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Enhancement of 4-Acetylantroquinonol B Production by Supplementation of Its Precursor during Submerged Fermentation of *Antrodia cinnamomea*

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ABSTRACT: The antiproliferation activity of the ethanol extract of *A. cinnamomea* mycelium on hepatocellular cancer cells HepG2 was found to be associated with aroma intensity of the broth during fermentation. We hypothesized that some of the volatile compounds are the precursors of the key bioactive component 4-acetylantroquinonol B of this fungus. The major volatile compounds of *A. cinnamomea* were identified by GC/MS, and they are oct-1-en-3-ol, linalool, methyl phenylacetate, nerolidol, γ-cadinene and 2,4,5-trimethoxybenzaldehyde (TMBA). TMBA and nerolidol were further selected and used as supplements during fermentation. It was found that both of them could increase the production of 4-acetylantroquinonol B and enhance the antiproliferation activity of the fungus. In addition, the TMBA was identified as the most promising supplement for increasing the bioactivity of *A. cinnamomea* during cultivation.

KEYWORDS: *Antrodia cinnamomea, volatile compounds, submerged fermentation, GC/MS, 4-acetylantroquinonol B, nerolidol, 2, 4, 5-trimethoxybenzaldehyde*

1. INTRODUCTION

Antrodia cinnamomea is a well-known medicinal fungus often produced commercially by submerged fermentation in Taiwan. This fungus is known for its potent antihepatocellular carcinoma function.[1−8] In the previous study we have isolated and identified the key antihepatic compound, 4-acetylantroquinonol B, from the mycelium of *A. cinnamomea*, which could inhibit proliferation of hepatocellular carcinoma cells HepG2 with IC_{50} 0.1 μg/mL.[9] When the HepG2 cells were treated with 4-acetylantroquinonol B, it resulted in the decreases of CDK2 and CDK4, and increase of p27 in a dose-dependent manner. The levels of p53 and p21 proteins also increased due to the treatment.[10]

Previous study found that the IC_{50} values of filtrate and ethanol extract of mycelium of *A. cinnamomea* on HepG2 cells decreased with increasing fermentation time. The cultivation of *A. cinnamomea* in either 5 L agitated or air-lift fermentors reached stationary phase in 4 weeks, and the IC_{50} of the mycelium extract also reached its lowest point at almost the same time.[11,12] It was also noticed that the fermentation broth of *A. cinnamomea* started to produce a unique aroma in 3 to 4 weeks, and the aroma intensity increased with fermentation time. We suspected that the aroma of *A. cinnamomea* is closely related with its antihepatoma activity.

There are some reports regarding the aroma and volatile compounds of *A. cinnamomea* in recent years. For fruiting body of *A. cinnamomea*, Chen et al. (2007) have identified that 2,4,5-trimethoxybenzaldehyde and β-nerolidol are the main volatile compounds.[13] The volatile compounds of *A. cinnamomea* during submerged culture were analyzed by Liu et al., and ethyl acetate, c-undecalactone, linalool, and 3-hydroxy-2-butanone had the highest intensity.[14] Lu et al. (2011) analyzed the volatile compounds of *A. cinnamomea* during submerged culture, and nerolidol was identified in both culture broth and the mycelia.[15] The volatile compounds found during solid state fermentation of *A. cinnamomea* were oct-1-en-3-ol, octan-3-one, and methyl 2-phenylacetate in exponential growth phase, and octan-3-one and methyl 2-phenylacetate in stationary phase. In addition, they detected *cis*-nerolidol at day 15, but this compound diappeared afterward.[16]

It has been reported that production of benzaldehyde during solid state fermentation of *Rhizopus oligosporus* USM R1 could be increased to 7.5-fold by the supplementation of L-phenylalanine, a precursor for benzaldehyde biosynthesis.[17] The hypothesis of this study was that some of the volatile compounds produced during submerged fermentation of *A. cinnamomea* are actually the precursors of the antihepatoma bioactive compound 4-acetylantroquinonol B. Therefore, we first collected and analyzed the volatile compounds produced during fermentation. Then, we selected the most likely precursors of the 4-acetylantroquinonol B and added them into the medium during submerged fermentation to confirm our hypothesis.

