Foods 2021

MDPI

Proceeding Paper

Tyrosinase Inhibition Ability Provided by Hop Tannins: A Mechanistic Investigation †

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† Presented at the 2nd International Electronic Conference on Foods, 15–30 October 2021; Available online: https://foods2021.sciforum.net/.

Abstract: The hop is rich in tannins meanwhile used as a conventional additive in beer industry, but other application is limited. This study investigated the tyrosinase inhibition activity of extracted hop tannins and the associated structure-function activity. The tannins were extracted and subjected to a gel permeation chromatography (GPC), a nuclear magnetic resonance (NMR) and an acid-cleavage coupled HPLC-ESI-MS/MS analysis to obtain the structural information of the tannins. Then tyrosinase inhibition kinetic assays, inductively coupled plasma optical emission spectrometer and antioxidant (ICP-OES), circular dichroism (CD) as well as molecular docking analysis were applied to investigate the inhibition mechanism. Furthermore, the intracellular inhibition ability of hop tannins was assessed with B16-F10 cells. The results indicated that, hop tannins were composed of (epi)catechin as extensional units and (epi)gallocatechin as terminal units and can be classified as prodelphenidins. The tyrosinase inhibition assays showed the hop tannin was 6.4 times more effective than hydroquinone, meanwhile it inhibited the tyrosinase through competitive-noncompetitive mixed way. The tannins were found to bind on the surface of tyrosinase via forming hydrogen bonding and consequently changed the secondary structure of tyrosinase. The fluorescence and antioxidant assay indicated the tannin had both copper ion chelating and antioxidant ability which may also contribute to the inhibition. The intracellular inhibition analysis showed activity of tyrosinase were reduced by 66.67% and melanin production were found reduced by 34.50% while 10uM hop tannins were applied. These results indicated that, the hops are not only important in beer industry, the hop tannins can be also applied as whitening agents in cosmetic industry.

Keywords: hop tannins; tyrosinase; enzyme binding; enzyme inhibition; inhibition mechanism; condensed tannins; B16F10 melanoma cells

Citation: Liu, J.; Chen, Y.; Zhang, X.; Zheng, J.; Wang, J.; Hu, W.; Teng, B. Tyrosinase Inhibition Ability Provided by Hop Tannins: A Mechanistic Investigation. **2021**, *1*, x. https://doi.org/10.3390/xxxxx

Academic Editor(s):

Published: date

1. Introduction

Tyrosinase is a copper-containing enzyme that is involved in melanin hyperpigmentation in humans, molting in insects, and undesirable browning in fruits and vegetables [1]. At present, a variety of tyrosinase inhibitors have been developed and widely used in the market. But due to safety and stability factors, only a few inhibitors were used in commercial production [2].

Condensed tannins are classified to polyphenols which widely distributed in plant tissues [3]. The hop is rich in tannins meanwhile used as a conventional additive in beer industry, but other application is limited. In order to expand the industrial utilization of the hops, also provide a new tyrosinase inhibitor for cosmetic industry, the structure related tyrosinase inhibition mechanisms of the hop tannins were studied. The tannins were extracted and purified with a Sephadex LH-20 column, then structure of the hop tannins

were characterized through a gel permeation chromatography (GPC), a nuclear magnetic resonance (NMR) and an acid-cleavage coupled HPLC-ESI-MS/MS analysis. Then inhibition mechanism of the hop tannins was evaluated through tyrosinase inhibition kinetic assay, Circular Dichroism (CD) Spectroscopy, molecular docking (MD), antioxidant assay as well as an inductively coupled plasma-optical emission spectroscopy (ICP-OES). The tyrosinase inhibition ability was also observed on the B16 melanoma cell model.

2. Material and Methods

2.1. Preparation of Hop Tannins Extract

The method for extraction and purification of the hop tannins was the same as our previous report [4].

2.2. GPC

GPC was used to obtain the average molecular mass of the purified tannin according to our previous report and Kennedy's report [5,6].

2.3. ^{13}C-NMR Analysis

Purified hops powder (30 mg) was dissolved with 750μL CD_4O:D_2O (1:1, *v*/*v*), and loaded into a nuclear magnetic tube for ^{13}C-NMR analysis at a frequency of 100.60 MHz, acquisition time of 1.36 s.

