Efficient Production of 4-Acetylantroquinonol B in Submerged Cultures of *Antrodia cinnamomea via* Addition of Chinese Herbal Medicine Extracts

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Antrodia cinnamomea is a valuable fungus. The 4-acetylantroquinonol B (4-AAQB) contained in it has strong anticancer activity. In this study, five kinds of herbs, burdock, wolfberry, coix seed, hawthorn, and tangerine peel were selected and processed into powders, aqueous extracts, and ethanolic extracts to investigate the effects on the production of 4-AAQB by *A. cinnamomea*. A combination strategy was conducted by adding burdock and tangerine peel aqueous extract. By this means, the production of 4-AAQB was improved to 54.5 mg/L, which was approximately 33-fold higher than the control. After analysis of components of the tangerine peel aqueous extract and the addition of the major components, vanillic acid, protocatechuic acid, and their analogs played an important role in the synthesis of 4-AAQB. It was demonstrated that the addition of Chinese herbs facilitated both cell growth of *A. cinnamomea* and 4-AAQB production, providing a feasible way to increase the yield of 4-AAQB.

DOI: 10.15376/biores.18.3.5368-5384

Keywords: Antrodia cinnamomea; 4-Acetylantroquinonol B; Fermentation; Aqueous extract; Burdock; Tangerine peel

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INTRODUCTION

Antrodia cinnamomea is a type of Chinese medicinal fungi with a long history. In Taiwan, the fruiting body of *A. cinnamomea* has been used as herbal medicine to treat hepatitis and alcoholic liver and is regarded as the "ruby in the forest" because of its excellent medicinal value (Hsu *et al.* 2005; Song *et al.* 2005; Ao *et al.* 2009; Yen *et al.* 2020; Liu *et al.* 2021; Wang *et al.* 2021). Thus, it became one of the most researched Chinese herbal medicines.

With the analysis of the chemicals in *A. cinnamomea*, there are numerous active compounds in *A. cinnamomea*, including terpenoids, benzenoids, steroids, benzoquinone derivatives, sesquiterpene lactone, and intracellular polysaccharides (Geethangili and Tzeng 2009; Chen *et al.* 2011). Some of these compounds have been shown to have inhibitory functions against cancer cells (Hseu *et al.* 2004; Chen *et al.* 2009; Hong *et al.* 2010; Lien *et al.* 2011). 4-AAQB is a type of ubiquinone, which was purified and identified in 2010 (Lin *et al.* 2010). Lin first found that 4-AAQB could significantly inhibit the proliferation of HepG2 cells by affecting the p53, p21, and p27-induced signal pathways (Lin and Chiang 2011). Subsequently, 4-AAQB proved to have inhibitory effects on

varieties of cancer cells, such as liver cancer cells, epithelial cancer cells, prostate cancer cells, and colorectal cancer cells, with almost no side effects detected (Lin *et al.* 2010; Ching 2013; Chang *et al.* 2015a, 2015b; Liu *et al.* 2017; Oluwaseun *et al.* 2018). Therefore, 4-AAQB was considered as one of the potential anti-cancer drugs and has garnered more attention in recent years.

However, *A. cinnamomea* only grew on the special plant *Cinnamomum kanehirae* Hay. This species has a long growth cycle, which led to the shortage of production and a high market price of *A. cinnamomea* fruit body (Wu and Ryvarden 1997; Ao *et al.* 2009). An artificial cultivation strategy was then proposed, in which the basswood cultivation method, the plate cultivation method, the solid cultivation method, and liquid submerged cultivation method were applied. The plate cultivation method is widely used in passage culture. It was indicated that the cultivation of *A. cinnamomea* on wood and solid medium also required a long time to obtain *A. cinnamomea* (3 months to 3 years), while an amount of *A. cinnamomea* mycelia could be quickly obtained in the submerged cultivation process (9 to 30 days) (Lin *et al.* 2011).

It has been verified that 4-AAQB was successfully detected in the mycelia of *A. cinnamomea*, and it is clear that submerged cultivation of *A. cinnamomea* is an effective approach to produce 4-AAQB. To further improve the production of 4-AAQB, Chiang *et al.* (2013) analyzed the volatile components in the liquid fermentation process and then added the main compounds into the fermentation medium. The result showed that the addition of the compound 2,4,5-trimethoxybenzaldehyde (TMBA) could increase the content of 4-AAQB to about 0.75 mg/g dry cell weight (DCW) after 6 weeks of fermentation (Chiang *et al.* 2013). Yang *et al.* (2017) added CoQ0 and 4-hydroxybenzoic acid (4-HBA) to the fermentation medium and the content of 4-AAQB increased to approximately 27.80 mg/g DCW, 16 times higher than the control group. Chou *et al.* (2017, 2019) used the isotope dilution method to speculate on the potential synthesis pathway of 4-AAQB and confirmed that orsellinic acid was the precursor for 4-AAQB synthesis. In the authors' previous studies, the two-stage cultivation method was developed for the fermentation process combined with the immobilization of the mycelium on a fiber net. This proved efficient for the improvement of cell growth and promoted the production of 4-AAQB, with the 4-AAQB yield increasing to 16.3 mg/L, which as 6.9 times higher than the control (Jin *et al.* 2021).

Chinese herbs are a precious resource that contain different active components and have diverse medicinal effects (Yang *et al.* 2003; Liu and Zhang 2007; Yang and Tang 2009). Adding traditional Chinese herbs into the fermentation medium is a proven positive approach to improving the target component's production. Yang *et al.* (2003) summarised the effects of different Chinese medicines on the submerged culture of medicinal fungi, including *Ganoderma lucidum*, *Grifola frondosa*, *Cordyceps sinensis*, and *Coprinus comatus*. In summary, the addition of most Chinese herbal medicines can promote the production of fungal active ingredients (Yang and Tang 2009). Yang *et al.* (2003) explored the aqueous extract and alcohol extract of 10 different Chinese medicines on the cell growth and exopolysaccharide production by *G. lucidum* in the submerged cultivation process, and the result showed that most Chinese medicines could improve the culture of *G. lucidum* (Yang *et al.* 2003). Some insects were used as traditional Chinese medicine in China. Liu and Zhang (2007) added ethyl acetate extract from *Eupolyphaga sinensis* and *Catharsius molossus* into the fermentation medium of *G. lucidum* to enhance polysaccharides production. These results suggest that when adding ethyl acetate extract of *E. sinensis* and *Catharsius molossus*, there was a considerable increase of biomass,

intracellular polysaccharides, and extracellular polysaccharides of *Ganoderma lucidum* (Liu and Zhang 2007).

