruiting bodies [9-13]. Owing to exhibiting such physiological functions, the determination of cultivation strategies by controlling culture conditions or modifying media compositions deserves detailed study in order to improve the production of triterpenoid.

Since natural *A. cinnamomea* in the wild grows very slowly and takes many years to form fruiting bodies, submerged cultivation has developed as an alternative to commercial practice at the present time. The use of submerged cultures provides some potential advantages, including higher mycelia production rate in a more-compact space over a shorter time with a lower chance of contamination; it is the preferred route for the production of some valuable metabolites. In order to enhance production efficiency, the modification of media compositions or the control of environmental conditions is vital. The research of Chang and Wang demonstrated that the addition of bark extract or wood chips of *Cinnamomum kanehirai* Hay was beneficial to the growth of *A. cinnamomea* in mycelia cultures [14-17]. However, this idea would never be practical due to the slow growth of *Cinnamomum*

kanehirai Hay. Therefore, finding a substitute for wood chips deserves further study.

Our previous work demonstrated that citrus peel extract additive can effectively enhance the production of biomass and bioactive metabolites in the submerged cultures or solid-state fermentation of *A. cinnamomea* [18-20]. Dry citrus peel has been used as a kind of Chinese herbal medicine. Most Chinese herbs that have a special smell are the most similar to bark extract or the wood chips of *C. kanehirai* Hay. Chinese herbs have been used as food and for medicinal purposes for centuries, in which contain many compounds that may be relevant to the medicine's putative activity. Research interest has focused on various herbs that possess antitumor, hypolipidemic or immune-stimulating properties that may be useful adjuncts in helping to reduce the risk of cancer and cardiovascular disease. Traditionally, Chinese herbs have a history of safe and effective treatment of many diseases [21-23]. Among many sources, Chinese herbs are obviously the safest and healthiest source.

Rice spirit is a distilled alcoholic beverage in Taiwan, and has been mainly used for Chinese cooking. During its manufacture process, large volumes of wastewater were produced and known as thin stillage, which is rich in carbon sources and organic acids. Owing to the characteristics of high organic content and low pH, thin stillage is difficult to treat. Therefore, developing an effective process for reutilization of the distillery wastewater is highly desired [24-28]. In the study, thin stillage

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was reused for *A. cinnamomea* mycelial culture to investigate the influences of adding Chinese herb water extract on the mycelia growth and bioactive component production.

Materials and Methods

Organism and inoculum

The organism used in this study was *Antrodia cinnamomea* CCRC35396, which was obtained from the Bioresources Collection and Research Center (BCRC) (Hsinchu, Taiwan). The strain was maintained on potato dextrose agar (PDA) slants, which were incubated at 25°C for 7 days. The organism was then stored at 4°C and usually transferred to a fresh agar plate every two months.

Inoculum preparation

The liquid medium for seed culture was made up of the following components (w/v): glucose 2.0%, malt extract 2.0% and peptone 0.1%. After sterilization at 121°C for 20 min., *A. cinnamomea* mycelia were transferred to the liquid medium by punching out 0.7 cm diameter agar discs from mycelia culture grown on PDA plates. Four discs were used to inoculate 100 mL liquid media in a 250 mL shake flask and the seed culture was then carried out at 25°C, 100 rpm for 7 days.

Shake flask cultures

The shake flask cultures were carried out in 250 mL Erlenmeyer flasks containing 100 mL of basal medium inoculated with 10% (v/v) of the seed culture. The thin stillage was provided by Taichung Distillery (Taiwin Tobacco and Liquor Corporation) and prepared by filtration with filter pore size of 6 μm, which was then used directly as the basal medium in a flask culture. Apart from the basal medium, various kinds or concentrations of Chinese herb water extracts were added into the medium to study their influence on the production of bioactive metabolites. After sterilization at 121°C for 20 min., the culture was incubated at 25°C, 100 rpm for 35 days, and samples were collected at various intervals for analyzing biomass concentration, intracellular polysaccharides (IPS) concentration and the contents of total polyphenol and triterpenoids. The data shown are the means of triplicate determinations.