2.4. Acid-Cleavage Coupled with HPLC-ESI-MS/MS Analysis

The acid-cleavage and reversed-phase HPLC-ESI-MS/MS analysis was applied, MS detection was done using a positive ionization in multiple-reaction monitoring (MRM) mode.

2.5. Tyrosinase Inhibition Kinetic Assay

The effects of the hops tannins on mushroom tyrosinase activity was assayed by a spectrophotometric method according to our previous report [4].

2.6. CD

The CD spectra were obtained on a MOS-450 AF-CD CD spectropolarimeter to investigate the influence of hop tannins on the secondary structure in mushroom tyrosinase [8].

2.7. MD

Enzyme-substrate molecule docking experiments were performed using AutoDock Vina software. AutoDock Vina was selected as docking method to perform the blind docking simulations within a grid box which was centred on the two copper ions of the active site All visualizations were analysed with PyMOL 2.2.

2.8. Antioxidant Ability

The antioxidant activity of the hops tannin was assessed through 3-ethylbenzthiazolin-6-sulfonic acid (ABTS) and 2,2-diphenyl-1-picrylhydrazyl (DPPH). The hop tannins against DPPH radical was analysed by the method described in Brand-Williams's report [10], while the ABTS radical was assessed as described by Re et al. [11].

2.9. ICP-OES

The chelating ability between Cu^{2+} and hops tannin was measured using ICP-OES as previously described [12].

2.10. Cell Culture and Cytotoxicity Assay

B16-F10 cells were grown in Dulbecco's modified eagle medium supplemented with 10% (*v*/*v*) heat-inactivated fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin and cultured in a cell incubator with humidified atmosphere of 37 °C and 5% CO_2. The medium was changed every 2 days. Cells were digested with 0.25% trypsin and subcultured when the cells were about 80% confluent. The cytotoxicity levels of hop tannins on melanoma B16F10 cells were determined according to the standard method of CCK-8 assay.

2.11. The Effect of Hop Tannins on Intracellular Melanin Production and Tyrosinase Activity of B16F10 Melanoma Cells

Melanin content assay was performed to evaluate the inhibitory effect of the hop tannins on melanogenesis according to method of Si [13]. The biochemical enzyme method used was as described by Si et al. [13] with some modifications. 2 mL of B16F10 mouse melanoma cells (1×10^6 cells) were seeded in a 6-well plate. After cell adherence, the hops tannins were added for 48 h. After that, the cells were washed thice with PBS, and then broken with 500 μL of PBS buffer (pH 6.8) containing 1% (*w*/*v*) Triton X-100.

2.12. Statistical Analysis

One-way analysis of variance (ANOVA) was performed to determine differences between the experimental samples (triplicated) using Minitab 18 (Minitab Inc., PA, USA). Tukey's post-hoc test was used to infer specific difference between the individual samples, different letters indicate a significant difference where $p < 0.05$.

3. Results and Discussions

3.1. Structural Analysis

Hop tannin structure was elucidated using ^{13}C NMR to obtain the composition of the structural moieties. Characteristic signals of (epi)catechin and (epi)gallocatechin were detected. Acid-cleavage analysis coupled HPLC-ESI-MS/MS results indicated hop tannins were composed of (epi)catechin as extensional units and (epi)gallocatechin as terminal units. GPC result showed the average molecular weight of hop tannins were 3581 g/mol.

3.2. Inhibitory Effect, Mechanism, and Type of Hop Tannins on the Tyrosinase

Figure 1A showed that catalytic activity of the tyrosinase were reduced while increasing hop tannin concentrations, implied the inhibition ability from hop tannins. The IC_{50} of hop tannins was determined as 6.03 ± 0.14 mg/mL (Table 1), which was about 6.4 times more effective than hydroquinone. The inhibition kinetic analysis showed a set of straight lines that all passed through the origin (Figure 1B), indicating the hop tannins inhibit the tyrosinase through a reversible inhibition. The inhibitory type was further evaluated through Lineweaver–Burk plot (Figure 1C), second quadrant intersected straight lines were observed. It implied hop tannins inhibit tyrosinase through competitive-noncompetitive mixed way. K_I (1.79 ± 0.12) showed smaller value than K_{IS} (2.61 ± 0.16), indicated that the affinity of the inhibitor for free enzyme was stronger than enzyme–substrate complex.