A. cinnamomea is similar to *G. lucidum*, belonging to medicinal fungi, but only two works reported adding the extract of Chinese herbs to culture medium of *A. cinnamomea*. Ma *et al.* (2014) added the extract of citrus peel to enhance the triterpenoid production in the submerged cultures of *A. cinnamomea*. The addition of the extract of citrus peel not only increases the content of triterpenoids, but it also enriches the kinds of triterpenoids (Ma *et al.* 2014). Lu *et al.* (2014) added the extract of *Cinnamomum camphora*, the host-related species of *A. cinnamomea*, and found that α-terpineol showed the greatest stimulatory effect on the triterpenoid content in *Cinnamomum camphora*.

In this study, the effect of Chinese medicines on the growth of *A. cinnamomea* as well as 4-AAQB accumulation was explored *via* adding the powder, aqueous extract, and alcohol extract of five different Chinese medicines. The composition of the tangerine peel aqueous extract was further analyzed to explore the effect of the major components on the production of 4-AAQB. Further, it was demonstrated that Chinese herbs also had a remarkable promotion effect on *A. cinnamomea*, which provided a feasible way to produce natural active products.

EXPERIMENTAL

Microorganism and Culture Medium

A. cinnamomea (YMT 1002) was obtained from Chaoyang University of Science and Technology (Taizhong, Taiwan). The strain was maintained on agar slant culture medium by periodical subculture following incubation at 30 ℃ for 20 days and stored at 4 ℃. To promote the production of 4-AAQB, the submerged culture process was divided into the seed culture stage and the fermentation stage (Jin *et al.* 2021). The compositions of the agar slant culture medium were glucose 20 g/L, peptone 2 g/L, malt extract 10 g/L, and ager 25 g/L. The compositions of the seed medium were glucose 20 g/L, peptone 10 g/L, KH_2PO_4 0.75 g/L, and Mg_2SO_4 1.5 g/L. Compositions of the fermentation medium of *A. cinnamomea* were: glucose 40 g/L, peptone 5 g/L, K_2HPO_4 0.5 g/L, and Mg_2SO_4 0.5 g/L. All general materials were from Shanghai Yuanye Bio-Technology Co., Ltd., China. The grade of peptone was biological reagent (CAS: 91079-38-8). Chinese herbs were from Beijing Tongrentang, China.

Spore Inoculum Preparation and Seed Culture Condition

The spores of *A. cinnamomea* (YMT 1002) were washed and collected from a slant culture medium using 10 to 15 mL sterilized water. A hemocytometer was used for counting spores under the microscope, and the spore inoculum concentration was adjusted to about $5*10^7$ per milliliter. A total of 0.6 mL prepared spore inoculum was then put into 250-mL shake flasks containing 100 mL seed medium in each flask that would be incubated for 5 days in the rotary shaker at 28 ℃ and 150 rpm to finish the seed culture process. Then, a homogeneous seed inoculum was obtained and the pellets mycelia formed with the diameter of about 0.7 mm (Jin *et al.* 2021).

Fermentation Process Conditions

A total of 80 mL fermentation medium was added into 250 mL shake flasks and sterilized at 115 ℃, 25 min. Then, 20 mL seed inoculum was inoculated into 80 mL of

fermentation medium and cultured at 28 ℃, 150 rpm for 16 days in shaker instrument (HZC-280, Peiying, China). All experiments were duplicated three times and average data were reported (Jin *et al.* 2021).

Pretreatment of Chinese Herbs

Preparation of the powder of Chinese herbs

The Chinese herbs were placed in a constant temperature oven and dried to constant weight under 65 °C for 16 h, which were then milled into a particle size of about 100 to 300 μm. The powders of these Chinese herbs were added into the fermentation medium with different levels to explore the effects on the growth of *A. cinnamomea* and the accumulation of 4-AAQB.

Preparation of aqueous extract from Chinese herbs

A total of 100 g powder of Chinese herbs above was added into 2000 mL deionized water. The mixture was boiled for 2 min and subjected to ultrasonic extraction for 15 min. Thereafter, the mixed decoction was filtered and concentrated to 100 mL. The aqueous extract of the herbs was prepared and filtration sterilized. Different volumes of the aqueous extract were added into the fermentation medium to explore the effects on the growth of *A. cinnamomea* and the accumulation of 4-AAQB.

Preparation of ethanol extract from Chinese herbs

Next, 100 g herbs powder above was added to 200 mL ethanol and repeatedly extracted six times using a Soxhlet extractor. Then, the mixture was filtered and concentrated to 100 mL. The ethanol we used was food grade. The ethanol extract from the Chinese herbs was prepared and filtration sterilized. Different volumes of the ethanol extract were added into the fermentation medium to explore the effects on the growth of *A. cinnamomea* and the accumulation of 4-AAQB.

Analytical Methods

The mycelium in the fermentation broth was recovered by filtration and washed twice with distilled water. The mycelium was then dried at 60 ℃ for 24 h to a constant weight to determine the biomass of *A. cinnamomea*.

A total of 0.1 g dried mycelium was mixed with 2 mL 90% anhydrous ethanol to extract 4-AAQB in *A. cinnamomea* cells at 30 ℃ for 2 h using constant temperature oscillation.

Standard 4-AAQB was obtained from Chaoyang University of Science and Technology (Taizhong, Taiwan). The 4-AAQB was detected using the Agilent 1100 HPLC system equipped with a UV detector and ODS Hypersil C18 column (250 × 4.6 mm^2, 5 μm, Thermo Scientific, Waltham, MA, USA). Elution was performed at a flow rate of 0.8 mL/min with a column temperature of 30 ℃ and UV wavelength of 248 nm with acetonitrile (A) and 0.2% acetic acid solution (B) as the mobile phase. The mobile-phase composition was changed linearly from 60% A to 75% A in 30 min. All experiments were repeated three times and average data were reported.

The yield of 4-AAQB in the liquid culture medium was calculated from the content of 4-AAQB in dry *A. cinnamomea* and the DCW of *A. cinnamomea* in the liquid culture medium.