2. MATERIALS AND METHODS

2.1. Microorganism and Reagents. *Antrodia cinnamomea* BCRC35716 was obtained from the Bioresoures Collection and Research Center (BCRC) in Food Industry Research and Development Institute (Hsinchu, Taiwan).

Potato dextrose agar (PDA), malt extract, and peptone were obtained from Difico (Sparks, MD, USA). 2,4,5-Trimethoxybenzaldehyde (TMA), nerolidol (a mixture of *cis*- and *trans*-nerolidol), oct-1-en-3-ol, and methyl phenylacetate were from Aldrich Chemical (Milwaukee, WI, USA). Sodium hydrogen carbonate ($NaHCO_3$) and linalool were

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provided by Merck Chemical (USA). Trpysin, Dulbecco's modified Eagle's medium (DMEM), and fetal calf serum (FCS) were from Hyclone (Logan, UT, USA). Dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT), and other reagents used in the study were from Sigma Chemical (St Louis, MO, USA).

2.2. Submerged fermentation of *A. cinnamomea*. The seed culture was maintained on 39 g L^{-1} potato dextrose agar at 25 °C and transferred to a fresh agar plate every month. To prepare the inoculum, the mycelium of *A. cinnamomea* was transferred from Petri dish to 250 mL flask containing 100 mL of medium (components: glucose 2.0%, malt extract 2.0%, and peptone 0.1%) and incubated at 25 °C for 7 days for mycelium growth. Then, 50 mL of the flask culture was transferred to a 5 L stirred tank reactor (1% inoculation) (Firstek Scientific Co. Taipei, Taiwan) and the fermentation was carried out at 22 °C, 0.5 vvm, and 100 rpm. The fermentation broth was sampled every four days for analyzing the volatile compounds.

2.3. Solid Phase Microextraction (SPME)−GC/MS Analysis. The fermentation broth was separated into mycelium and filtrate by filtering through Whatman No. 1 filter paper. After dissolving 4 g of sodium chloride in 8 mL of filtrate in a vial, the vial was tightly capped and then heated at 60 °C for 30 min. The hydrocarbon compound acenaphthene-d_{10} (t_R = 10.85 min) was added to each vial as internal standard. The volatile compounds in the filtrate were absorbed by the polydimethylsiloxane/divinylbenzene (PDMS/DVB) fiber (65 μm, Supelco, USA) and then analyzed by GC/MS. The relative concentration of the identified volatile compound was estimated by (peak area of the volatile compound)/(peak area of internal standard) × 100%.

The GC/MS instrument consists of HP 5890 series II gas chromatograph with HP 5972 mass-selective detector equipped with DB5 column (30 m × 0.25 mm, 0.25 μm) (Agilent Technologies Inc., USA). Helium was used as the carrier gas at a flow rate of 0.8 mL/min. The electronic impact (EI) mode was used, and ionization energy was 70 eV. The injection temperature was 250 °C. The GC oven temperature was programmed as follows: initial temperature 50 °C for 1 min, increased to 130 °C at 20 °C/min and maintained for 1 min, increased to 230 °C at 8 °C/min and maintained for 1 min, and then increased to 300 °C at 20 °C/min and maintained for 2 min. Components were identified by comparing the mass spectra obtained with NIST 05 library base. Pure compounds of TMBA, oct-1-en-3-ol, linalool, methyl phenylacetate, and nerolidol were also used as standards for checking the identities of the volatile compounds during GC/MS analyses.

2.4. Using the Main Volatile Compounds as Precursor. The *A. cinnamomea* was cultivated in 250 mL shake flasks with 100 mL of medium composed of glucose 2.0%, malt extract 2.0%, and peptone 0.1%. Then, 0.05, 0.1, or 0.2% TMBA (2,4,5-trimethoxybenzaldehyde) or 0.25, 0.5, or 1% nerolidol was added. Both of the aforementioned compounds were identified as precursors of 4-acetylantroquinonol B. The pH value of the medium was adjusted to 5 by adding 0.1 N NaOH or 0.1 N HCl, and then sterilized at 121 °C for 20 min. The fermentation was operated with 10% inoculum and carried out at 25 °C and 100 rpm for 6 weeks.