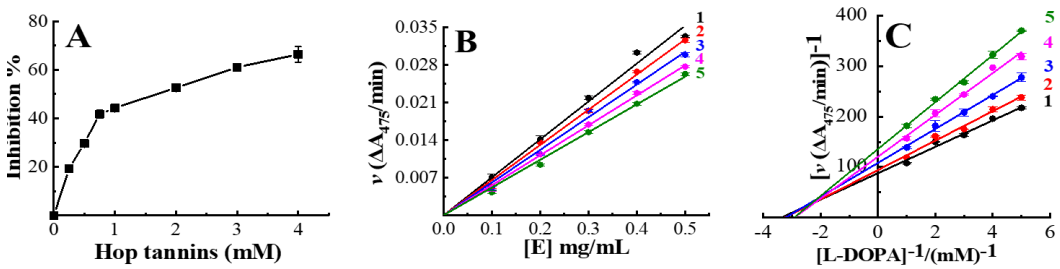

Figure 1. Inhibition effect (**A**), inhibitory mechanism (**B**) and inhibitory type (**C**) of hop tannins on tyrosinase. Concentrations of condensed tannins for curves 1–5 were 0, 0.3, 0.6, 0.9, and 1.5 μM, respectively.

Table 1. Tyrosinase inhibition activity of the hop tannins.

	IC_{50} (mg/mL)	Inhibition Mechanism	Inhibition Type	Inhibition Constants (mM)	
Hop tannin	6.03 ± 0.14 [a]	reversible	mixed	K_{IS} = 2.61 ± 0.16	K_I = 1.79 ± 0.12
Hydroquinone	38.88 ± 0.58 [b]	--	--	--	--

3.3. CD

Structural conformations are crucial for function and activity of tyrosinase. However, the secondary structure of tyrosinase were changed after interact with hop tannins (Figure 2). This conformation changes may lead by the non-selective binding of tannin and result in a decrease of tyrosinase catalytic activity [14].

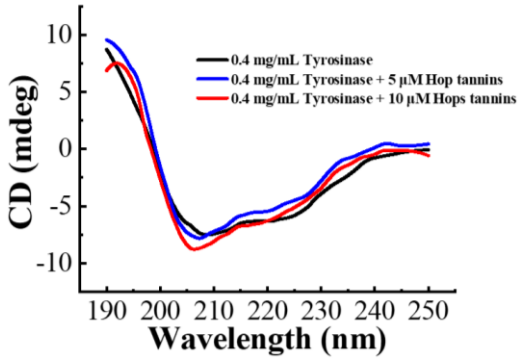

Figure 2. CD spectrum of tyrosinase before and after binding with hop tannins.

3.4. Copper Ion Chelating and Antioxidant Abilities

The copper ions are responsible for: (1) remaining the conformation of active cite of tyrosinase and; (2) participate in the catalyze reaction (redox reaction). In current study, the hop tannins not only showed antioxidant abilities but also found able to chelate with copper ions (Table 2). These results indicated hop tannins could either alter the conformation of tyrosinase by forming tannin-copper complexes, or hinder the redox reaction.

Table 2. Copper ion chelating and antioxidant abilities of hop tannins.

	Cu^{2+} Chelating (%)	DPPH Free Radical Scavenging Activity (IC_{50} mM)	ABTS Free Radical Scavenging Activity (IC_{50} mM)
Hops tannin	44.77 ± 0.45	0.051 ± 0.002 [b]	0.040 ± 0.000 [b]
Ascorbic acid	-	0.654 ± 0.010 [a]	0.730 ± 0.008 [a]

3.5. Molecular Docking Analysis

Molecular docking analysis suggested that, the structural units of hop tannins were observed embedding into the target active cavity of the tyrosinase and interacted with the surrounding amino acid residues (Figure 3). The results also found that the hydrogen bonds were mainly responsible for the tannin-tyrosinase binding.