Preparation of Chinese herb water extracts

Five kinds of Chinese herbs (Star anise, Fagara, Astragalus, Turmeric and Cinnamon) were purchased from a local Chinese herbal medicine shop; 50 g of Chinese herb were mixed with one liter of water, and the mixture was then boiled for 3 h. After filtration with 100 mesh sieves, the extract was concentrated to 10-fold by using a vacuum evaporator and then stored at 4°C.

Determination of Intracellular Polysaccharides (IPS)

Intracellular polysaccharides were extracted from dried mycelia (100 mg) by suspending the mycelia in 10 mL of distilled water and autoclaving at 15 psi, 121°C for 20 min [27]. Samples from the shake flasks were centrifuged at 8000 rpm for 10 min, and the supernatant obtained was then mixed with four volumes of 95% (v/v) ethanol, stirred vigorously and left overnight at 4°C. The precipitated polysaccharide was collected by centrifugation at 8000 rpm for 10 min and then lyophilized to remove residual ethanol. Total polysaccharide in the culture medium was determined by phenol-sulfuric acid assay according to Dubois et al. [29].

Measurements of crude triterpenoid and total polyphenol

The dried mycelia (100 mg) were extracted by 50% (v/v) ethanol (3 mL) for one week (twice) for the determination of crude triterpenoid. The crude triterpenoid content was analyzed by the method described in the paper of Tsujikura et al. [30]. For the assay of total polyphenol, the dried mycelia (100 mg) were extracted by methanol (20 mL) for 12 h at 50°C. The total polyphenol content was analyzed according to the method of Singleton and Rossi [31] with a slight modification. The detailed analysis procedures were the same as those described in our previous papers [18-20].

HPLC system for the analysis of triterpenoid

The dried mycelia (100 mg) were extracted by 50% (v/v) ethanol (3 mL) for 12 h, and the extraction was assisted by successive sonication using Delta D200H Sonication Cleaner for the HPLC analysis of triterpenoid. The pretreatment procedures were the same as those described in our previous papers [19,20].

According to the paper by Chang et al. in 2011 [32], the method for the analysis of triterpenoid was modified and HPLC analysis was performed on an Agilent 1100 series with UV detection. The detection wavelength was set to 254 nm. Separation was obtained with a reversed-phase column (Cosmosil 5C18- 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% H₃PO₄ in H₂O) and B (acetonitrile). The column was eluted according to the following profile: 0~65 min, 30~47% B, 65~110 min, 47~47% B, 110~140 min, 47~100% B, 140~160 min, 100~100% B, 160~165 min, 100~30% B, 165~180 min, 30~30% B. The column temperature was set to 30°C. The injection volume was 20 μL.

Statistical analysis

Variations between experiments were displayed from standard deviations, and the statistical significance of changes in the physiological parameters was also calculated using the Student's t-test. In all the graphs, the symbol * indicates a significant difference to the untreated groups, p<0.05; ** indicates a significant difference to the untreated groups, p<0.01. All data were the means of three measurements.

Results and Discussion

Batch flask culture

The kinetics of mycelia growth and metabolite accumulation by *A. cinnamomea* in shake flask cultures is displayed in Figure 1. The control

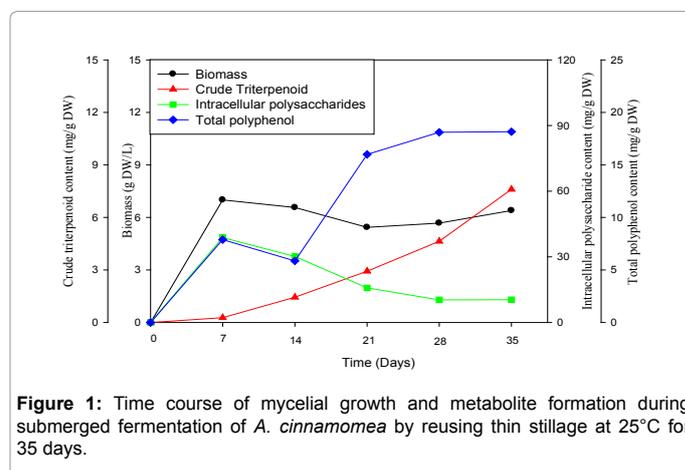


Figure 1: Time course of mycelial growth and metabolite formation during submerged fermentation of *A. cinnamomea* by reusing thin stillage at 25°C for 35 days.