2.5. Determination of Biomass and 4-Acetylantroquinonol B Content. The mycelium in the fermentation broth was recovered by filtering the broth through Whatman No. 1 filter paper and washed twice with distilled water. Biomass was then determined by vacuum-drying (50 °C) until a constant weight was obtained.

The ethanol extract of the mycelium (EEM) of *A. cinnamomea* was obtained by extracting vacuum-dried mycelium (0.5 g) with 95% ethanol (100 mL) at 25 °C for 48 h. The extract was filtered through Whatman No. 1 filter paper and then vacuum-dried at 50 °C to obtain EEM. The EEM was further separated by HPLC using the Agilent 1100 HPLC system equipped with a UV detector. The ODS Hypersil C18 column (250 × 4.6 mm, 5 μm, Thermo Scientific) was used for separation using gradient elution composed of 0.3% CH_3COOH (A) and MeOH (B). The gradient elution profile was as follows: 0−10 min, A:B = 95:5 to A:B = 20:80 (linear gradient); 10−20 min, A:B = 20:80 to A:B = 10:90 (linear gradient); 20−35 min, A:B = 10:90 (isocratic); 35−40 min, A:B = 10:90 to A:B = 95:5 (linear gradient); the flow rate was 1 mL/min, and the detector wavelength was set at 254 nm. Pure 4-acetylantroquinonol B isolated from the mycelium of *A.cinnamomea* during our previous study[10] was diluted to 125, 250, 500, 1000, and 2000 μg/mL and used for constructing the standard curve (R^2 = 0.998).

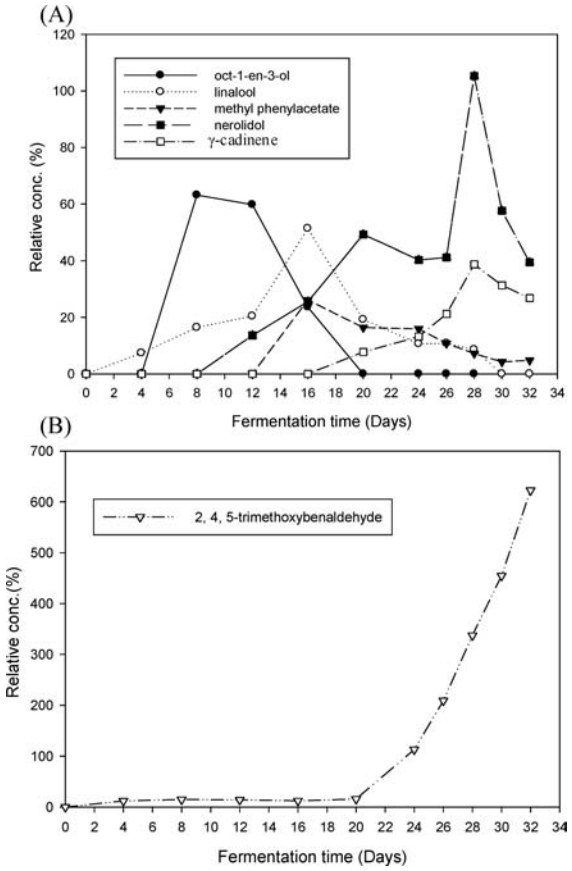

Figure 1. The main volatile compounds profile of the fermentation broth of *Antrodia cinnamomea* during submerged culture in a 5 L stirred tank reactor.