RESULTS AND DISCUSSION

Addition of Herbs Powders to Promote 4-AAQB Production by *A. cinnamomea*

Based on the preliminary work (In the preliminary work, 25 inexpensive and easily available Chinese medicines were selected and powdered to added to the medium at 20 g/L for the pre-experiment. The results are shown in supplementary materials Fig. S1), 5 kinds of traditional Chinese medicines were selected for the next experiments. Firstly, these five Chinese herbs were pretreated to a fine powder and added into the fermentation medium with different concentrations (10, 20, 30, 40, or 50 g/L) at the beginning of fermentation to investigate the effects on 4-AAQB production. As shown in Fig. 1, when the powders of these Chinese herbs were added, the dry cell weight (DCW) of *A. cinnamomea* mycelia all increased after being cultivated for 16 days. Moreover, the highest DCW was up to 16.7 g/L when 30 g/L of wolfberry powder was added into the medium. It was found that 50 g/L hawthorn powder also could promote the biomass accumulation of *A. cinnamomea*; about 16 g/L DCW was obtained. It was speculated that the polysaccharides contained in the herbs provided additional carbon sources for the growth of *A. cinnamomea*. Meanwhile, because most of the powder was difficult to dissolve completely in water, it might adhere to the surface of mycelia and impact the calculation of dry cell weight.

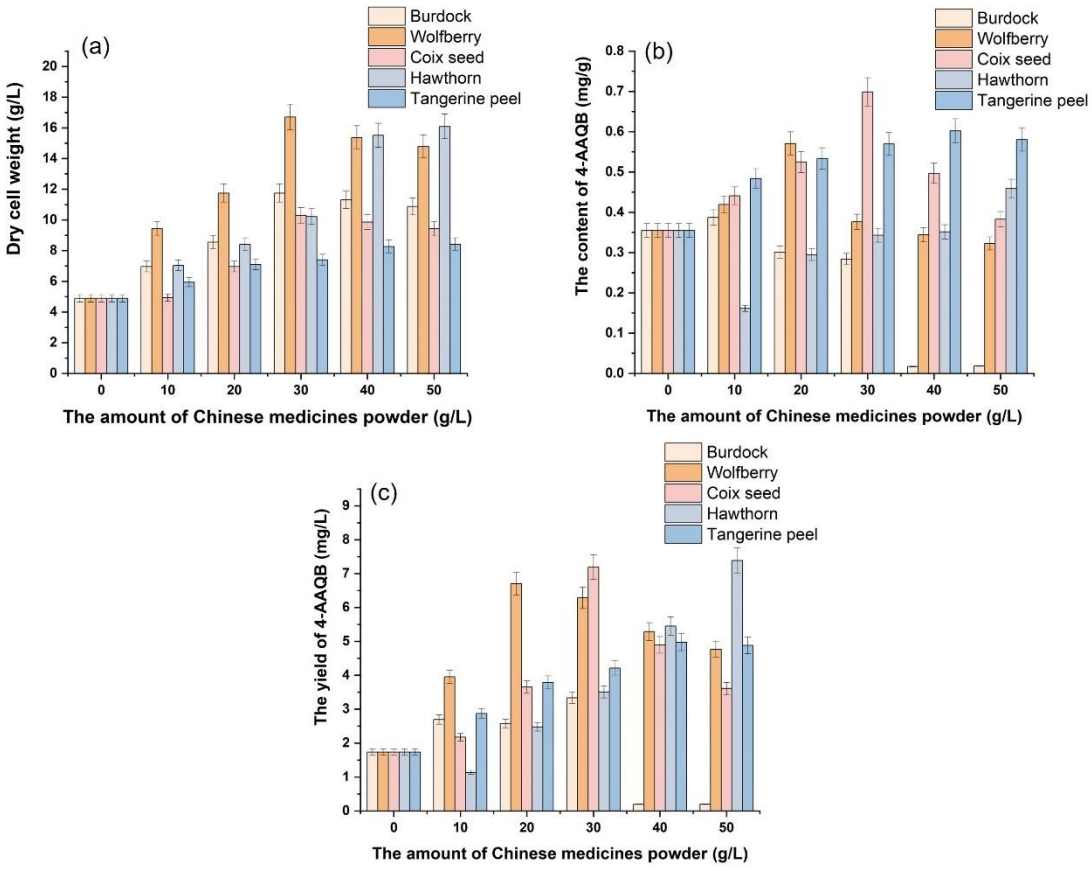

Fig. 1. The effect of herbs powders on the production of 4-AAQB by *A. cinnamomea.* (a) The effect of herbs powders on the dry cell weight of *A. cinnamomea.* (b) The effect of herbs powders on the content of 4-AAQB in *A. cinnamomea.* (c) The effect of herbs powders on the yield of 4-AAQB

After analyzing the 4-AAQB content, it was indicated that the highest yield of 4-AAQB reached 0.7 mg/g when 30 g/L coix seed power was added. This was about twice as high as the group without Chinese herbs powder, 0.355 mg/g. Moreover, tangerine peel powder also had a remarkable effect on increased 4-AAQB accumulation, which the addition of 40 g/L tangerine peel led to 4-AAQB content of 0.6 mg/g. Among these Chinese herbs, burdock showed the least promoting effect on 4-AAQB production. With the increase of the additional level of burdock, the yield of 4-AAQB gradually decreased. After calculating the yield of 4-AAQB, the hawthorn powder was the optimum among these five Chinese herbs for 4-AAQB production by *A. cinnamomea*. However, as the herbal powders could not dissolve in water completely, it was difficult to analyze their effect on the cell growth of *A. cinnamomea*. Therefore, the extract of these herbs was further prepared to explore the effects on the growth of *A. cinnamomea* and the production of 4-AAQB.

The effect of aqueous extract from Chinese herbs

First, the powders of Chinese medicines were processed to obtain the aqueous extract. The concentration of herbs was 1 g/mL. Then, the authors added the aqueous extract into the fermentation medium with the concentrations 10, 20, 30, 40, and 50 g/L (the concentration was the herbs in liquid culture medium) to promote 4-AAQB production by *A. cinnamomea*. As shown in Fig. 2, the aqueous extract from different herbs had disparate effects on the growth of cells and 4-AAQB production.