culture was carried out in a 250 ml flask with 100 mL of basal medium (thin stillage) at initial pH 3.5, 25°C and 100 rpm for 35 days. The data indicate that the biomass and intracellular polysaccharide concentrations increased rapidly within the first 7 days, during which time the cells were in the exponential growth phase. According to the results shown in Figure 1, intracellular polysaccharide appears to be a growth-associated product. The mycelia biomass and IPS concentrations achieved the highest levels on day 7, and the corresponding maximum values were 7.00 g DW/L and 38.80 mg/g DW, respectively. On the contrary, crude triterpenoid and total polyphenol did not formed proportionally to the mycelia growth and displayed a pattern of mixed-growth-associated products. Their concentrations rose to the highest values of 7.60 mg/g DW and 18.16 mg/g DW, respectively, on day 35.

Effect of addition of Astragalus water extract

The time profiles of concentrations of biomass and metabolite produced in the culture with the addition of Astragalus water extract of 2% (v/v) is displayed in Figure 2. Compared with the control demonstrated in Figure 1, it is interesting to note that there were great differences regarding the tendency of cell growth and bioactive metabolites formation during days 7 and 21. The mycelia even continued to grow until day 14, when the highest concentration of 8.80 g DW/L was obtained. The intracellular polysaccharide concentration rose to 71.16 mg/g DW on day 7 with a two-fold increase. Moreover, the amount of crude triterpenoid achieved 10.36 mg/g DW on day 21, with a more than three-fold increase (control on day 21). In contrast, the amount of total polyphenol reached 15.31 mg/g DW on day 21, which showed only a slight decrease. The data demonstrate that the addition of Astragalus water extract had a great influence on cell physiology, and could effectively enhance the formation of secondary metabolites.

Effect of various kinds of Chinese herb water extracts

In addition to Astragalus, the influences of other Chinese herb water extracts, including Star anise, Fagara, Turmeric and Cinnamon, were also studied for comparison. Figure 3 indicates the influence of different kinds of Chinese herb water extracts on mycelial growth, intracellular polysaccharide and total polyphenol production. All additives caused an extension of the exponential phase and seemed to be benefit the mycelial growth, but Turmeric and Cinnamon water extracts were found to exhibit no effect on the production of polysaccharide. In contrast, all the additives seemed to slightly decrease the content of total polyphenol. Among the five Chinese herb water extracts tested, Star anise and Astragalus water extracts demonstrated to be the most valid in improving the production of bioactive metabolite. The content of intracellular polysaccharide content reached 73.32 mg /g DW (Star

anise) and 71.16 mg /g DW (Astragalus), representing 89% and 84% increase, respectively, compared with the control group.

Based on the data obtained on day 21, Figure 4 indicates the influence of various kinds of Chinese herb water extracts on the content and production of crude triterpenoid. With the exception of Fagara, the addition of Chinese herb water extracts appeared beneficial in regard to the crude triterpenoid content in the mycelium. Among the five Chinese herb water extracts tested, Astragalus water extracts was the most effective in enhancing the production of crude triterpenoid. Crude triterpenoid content reached 10.36 mg /g DW, representing nearly a 3.5-fold increase compared with the control group. Moreover, the production of crude triterpenoid rose from 16.00 mg /L in the control to 85.28 mg /L, with a more than five-fold increase. The test demonstrated that Chinese herb water extracts tended to prolong the exponential phase and also accelerated the formation of crude triterpenoid in mycelium on day 21.

Effect of concentrations of Astragalus water extracts

Different ratios of Astragalus water extract, ranging from 0.5 to 4% (v/v), were added into the media to study the effect of concentration, and also to determine an appropriate amount. In accordance with the results displayed in Figure 5A, the mycelia can grow with various amounts of Astragalus water extract, and total polyphenol content still reached a similar level to that of the control. Figure 5B exhibits that total polyphenol production reached the highest concentration of 125.87 mg/L at an addition level of 2% (v/v) on day 21, representing a 46% increase as compared with the control group.

The effect of the concentration on the formation of crude triterpenoid is presented in Figure 6. A 2% (v/v) addition of Astragalus water extract demonstrated to be the most effective concentration; it could achieve the highest crude triterpenoid content of 10.36 mg/g DW and maximum crude triterpenoid production of 85.28 mg/L on day 21.