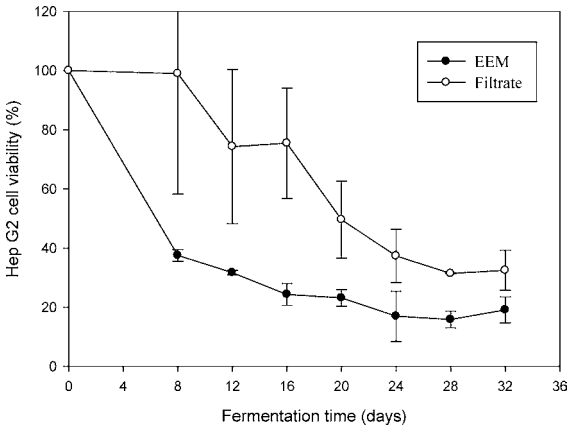

Figure 2. Effect of the filtrate (500 μg/mL) and the ethanol extract of mycelium (50 μg/mL) of *A. cinnamomea* on the growth of HepG2 cells during fermentation. Each value is the mean of six values, and the error bars indicate the standard deviations. Two batches of 5 L fermentation were carried out, and three samples were taken from each batch and analyzed.

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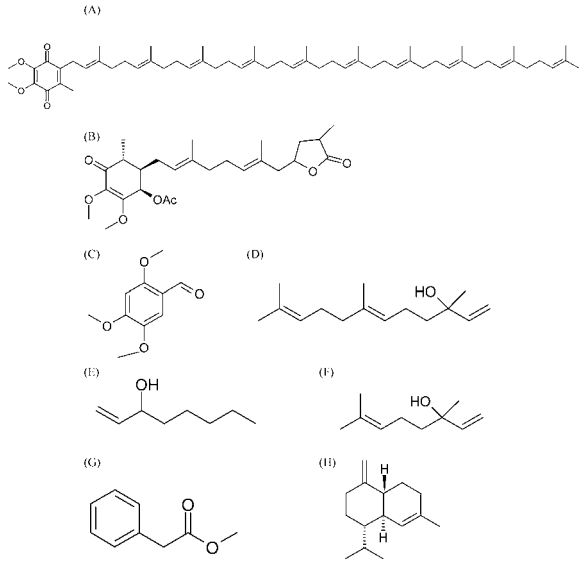

Figure 3. The structures of ubiquinone (A), 4-acetylantroquinonol B (B), 2,4,5-trimethoxybenzaldehyde (C), nerolidol (D), oct-1-en-3-ol (E), linalool (F), methyl phenylacetate (G), and γ-cadinene (H).

2.6. Cell Viability Assay. The human hepatocellular carcinoma cells (HepG2 cells) were obtained from the Bioresoures Collection and Research Center (BCRC) in Food Industry Research and Development Institute (Hsinchu, Taiwan). These cells were maintained in Dulbecco's modified Eagle's medium (Hyclone, USA) supplemented with 10% fetal bovine serum, 1% penicillin–streptomycin in a humidified incubator (5% CO_2 in air at 37 °C). Cells were seeded in 96-well plates prior to the addition of broth filtrate or EEM. The HepG2 cells were incubated with broth filtrate or EEM at various concentrations for 72 h.

The tetrazolium dye colorimetric test (MTT test) is used to monitor cell growth indirectly, as indicated by the conversion of the tetrazolium salt to the colored product, formazan, the concentration of which can be measured spectrophotometrically. Briefly, the HepG2 cells numbers were counted by hemocytometer and seeded in 96-well microplates (1×10^4 cells/well in 200 μL of complete DMEM medium) for 24 h. Cells were then washed with PBS and incubated with the broth filtrate or EEM at various concentrations for 72 h. The medium was removed at the end of incubation; then 100 μL of 2 mg/mL MTT (Sigma, USA) was added to each well, and incubation was allowed to continue for 2 h. Finally, 100 μL of DMSO was added to each well and incubated for 15 min. The plate was read by a UV/vis spectrophotometer (Jasco model 7800, Japan) at a wavelength of 570 nm. The cell viability was calculated by dividing the absorbance of each experimental sample by the corresponding control sample (the medium).

2.7. Statistical Analysis. Statistical analysis was performed using one-way analysis of variance and Duncan's multiple-range test (SAS Institute Inc., Cary, NC, USA) to determine significant differences among means ($p < 0.05$).