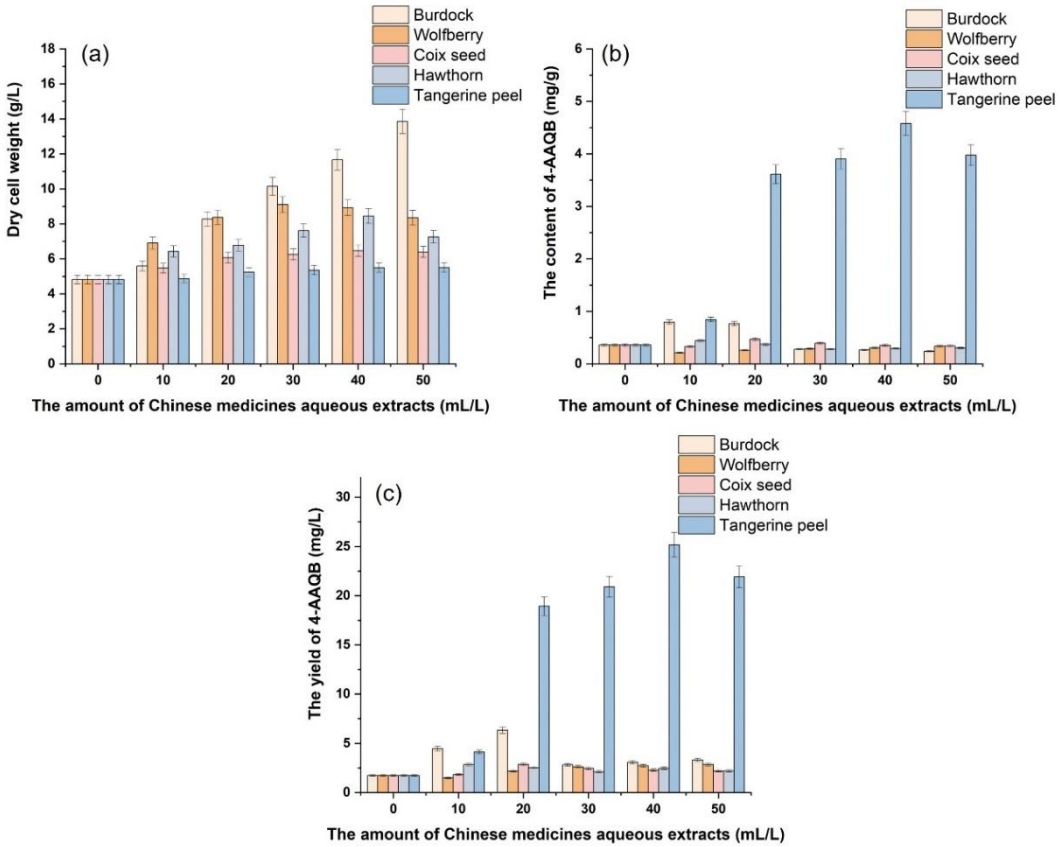

Fig. 2. The effect of aqueous extracts of herbs on the production of 4-AAQB by *A. cinnamomea.* (a) The effect of aqueous extracts of herbs on the dry cell weight of *A. cinnamomea.* (b) The effect of aqueous extracts of herbs on the content of 4-AAQB in *A. cinnamomea.* (c) The effect of aqueous extracts of herbs on the yield of 4-AAQB

It was worth mentioning that the aqueous extract of hawthorn did not promote as well as the hawthorn powder, probably because few polysaccharides of hawthorn could dissolve in water to provide an extra carbon source for *A. cinnamomea*. The aqueous extract of burdock showed remarkable promotion in the growth of cells, which led to the highest DCW 13.85 g/L when 50 mL/L aqueous extracts of burdock (corresponding to 50 g/L herbs powder) was added. It was found that the content of 4-AAQB would promote by the addition of burdock aqueous extract with 10 and 20 mL/L, but the higher addition of burdock aqueous extract showed an inhibitory effect on the accumulation of 4-AAQB. The aqueous extract of tangerine peel was the most beneficial for 4-AAQB production. The highest content of 4-AAQB reached 4.58 mg/g, and the yield of 4-AAQB was 25.18 mg/L when 40 mL/L tangerine peel aqueous extract was added, which was about 14.6 times higher than the control. According to the report, Chiang *et al.* (2013) collected nerolidol, which exists in citrus plants, and then added nerolidol into the fermentation medium of *A. cinnamomea*, it also showed a remarkable promotion on 4-AAQB production *A. cinnamomea*. Therefore, the addition of tangerine peel aqueous extract could substantially enhance the accumulation of 4-AAQB, where it was suspected that some volatile components contained in the tangerine peel were structural analogs of 4-AAQB and provided precursors for the synthesis of 4-AAQB.