Astragalus membranaceus are among the most popular health-promoting herbs in China; their use dates back more than 2000 years. *Astragalus* species are the richest source of cycloartanes; the unique triterpenoids have a characteristic 9,19-cyclopropane ring, and cycloastragenol, a key intermediate in the biosynthesis of different phyosterols [33-35]. In Kuban's study, a rearrangement to provides a novel triterpene framework via a ring cleavage (cycloastragenol) followed by a methyl migration, demonstrating once more the potential of the microbial systems and *Cunninghamella blakesleeana* for the transformation of bioactive molecules [36]. Cycloastragenol was subjected to microbial transformation studies using *C. blakesleeana* and *Glomerella fusarioides* fungi, and *Nocardia* sp., *Mycobacterium* sp. 3683 and *Mycobacterium* sp. bacteria. The two fungi mainly provided hydroxylated metabolites together with products formed by dehydrogenation, cyclization and oxidation of Baeyer-Villiger, leading to a ring cracking [35,37]. It is an important reason why the addition of Astragalus water extract achieved the highest crude triterpenoid content.

Comparison of fermentation kinetic parameters

Tables 1 and 2 indicate fermentation kinetics parameters of all the shake flask cultures for comparison. Chinese herb water extracts added into the media obviously caused a great influence on mycelia growth and metabolite formation. Concerning the crude triterpenoid production rate, as can be seen in Table 1, Fagara was the only Chinese herb that had a negative effect. In contrast, Astragalus water extract (2% (v/v)) can effectively enhance the production rate of crude triterpenoid from 1.35

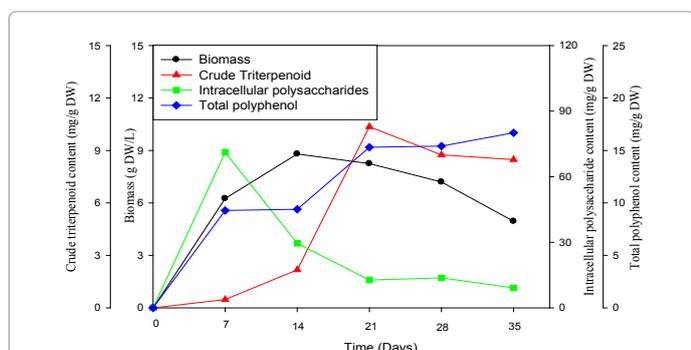


Figure 2: Time course of mycelial growth and metabolite formation of *A. cinnamomea* in a thin stillage submerged culture with the addition of Astragalus water extract (2% (v/v)) at 25°C for 35 days.

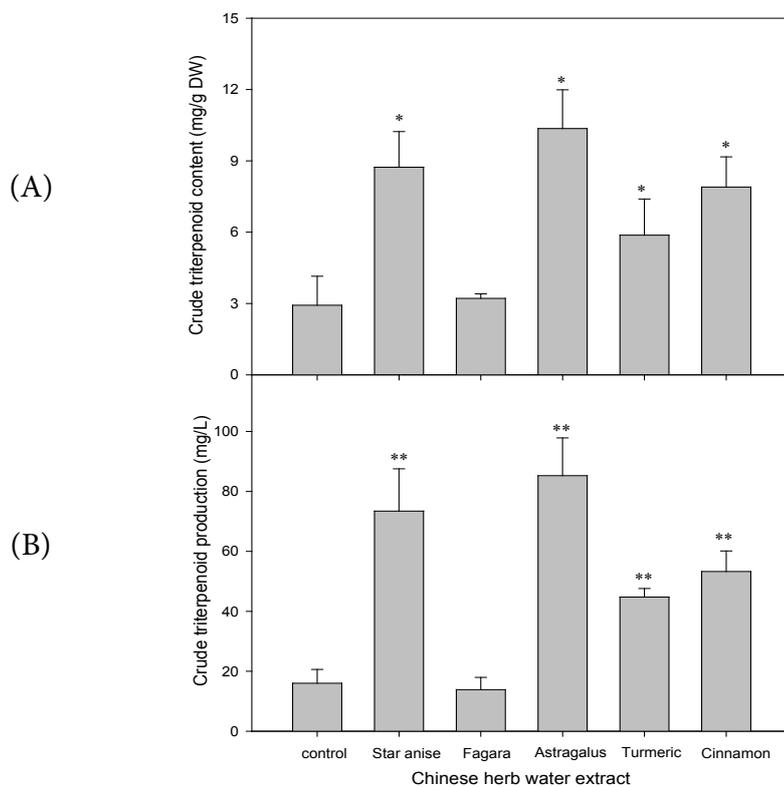


Figure 3: Effect of different kinds of 2% (v/v) Chinese herb water extract on the biomass for 14 days (A), Intracellular polysaccharides for 7 days (B) and total polyphenol for 21 days (C) by reusing thin stillage at 25°C.