3. RESULTS AND DISCUSSION

3.1. The Volatile Compounds Produced by *A. cinnamomea* during Fermentation. The main volatile compounds produced by *A. cinnamomea* during fermentation were identified by GC/MS, and they are oct-1-en-3-ol, linalool,

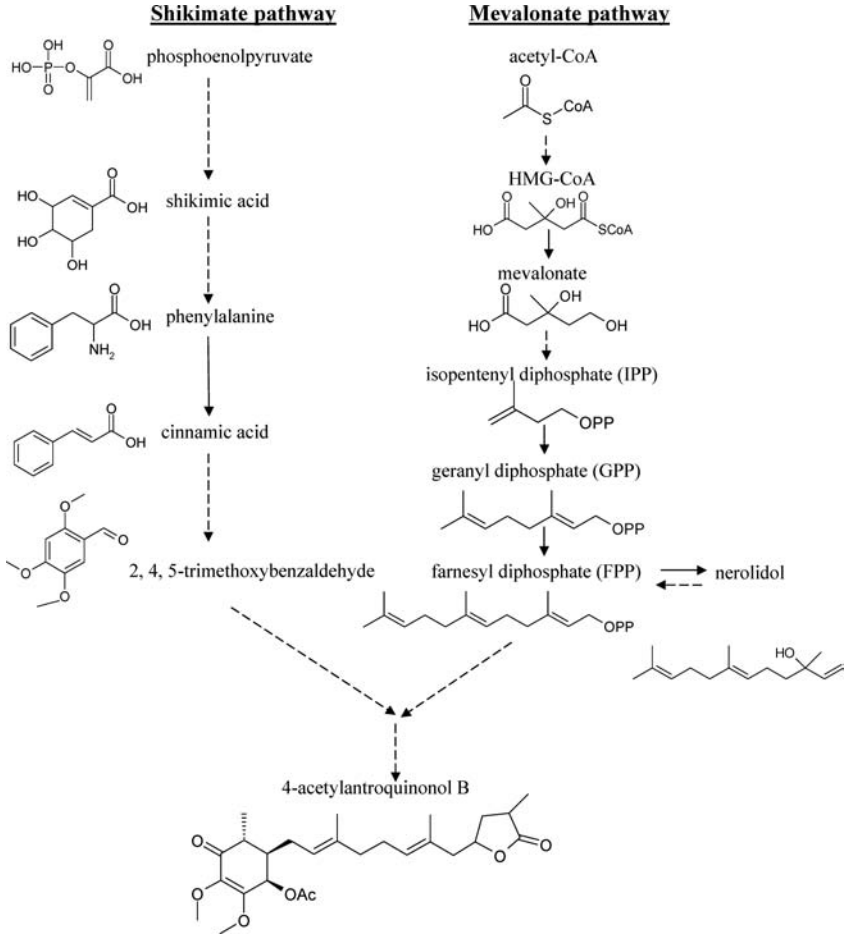

Figure 4. The proposed biosynthesis pathway of 4-acetylantroquinonol B.

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methyl phenylacetate, nerolidol, γ-cadinene, and 2,4,5-trimethoxybenzaldehyde. Changes of these volatile compounds during fermentation are given in Figure 1. The linalool is the first volatile compound that appeared during fermentation. It is a monoterpene with floral and citrus aroma.[18] However, this compound reached its highest concentration at day 16 and then decreased gradually. Similar to linalool, the volatile compounds oct-1-en-3-ol also appeared at the initial fermentation stage. This compound gives mushroom-like aroma.[18] But it reached the highest amount at day 8, then decreased, and became undetectable at day 20. The γ-cadinene was detected at day 20 and reached its highest concentration at day 28. The γ-cadinene is a sesquiterpene compound with a woody aroma.[18] The methyl phenylacetate appeared at day 16 but decreased gradually thereafter. Compared to other volatile compounds, the concentrations of γ-cadinene and methyl phenylacetate are relatively low.

Two other major volatile compounds detected during fermentation of *A. cinnamomea* were nerolidol and 2,4,5-trimethoxybenzaldehyde. Nerolidol is a sesquiterpene compound with floral and woody aroma.[18] It appeared at day 12 and reached more than 100% relative concentration at day 28. The 2,4,5-trimethoxybenzaldehyde appeared at an early stage of fermentation but stayed at low relative concentration (14.0%) until day 20. Thereafter, it increased rapidly and reached more than 620% relative concentration at day 32.