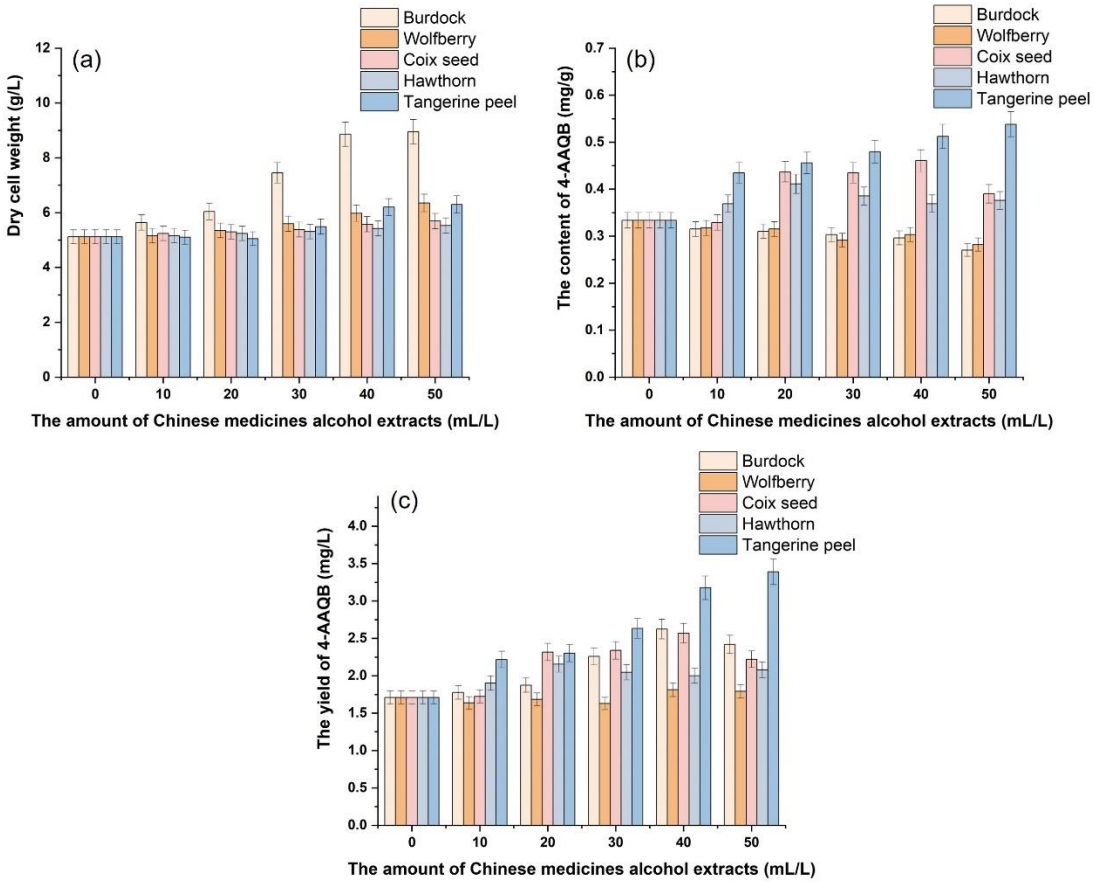

Fig. 3. The effect of alcohol extracts of herbs on the production of 4-AAQB by *A. cinnamomea.* (a) The effect of alcohol extracts of herbs on the dry cell weight of *A. cinnamomea.* (b) The effect of alcohol extracts of herbs on the content of 4-AAQB in *A. cinnamomea.* (c) The effect of alcohol extracts of herbs on the yield of 4-AAQB

The effect of ethanol extract from Chinese herbs

Considering that there were some non-water-soluble active products in herbs, the solvent was changed to ethanol for extraction to compare the effects of ethanol extract from Chinese herbs on *A. cinnamomea*. Different from the results *via* addition of the aqueous extract of herbs, the ethanol extract did not contribute much to both the cells growth and 4-AAQB production of *A. cinnamomea*, as shown in Fig. 3. The highest DCW was achieved at 8.95 g/L when 50 mL/L ethanol extract of burdock was added into the fermentation medium. This was consistent with the results above that both the aqueous extract and the ethanol extract of burdock could promote the cell growth of *A. cinnamomea*, but it could not improve the accumulation of 4-AAQB. Similarly, the tangerine peel was the most effective in increasing the production of 4-AAQB, which led to the content of 4-AAQB up to 0.54 mg/g with the yield was 3.4 mg/L when 50 mL/L ethanol extract was added.

Therefore, the active compounds in the extract of Chinese herbs had a positive effect on both the cell growth and the production of 4-AAQB by *A. cinnamomea*. Compared with the ethanol extract of herbs, the aqueous extract was more beneficial for *A. cinnamomea*. It was possible that the growth of *A. cinnamomea* required a stringent environment and ethanol as the extraction solvent added into the medium might inhibit the cell growth and negatively affect the 4-AAQB production by *A. cinnamomea*. Further, according to the results above, the aqueous extract burdock and tangerine peel were the most effective herbs to promote cells growth and 4-AAQB accumulation, respectively. To further enhance the production of 4-AAQB, a combination addition strategy of the aqueous extracts from these two herbs was explored.

Combined Addition of Herbs Extracts to Promote 4-AAQB Production

To increase the yield of 4-AAQB by increasing the DCW of *A. cinnamomea* and the content of 4-AAQB in *A. cinnamomea*, the combination addition strategy of the aqueous extracts was applied. Aqueous extracts of burdock and tangerine peel were added into the fermentation medium with different volumes (20, 30, 40, and 50 mL/L) to explore the optimum combination.

As shown in Fig. 4, the highest DCW of *A. cinnamomea* reached 13.04 g/L when the combination of 40 mL/L burdock aqueous extract and 40 mL/L tangerine peel aqueous extract were added. This was comparable to the result by adding the aqueous extract of burdock individually, which indicated that the combinational additions of tangerine peel aqueous extract did not have a great effect on the growth of *A. cinnamomea*. After analyzing the content of 4-AAQB, the optimum combination was a little different from the result above. It was shown that the best group was 20 mL/L tangerine peel aqueous extract combined with 40 ml/L burdock aqueous extract for the content of 4-AAQB. In this group, the content of 4-AAQB up to 4.50 mg/g. This yield was comparable to the level by adding 40 mL/L tangerine peel aqueous extract individually. These findings might be attributed to the synergistic effect of the two aqueous extracts, and the addition of the burdock aqueous extract promoted the cell growth to increase the production of 4-AAQB. When the volume of the tangerine peel aqueous extract was increased to 30 mL/L combined with 40 mL/L burdock aqueous extract, the content of 4-AAQB reached 4.27 mg/g. This was a lower content of 4-AAQB, but it achieved the highest yield of 4-AAQB, 54.49 mg/L, which was almost two times higher than the result by adding 40 mL tangerine peel aqueous extract individually. It was demonstrated that the combined addition strategy could remarkably improve the accumulation of 4-AAQB by *A. cinnamomea*. Meanwhile, compared with the

results in the control group without the addition of Chinese herbs, the DCW of *A. cinnamomea* was increased approximately 2.6 times and the production of 4-AAQB was increased approximately 33-fold. It was again demonstrated that the addition of Chinese herbs facilitated the improvement of both cell growth and 4-AAQB production in the submerged cultivation of *A. cinnamomea*, which provided a feasible way to increase the yield of 4-AAQB and laid a foundation for the development of more nutraceuticals from *A. cinnamomea*.

Based on the above results, the burdock aqueous extract facilitated the growth of *A. cinnamomea*, while the tangerine peel aqueous extract could promote the synthesis of 4-AAQB. According to the reports, burdock is rich in dietary fiber and inulin, which could be used as an additional carbon source (Liu *et al.* 2012). Meanwhile, the lignin in burdock was also a potential component to promote the cell growth of *A. cinnamomea* (Qin *et al.* 2019). The composition of the tangerine peel aqueous extracts was further analyzed to explore the effect of the major components on the production of 4-AAQB.