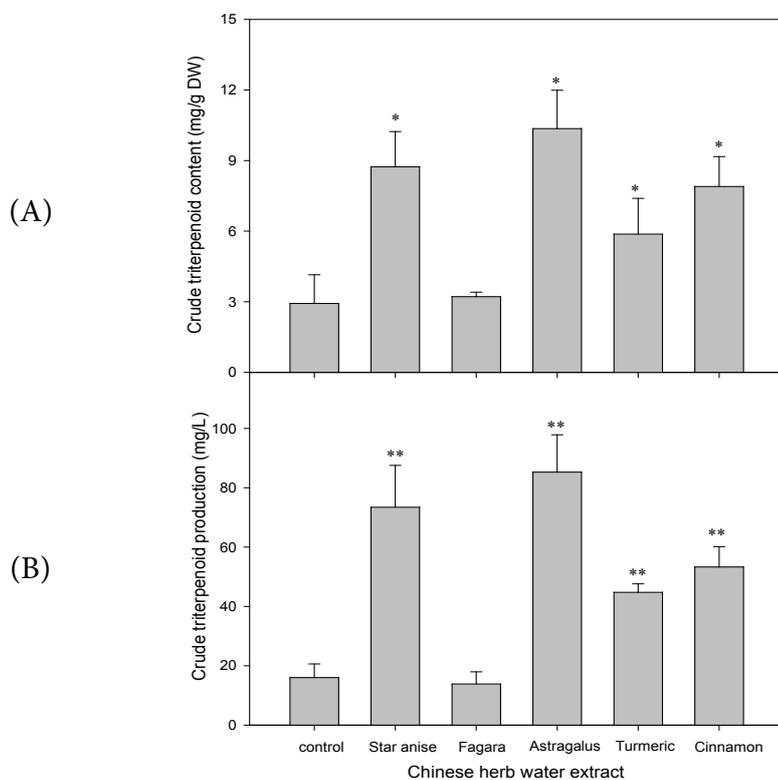


Figure 4: Effect of different kinds of 2% (v/v) Chinese herb water extract on the content (A) and production (B) of triterpenoid by reusing thin stillage at 25°C for 21 days.

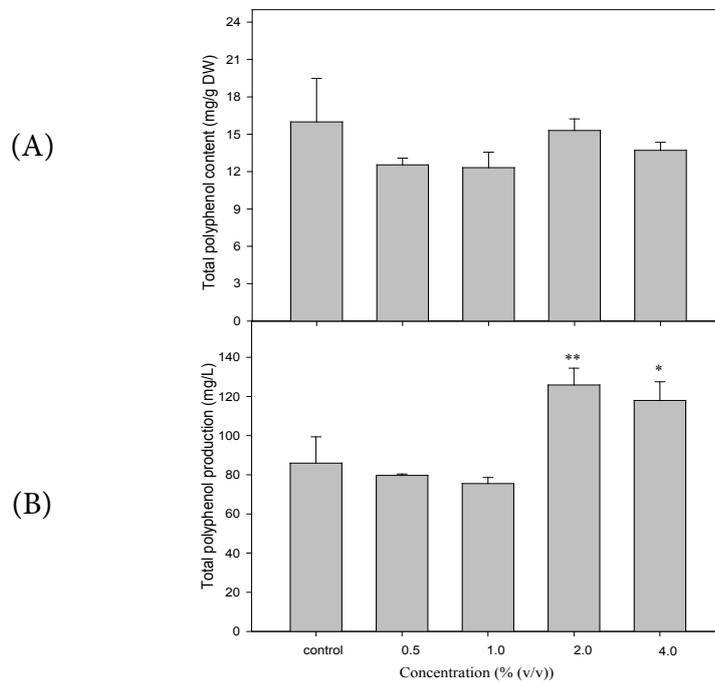


Figure 5: Effect of different concentrations of Astragalus water extract on the content (A) and production (B) of total polyphenol by reusing thin stillage at 25°C for 21 days.