3.2. The Relationship between Volatile Compounds and Antihepatoma Compound 4-Acetylantroquinonol B.

Effects of the broth filtrate and the ethanol extract of the mycelium obtained at different time periods during *A. cinnamomea* fermentation on the growth of HepG2 cells are shown in Figure 2. As expected, the cell viability decreased with increasing fermentation time. In a comparison of Figure 1 and Figure 2, it appears that the volatile compounds 2,4,5-trimethoxybenzaldehyde and nerolidol are closely related to the antihepatoma activity of the filtrate and mycelium extract.

It is known that the most potent antihepatoma compound produced by the *A. cinnamomea* is 4-acetylantroquinonol B.[9,10] Structures of this compound along with the major volatile compounds of *A. cinnamomea* are provided in Figure 3. It shows that 2,4,5-trimethoxybenzaldehyde is similar in structure with the quinone ring of 4-acetylantroquinonol B, and the structure of nerolidol resembles the isoprenoid chain and five-membered oxygen-containing ring of 4-acetylantroquinonol B.

Antroquinonol is a natural ubiquinone derivative.[19] The differences in the structures of antroquinonol and 4-acetylantroquinonol B are only in the five-membered oxygen-containing ring and the acetyl group on the quinone ring. Therefore, 4-acetylantroquinonol B should be closely related to ubiquinone during biosynthesis.

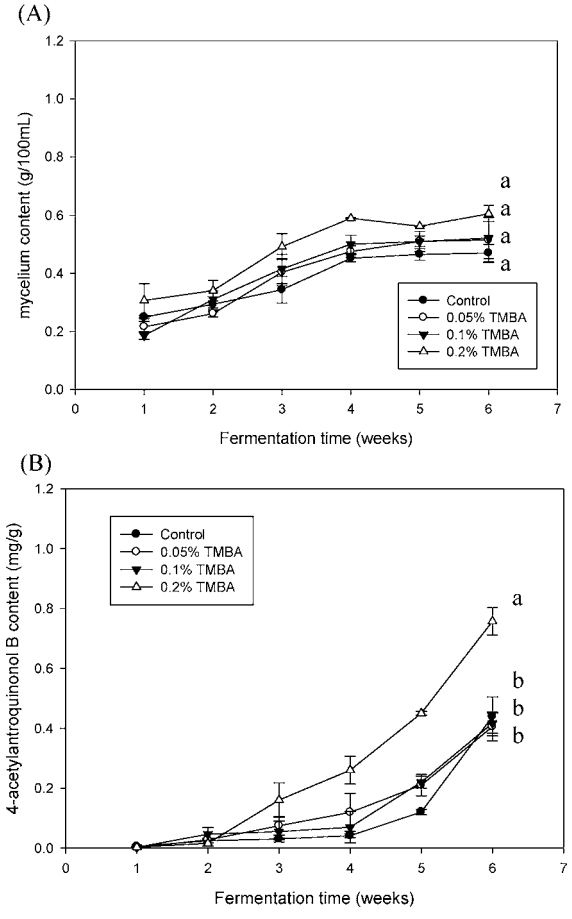

Figure 5. Effect of 2,4,5-trimethoxybenzaldehyde supplementation on the biomass (A) and 4-acetylantroquinonol B (B) production of *A. cinnamomea* in 250 mL shake flask cultures. Each value is the mean of three determinations, and the error bars indicate the standard deviations from three independent samples.