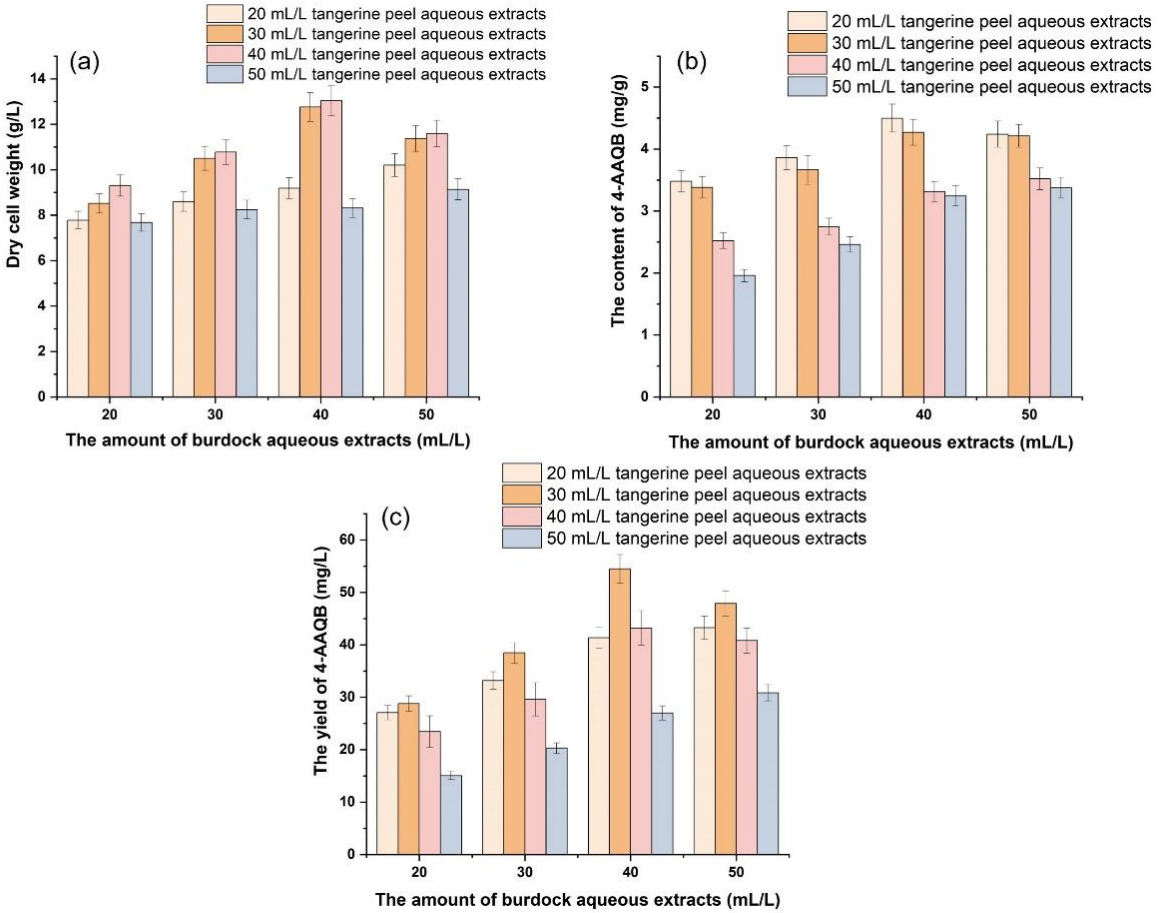

Fig. 4. The effect of combined addition of herbs extracts on the production of 4-AAQB by *A. cinnamomea.* (a) The effect of combined addition of herbs extracts on the dry cell weight of *A. cinnamomea.* (b) The effect of combined addition of herbs extracts on the content of 4-AAQB in *A. cinnamomea.* (c) The effect of combined addition of herbs extracts on the yield of 4-AAQB

Effect of the Major Components in the Tangerine Peel Aqueous Extracts on the Production of 4-AAQB by *A. cinnamomea*

Because tangerine peel aqueous extract showed an excellent promotion effect on 4-AAQB production, the composition of the tangerine peel aqueous extract was further analyzed to identify the potential active ingredients. The main compounds with similar chemical structures to 4-AAQB in tangerine peel aqueous extract, including hesperidin, nobiletin, tangerine, vanillic acid, protocatechuic acid, and others, are shown in Fig. 5. These compounds were added to the *A. cinnamomea* fermentation medium at the level of 0.01%, 0.05%, 0.1%, and 0.2% on the 10^{th} day to explore their effects on 4-AAQB production. As shown in Fig. 6, vanillic acid and protocatechuic acid were the main compounds in tangerine peel aqueous extract in the promotion of 4-AAQB production. When 0.05% vanillic acid were added, the yield of 4-AAQB could reach 7.95 mg/L. Protocatechuic acid could promote the content of 4-AAQB to 14.1 mg/L; compared with the control group, it improved 8.27 times. The DCW of *A. cinnamomea*, content of 4-AAQB, and the yield of 4-AAQB is shown in Fig. S2.

Chou *et al.* (2019) and Yang *et al.* (2017) found that the addition of 4-hydroxybenzoic acid (4-HBA) to the cultivation process remarkably enhanced 4-AAQB production. It was indicated that 4-HBA and orsellinic acid (OA) was the ring precursor of the competitor antroquinonol (AQ), while the ring precursor of 4-AAQB was only derived from OA (Yang *et al.* 2017; Chou *et al.* 2019). The addition of 4-HBA reduced the dependence of OA in the synthesis of AQ, which thereby promoted the accumulation of precursor for the production of 4-AAQB. Moreover, Chou *et al.* suggested that vanillic acid and 2,4-dihydroxybenzoic acid could promote the synthesis of 4-AAQB because lactonized farnesyl pyrophosphate could only be linked to quinone rings with hydroxylated or methylated modifications at the C-5 or C-6 positions (consider the connected carbon as C-3) (Chou *et al.* 2019). Therefore, vanillic acid and protocatechuic acid might be the ring precursors for the synthesis of 4-AAQB because of their structure to meet the requirement above. In addition, because both OA and 4-HBA could be the ring precursors for the synthesis of AQ, it was suggested that the farnesyltransferase in the AQ synthesis pathway was similar to Coq6 in yeast and the UbiA in *E. coli* without strict structural selectivity (Wessjohann and Sontag 1996; Pierrel *et al.* 2010; Xie *et al.* 2015). Therefore, vanillic acid and protocatechuic acid could also act as the ring precursors for the synthesis of AQ, thus alleviating the consumption of OA and enhancing the yield of 4-AAQB. Ferulic acid and caffeic acid also had a beneficial effect on the synthesis of 4-AAQB. It was reported that ferulic acid could be converted to vanillic acid in *Aspergillus niger* and *Sporotrichum thermophile* (Lesage-Meessen *et al.* 1996; Topakas *et al.* 2003). Thus, it was suspected that ferulic acid and caffeic acid could be converted to vanillic acid and protocatechuic acid by *A. cinnamomea* to generate more ring precursors for the synthesis of AQ and thereby promote the production of 4-AAQB. *A. cinnamomea* had the potential to utilize phenylalanine to synthesize AQ (Chou *et al.* 2019). Ferulic acid and caffeic acid, as phenylpropanoids may also act as precursors of benzoquinones in *A. cinnamomea*.