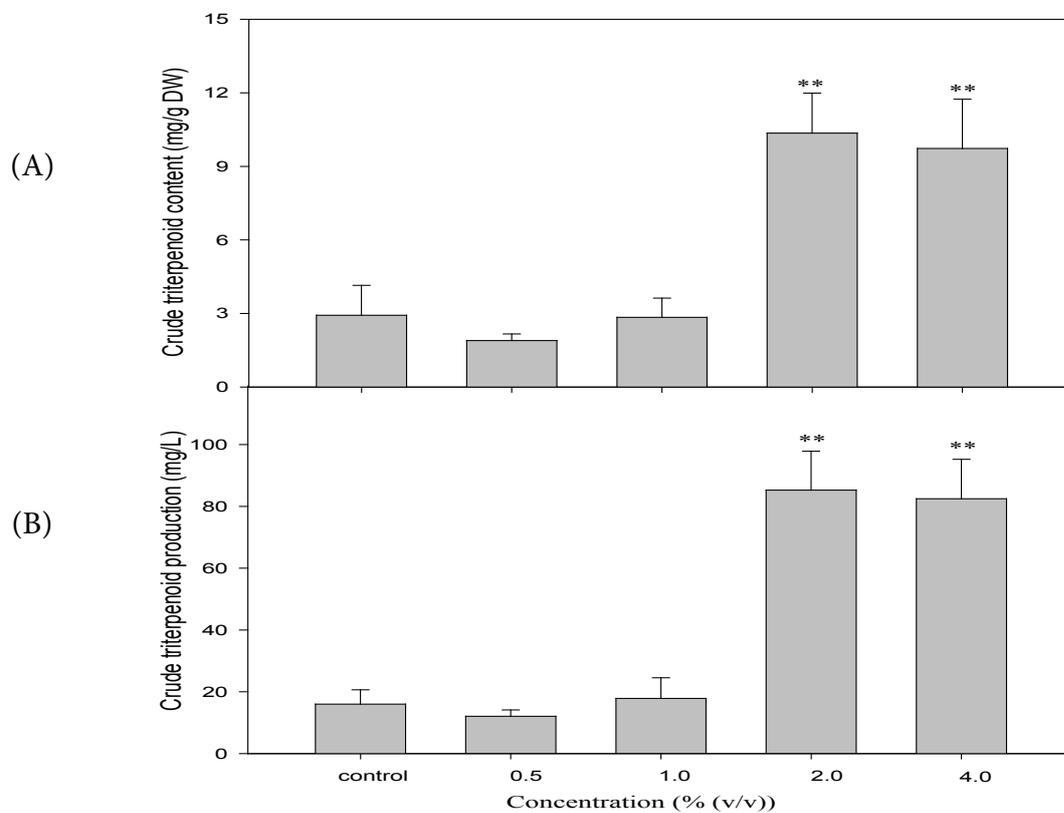


Figure 6: Effect of different concentrations of Astragalus water extract on the content (A) and production (B) of triterpenoid by reusing thin stillage at 25°C for 21 days.

Chinese herb kind					Total polyphenol				Intracellular polysaccharides			
	P1 _{max} (mg/L)	Q _{P1} (mg/L day)	Y _{P1/s} (mg/g)	t (day)	P2 _{max} (mg/L)	Q _{P21} (mg/L day)	Y _{P2/s} (mg/g)	t (day)	P3 _{max} (mg/L)	Q _{P3} (mg/L day)	Y _{P3/s} (mg/g)	t (day)
Control	47.25	1.35	--	35	115.6	3.21	--	35	271.6	38.80	--	7
Star anise	73.43	3.50	7.34	21	125.9	4.50	12.59	28	487.6	69.66	48.76	7
Fagara	13.87	0.66	1.39	21	95.2	2.72	9.52	35	342.1	48.87	34.21	7
Astragalus	85.28	4.06	8.53	21	126.3	6.02	12.63	21	441.6	63.09	44.16	7
Turmeric	44.77	2.13	4.48	21	93.8	2.68	9.38	35	248.2	35.46	24.82	7
Cinnamon	53.29	2.54	5.33	21	116.9	3.34	11.69	35	320.5	45.79	32.05	7

Table 1: Comparison of fermentation kinetic parameters in the cultures with the additions of different Chinese herb water extract.