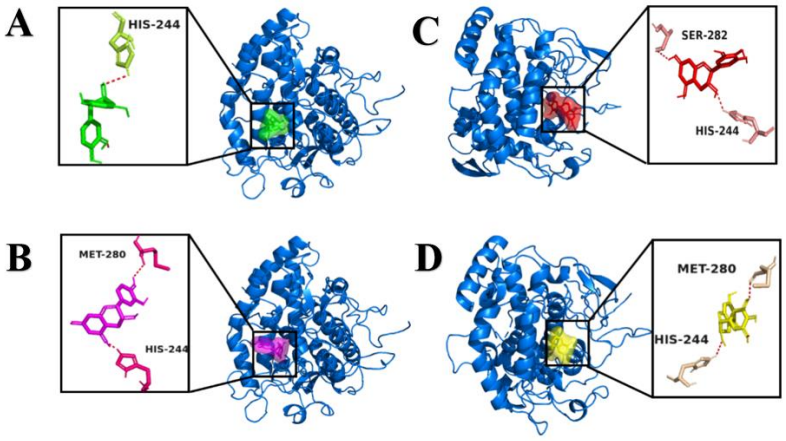

Figure 3. Docking poses of catechin–tyrosinase (**A**), epicatechin–tyrosinase (**B**), gallocatechin–tyrosinase (**C**), and epigallocatechin-tyrosinase (**D**). The dotted lines were hydrogen bond formed between tannin and tyrosinase.

3.6. Effect of Hop Tannin on Cell Viability, Melanin Synthesis, and Tyrosinase Activity in B16F10 Mouse Melanoma Cells

Within non-toxic dosages (0–10 μM, Figure 4A), melanin content and intracellular tyrosinase activity were observed reducing with increasing hop tannin concentrations. Especially when 10 μM hop tannins were applied, tyrosinase activity was reduced by 66.67% and melanin production were reduced by 34.50%. These findings demonstrated that hop tannins regulated tyrosinase and subsequently suppressed melanin synthesis in B16F10 mouse melanoma cells at nontoxic doses.

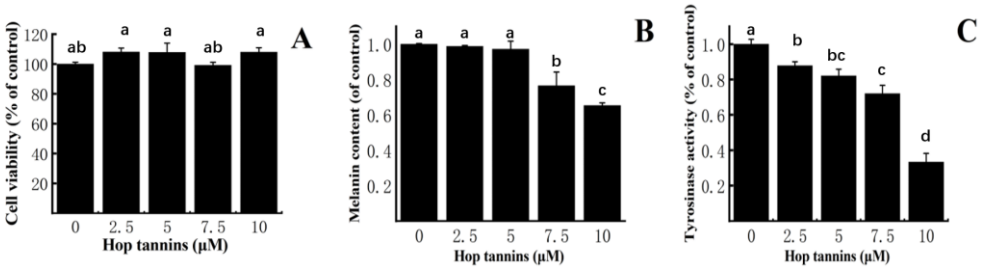

Figure 4. Effect of hop tannins on cell viability (**A**), melanin content (**B**), and tyrosinase activity (**C**) in B16F10 mouse melanoma cells.

4. Conclusions

Condensed tannins were extracted from hops and characterized as prodelphenidins ((epi)catechin as extensional units and (epi)gallocatechin as terminal units). The hop tannins showed outstanding tyrosinase inhibition ability (6.4 times better than hydroquinone) through competitive-noncompetitive mixed way. This inhibition ability can be attributed to: (1) changing the conformation of tyrosinase after binding process; (2) chelating with copper ions; (3) hinder the redox reaction of the copper ions; (4) blocking the active sites through forming hydrogen bonds with amino acid residues. On the aspect of melanoma cell, the hop tannins were also found able to regulate tyrosinase and subsequently suppressed melanin synthesis.

Author Contributions: Conceptualization, B.T. and Y.C.; methodology, B.T.; formal analysis, J.L., X.Z., J.Z. and J.W.; investigation, B.T.; resources, W.H.; writing—original draft preparation, B.T., J.L.; writing—review and editing, B.T.; supervision, B.T.; project administration, Y.C., B.T.; funding acquisition, Y.C. All authors have read and agreed to the published version of the manuscript.

Funding: This research was financially supported by Natural Science Foundation of Guangdong Province, China (2020A1515011357); the Department of Education of Guangdong Province, China, (2018KQNCX081) and (2018KCXTD012); the Key Program of Marine Economy Development (Six Marine Industries) Special Foundation of Department of Natural Resources of Guangdong Province (GDNRC [2020]076); and the Research and Development Start-up Foundation (NTF18031). 2020 Li Ka Shing Foundation Cross-Disciplinary Research Grant (2020LKSFG02E).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement:

Acknowledgments: Stella Kassara and Jacqui McRae, and Keren Bindon are thanked for their help and support during the experimental work and analysis.

Conflicts of Interest: The authors declare no conflict of interest.

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