However, the authors' results also indicated that the production of 4-AAQB *via* the addition of a single compound did not achieve similar yields with the addition of tangerine peel aqueous extract. This might be because the combination of multiple components was more efficient to promote the synthesis of 4-AAQB. Additionally, fructose played an important role in the promotion of 4-AAQB production in the authors' previous work. The high fructose content of the tangerine peel aqueous extract might also be one of the reasons for its promotion of 4-AAQB production (Jin *et al.* 2021).

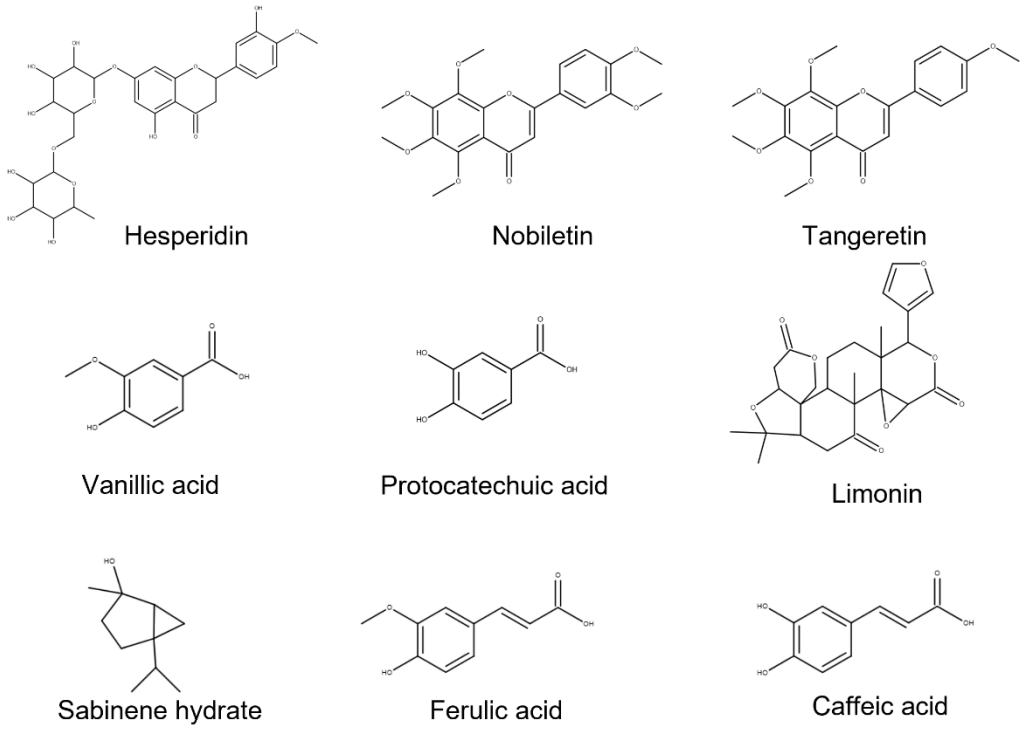

Fig. 5. The main compounds with similar chemical structures to 4-AAQB in tangerine peel aqueous extract

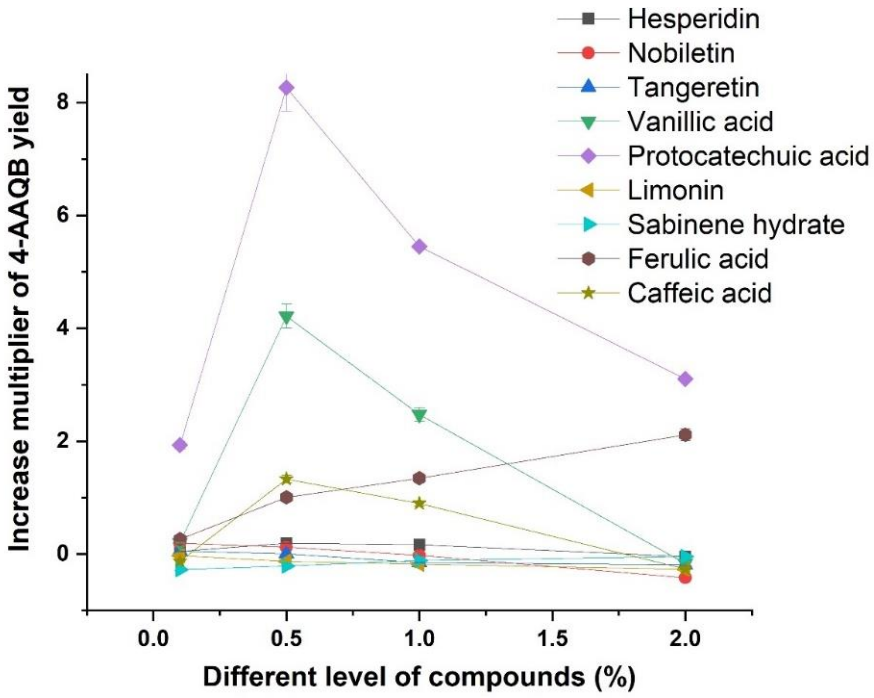

Fig. 6. Increase multiplier of 4-AAQB yield by *A. cinnamomea* with different level of different compounds

CONCLUSIONS

1. In this study, different types of Chinese herbs were processed into powders, aqueous extracts, and ethanolic extracts. They were added into the medium to investigate the effects on the cell growth and the production of 4-AAQB in the submerged cultivation of *A. cinnamomea*. It was indicated that burdock aqueous extract and tangerine peel aqueous extract were optimum to promote cell growth and 4-AAQB production, respectively. A combination strategy thus was applied, which led to the production of 4-AAQB increasing to 54.49 mg/L with 40 mL/L burdock aqueous extract and 30 mL/L tangerine peel aqueous extract added, which was approximately 33-fold higher than the control group without the addition of Chinese herbs.
2. Furthermore, the composition of the tangerine peel aqueous extract was analyzed. The major components were added to the fermentation medium. It was indicated that the addition of vanillic acid, protocatechuic acid, and their analogs facilitated the production of 4-AAQB, which were considered to play an important role in the synthesis of 4-AAQB.
3. The addition of Chinese herbs could remarkably promote cell growth and 4-AAQB production in the submerged cultivation of *A. cinnamomea*, which will provide new ideas for the cultivation of *A. cinnamomea* as well as other medicinal fungi. However, there are still some limitations in this study, such as lacking the experiments about amplified fermentation and different addition times of Chinese herbs, which should be explored in future work.