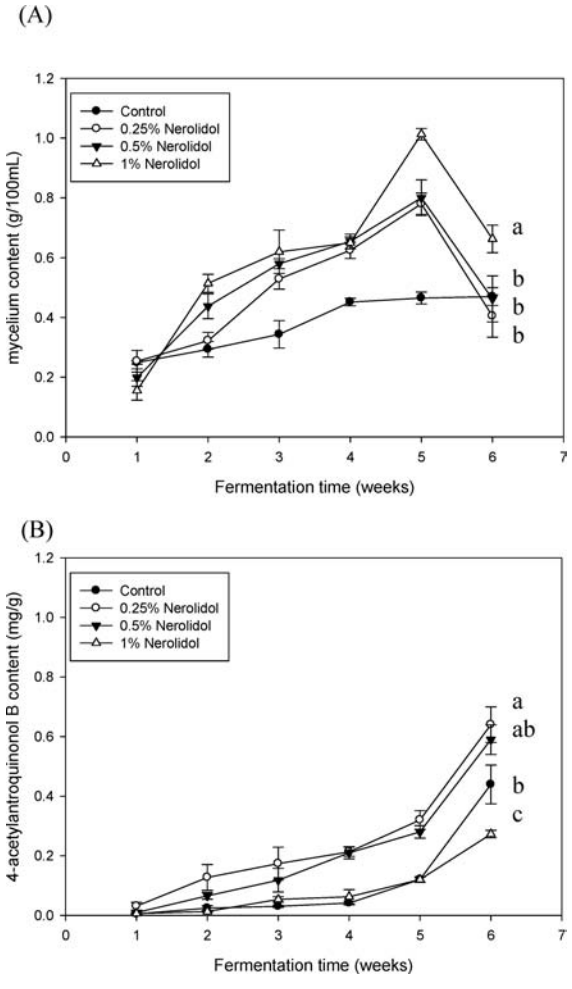

Figure 6. Effect of nerolidol supplementation on the biomass (A) and 4-acetylantroquinonol B (B) production of *A. cinnamomea* in 250 mL shake flask cultures. Each value is the mean of three determinations, and the error bars indicate the standard deviations from three independent samples.

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The 2,3,4-trimethoxybenzaldehyde is considered a promising material for the synthesis of ubiquinone. It can be converted to 2,3,4,5-tetramethoxytoluene via Dakin reaction, formylation, reduction, and methylation of the phenolic hydroxyl group. Then, 2,3,4,5-tetramethoxytoluene can be further converted to ubiquinone.[20]

It is known that nerolidol is a synthetic member of the coenzyme Q group.[21] Kijima et al. developed a process for synthesis of ubiquinone by reacting 2-methyl-4,5,6-trimethoxyphenol with an aryl boronic acid to obtain a 2-methyl-4,5,6-trimethoxyphenol ester and then reacting with an isoprenol, such as nerolidol, to obtain 2-methyl-3-substituted-4,5,6-trimethoxyphenol. Finally, the 2-methyl-3-substituted-4,5,6-trimethoxyphenol can be oxidized to obtain coenzyme Q.[22] Therefore, nerolidol may also be a precursor for the synthesis of ubiquinone or 4-acetylantroquinonol B.

The 4-acetylantroquinonol B was isolated and identified from *A. cinnamomea* in 2009,[23] later than the antroquinonol, which was identified in 2007.[24] Till today, the biosynthesis pathway of antroquinonol is still not clear, not to mention the biosynthesis pathway of 4-acetylantroquinonol B. However, the biosynthesis of 4-acetylantroquinonol B may relate to the biosynthesis pathway of ubiquinone.[25−30] According to the mevalonate pathway, we suspect that, in the presence of sufficient nerolidol, the farnesyl diphosphate (FPP) may accumulate and thus could be transferred to hydroxybenzoate or trimethoxybenzaldehyde. Based on the biosynthesis pathway of ubiquinone, the proposed biosynthesis pathway of 4-acetylantroquinonol B is given in Figure 4. The 2,4,5-trimethoxybenzaldehyde (TMBA) may be produced from phosphoenolpyruvate via the Shikimate pathway and finally converted to TMBA. The TMBA reacts with farnesyl diphosphate or its derivative to synthesize 4-acetylantroquinonol B.

Since both 2,4,5-trimethoxybenzaldehyde and nerolidol could be the precursor of 4-acetylantroquinonol B, these two volatile compounds were added to the fermentation medium for enhancing the production of 4-acetylantroquinonol B during submerged cultivation of *A. cinnamomea*.