Chinese herb kind	Triterpenoid				Total polyphenol			
	P1 _{max} (mg/L)	Q _{P1} (mg/L day)	Y _{P1/s} (mg/g)	t (day)	P2 _{max} (mg/L)	Q _{P21} (mg/L day)	Y _{P2/s} (mg/g)	t (day)
Control	47.25	1.35	--	35	115.6	3.21	--	35
0.5	12.08	0.58	4.83	21	79.7	3.80	31.88	21
1	17.84	0.85	3.57	21	75.5	3.60	15.10	21
2	85.28	4.06	8.53	21	126.3	6.02	12.63	21
4	82.46	3.93	4.12	21	117.9	5.61	5.90	21

Table 2: Comparison of fermentation kinetic parameters in the cultures with the additions of different concentrations of Astragalus water extract.

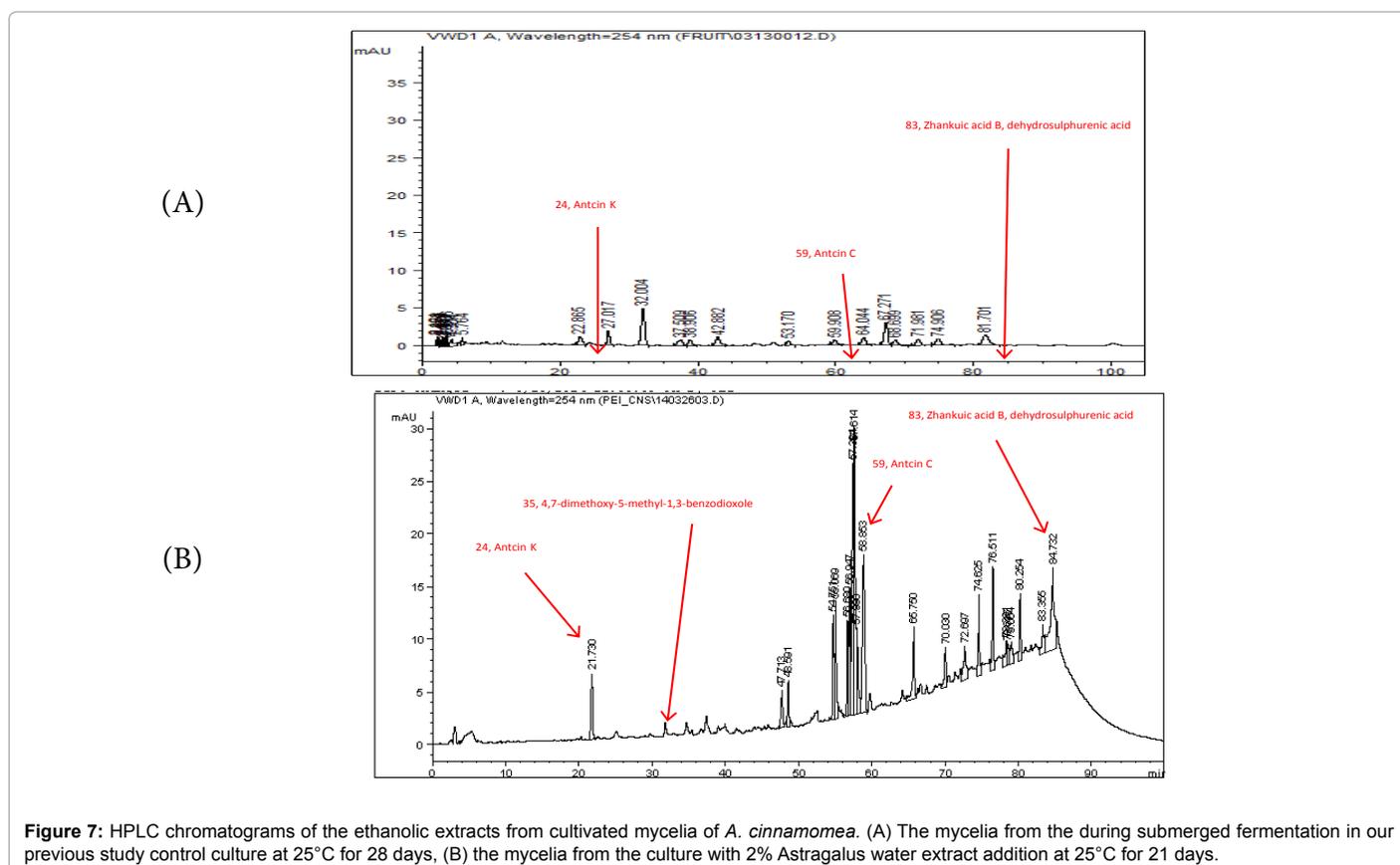


Figure 7: HPLC chromatograms of the ethanolic extracts from cultivated mycelia of *A. cinnamomea*. (A) The mycelia from the during submerged fermentation in our previous study control culture at 25°C for 28 days, (B) the mycelia from the culture with 2% Astragalus water extract addition at 25°C for 21 days.

mg/L day (the control) to 4.06 mg/L day, resulting in more than a three-fold increase. Yield coefficient also increased to 8.53 mg/g.

With respect to another metabolite of total polyphenol, the Chinese herb water extracts of Star anise and Astragalus were demonstrated to have a positive influence on the formation of total polyphenol. The addition of Astragalus water extract (2% (v/v)) was able to raise the production rate parameter to 6.02 mg/L day, compared with the control of 3.21 mg/L, and had a two-fold increase. The yield coefficient parameter increased to 12.63 mg/g. Based on the results indicated

in Table 1, with respect to the other metabolite of intracellular polysaccharides, Turmeric was the only Chinese herb that had a negative effect. In contrast, Star anise water extract (2% (v/v)) was able to effectively enhance the IPS production rate from 38.8 mg/L day (the control) to 69.66 mg/L day, and had more than a 1.8-fold increase.

As the results indicated in Table 2, when different addition amounts of Astragalus water extract ranging from 0.5 to 4% (v/v) were compared, the most appropriate level was still found to be around 2%.

HPLC analysis of triterpenoids