ACKNOWLEDGMENTS

This research was financially supported by National Key research program (2016YFD0400601, 2017YFB03069043), the Hong Kong, Macao, and Taiwan Scientific and Technological cooperation projects (2015DFT30050), and the National Natural Science Foundation of China (21978019，21978020).

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Article submitted: April 17, 2023; Peer review completed: June 17, 2023; Revised version received and accepted: June 23, 2023; Published: June 27, 2023.
DOI: 10.15376/biores.18.3.5368-5384

SUPPLEMENTARY MATERIAL

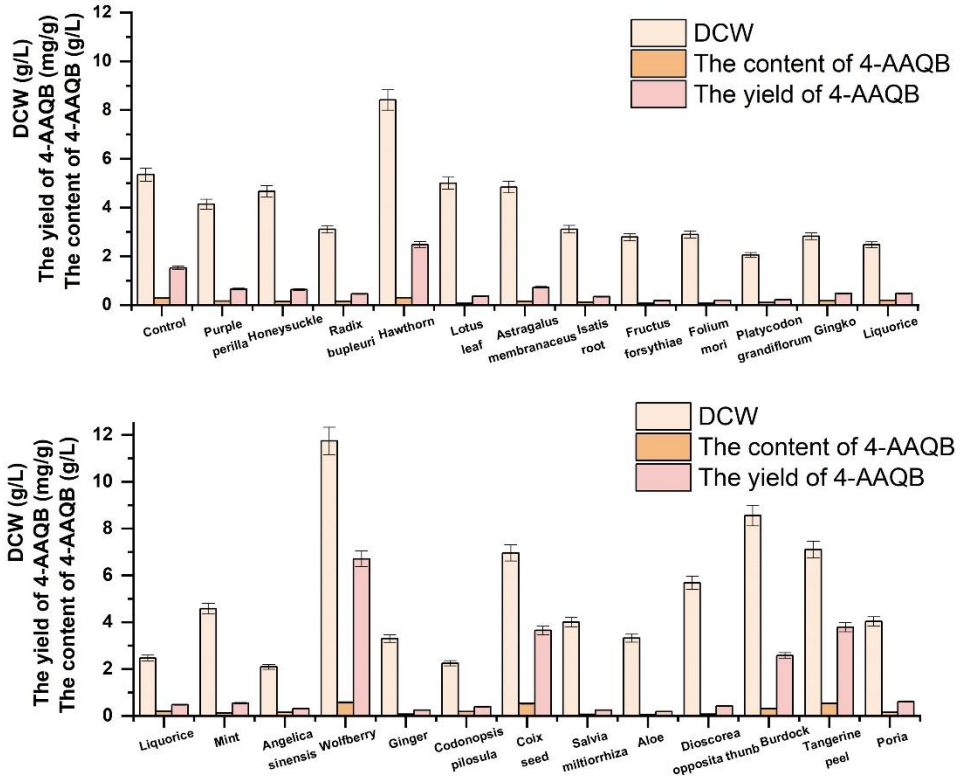

Fig. S1. Results of previous experiment-the effect of 25 kinds of Chinese medicines on the production of 4-AAQB by *A. cinnamomea*

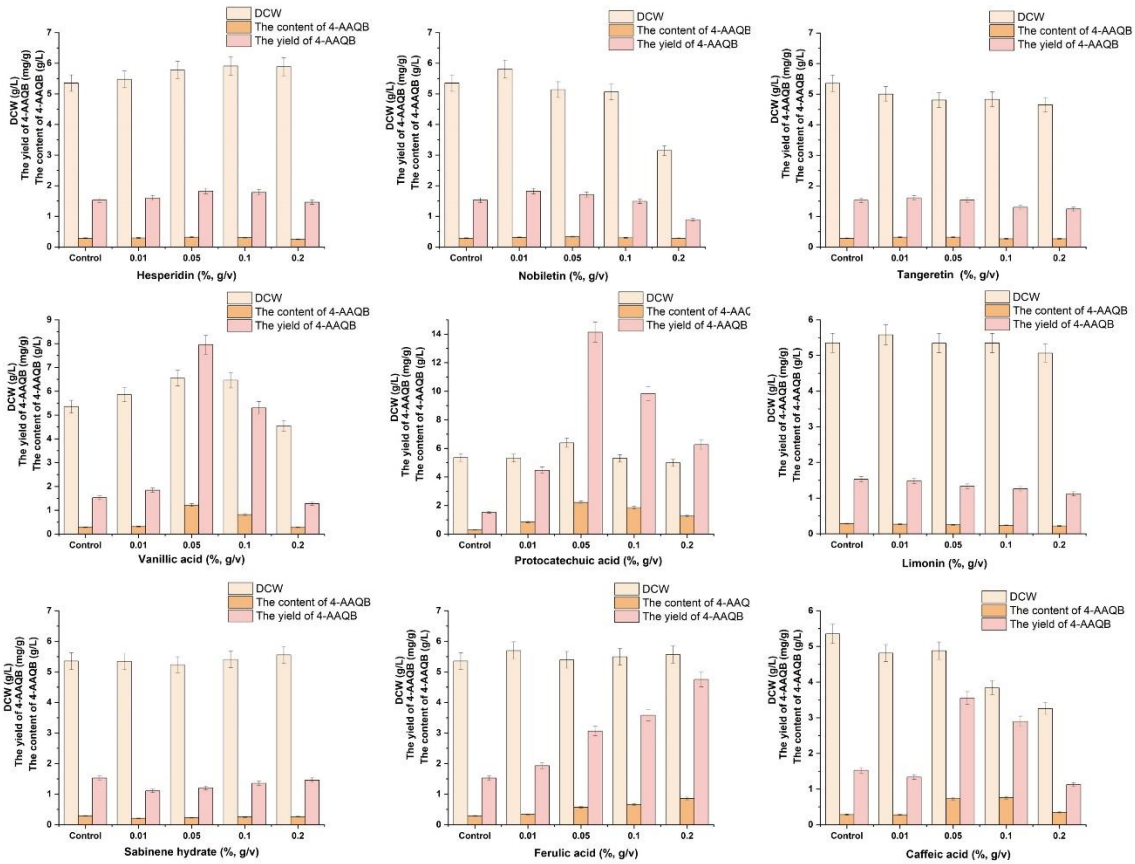

Fig. S2. The effect of different level of different compounds in tangerine peel aqueous extract on the production of 4-AAQB by *A. cinnamomea*