3.3. Effect of Nerolidol and TMBA on 4-Acetylantroquinonol B Production. Effects of 2,4,5-trimethoxybenzaldehyde (0.05, 0.1, 0.2%) on the production of biomass and 4-acetylantroquinonol B are shown in Figure 5. As expected, the fermentation entered into stationary phase at the fourth week, and supplementation of 0.2% TMBA in the culture medium resulted in a significantly higher production of 4-acetylantroquinonol B as compared with control ($p < 0.05$).

The effect of nerolidol supplementation (0.25, 0.5, and 1%) on the biomass and 4-acetylantroquinonol B production during fermentation is given in Figure 6. Supplementation of 0.25% nerolidol in the culture medium could significantly increase the 4-acetylantroquinonol B production as compared with control ($p < 0.05$). However, we found that supplementation of the nerolidol during fermentation to a high level, such as 1%, actually decreased the 4-acetylantroquinonol B production. We suspect that the existence of too much nerolidol would result in a back-inhibition effect on the mevalonate pathway and, thus, decrease the biosynthesis of 4-acetylantroquinonol B. It was also found that nerolidol increased the biomass production in a dosage dependent manner. The fungus *A. cinnamomea* forms fruit body naturally on the wood of *Cinnamomum kanehirai* Hay. Nerolidol is also a compound found in *Cinnamomum kanehirai* wood;[13] therefore, it may be a carbon source for the growth of *A. cinnamomea*. This is probably why supplementation of nerolidol could increase the biomass production. The fermentation broth had the highest biomass content at the fifth week and then decreased, indicating that the fungus has entered into death phase. We also observed that the pH of the broth at the sixth week was higher than that of the fifth week (data not shown); the same condition was found in our previous study.[12]

3.4. Antihepatoma Activity. Our previous study has shown that 4-acetylantroquinonol B is the key compound responsible for the antiproliferation activity of *A. cinnamomea*.[9] This study has also proved that supplementation of TMBA or nerolidol during cultivation of *A. cinnamomea* could increase the 4-acetylantroquinonol B content in the mycelium of this fungus. To further confirm the antihepatoma activity, we compared the effects of ethanol extracts of the mycelia (EEM) of *A. cinnamomea* cultivated with or without supplementation of 0.2% TMBA or 0.5% nerolidol on the growth of HepG2 cells. It was found that EEM extracted from the mycelium cultivated with TMBA or nerolidol indeed had higher antiproliferation activity toward HepG2 cells as compared with control (Figure 7).

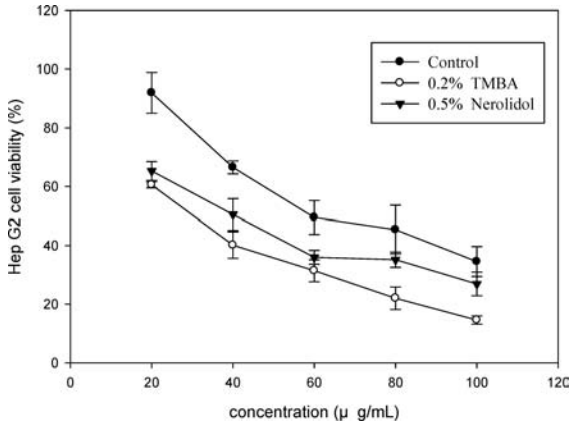

Figure 7. Effect of the ethanol extract of the *A. cinnamomea* mycelium cultivated with TMA and nerolidol in 250 mL shake flasks at 25 °C and 100 rpm for 6 weeks on the growth of HepG2 cells. Each value is the mean of three determinations, and the error bars indicate the standard deviations from three independent samples.

It appeared that TMBA is a more useful supplement for enhancing the 4-acetylantroquinonol B production than the nerolidol. Since the emergence of TMBA during cultivation of *A. cinnamomea* is more synchronous with the increase of 4-acetylantroquinonol B content in the mycelium than that of nerolidol (Figure 1), and the nerolidol may have a back-inhibition effect on the biosynthesis of 4-acetylantroquinonol B, we believe that TMBA is a better candidate as a supplement for enhancing 4-acetylantroquinonol B production.

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Notes

The authors declare no competing financial interest.

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