HPLC analyses were according to Chang's paper it displays that the formation of ergostane triterpenoids, such as ancincins C and K, and zhankuic acids A, B and C, is strongly related to the basidiomatal formation of *A. cinnamomea* [32]. HPLC analyses were performed on mycelia extracts from the shake flask cultures. The resulting profiles are given in Figure 7A and 7B. Figure 7A shows the results of the mycelia extract from our previous study control culture [19]. In the mycelium extract, three ergostane triterpenoid peaks appeared at the retention times of 24, 59 and 83 min (Ancinc K, Ancinc C and zhankuic acids B). These products were formed after 28 days' fermentation, but the content was very little. HPLC chromatograms of the mycelia extract from the addition of 2% Astragalus water extract culture is presented in Figure 7B after 21 days of fermentation. Compared with the mycelia of our previous study control culture, the profiles of HPLC analysis showed that the mycelia cultured with Astragalus water extract addition contained more sorts of triterpenoid. In other words, this result demonstrates that the addition of Astragalus water extract can effectively enhance the production of bioactive metabolites in the submerged culture of *A. cinnamomea*.

Conclusions

Since a fruiting body of *A. cinnamomea* grows very slow and its market value reaches approximately twenty thousand US dollars per kilogram, the production of mycelia and bioactive metabolites by *A. cinnamomea* in submerged cultures has prospered in Taiwan in recent years. In order to enhance production efficiency, the control of environmental conditions or the modification of media composition is vital. Some published papers have demonstrated that the additives extracted from the bark of *Cinnamomum kanehirai* Hay are effective in enhancing mycelia growth and the production of bioactive compounds [14-17]. However, from plant conservation and large-scale production points of view, finding substitutes for *Cinnamomum kanehirai* Hay bark are definitely necessary. Among many sources, Chinese herbs are obviously the safest and healthiest source. From a series of experiments, our study demonstrated that the addition of Chinese herb water extracts can effectively enhance the production of biomass and triterpenoid in submerged cultures. In addition, these results demonstrate the feasibility of reusing thin stillage for *A. cinnamomea* mycelia culture.

Acknowledgements

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