Discovery and identification of potential anti-melanogenic active constituents of *Bletilla striata* by zebrafish model and molecular docking

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Research Article

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Abstract

Background

Bletilla striata was the main medicine of many skin whitening classic formulas in traditional Chinese medicine (TCM) and was widely used in cosmetic industry recently. However, its active ingredient was still unclear and its fibrous roots were not used effectively. The aim of the present study was to discover and identify its potential anti-melanogenic active constituents by zebrafish model and molecular docking.

Methods

The antioxidant activities were evaluated by 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity, 2,2'-azino-bis-(3-ethylbenthiazoline-6-sulphonic acid) (ABTS) radical scavenging activity and ferric reducing antioxidant power (FRAP) assay. The anti-melanogenic activity was assessed by tyrosinase inhibitory activity *in vitro* and melanin inhibitory in zebrafish. The chemical profiles were performed by ultra-high-performance liquid chromatography combined with quadrupole time-of-flight tandem mass spectrometry (UPLC-Q-TOF-MS/MS). Meanwhile, the potential anti-melanogenic active constituents were temporary identified by molecular docking.

Results

The 95% ethanol extract of *B. striata* fibrous roots (EFB) possesses the strongest DPPH, ABTS, FRAP and tyrosinase inhibition activity, with IC_{50} 5.94 mg/L, 11.69 mg/L, 6.92 mmol $FeSO_4$ /g, and 58.92 mg/L, respectively. In addition, EFB and 95% ethanol extract of *B. striata* tuber (ETB) can significantly reduce the melanin synthesis of zebrafish embryos in dose-dependent manner. 39 chemical compositions, including 24 stilbenoids were tentatively identified from EFB and ETB. Molecular docking indicated that there were 83 (including 60 stilbenoids) and 85 (including 70 stilbenoids) compounds exhibit stronger binding affinities toward tyrosinase and adenylate cyclase.

Conclusion

The present findings supported the rationale for the use of EFB and ETB as natural skin-whitening agents in pharmaceutical and cosmetic industries.

Background

Bletilla striata (Thunb.) Reichb. f. is a herbaceous perennial plant widely distributed in Asia, such as Korea, China, and Japan [1]. The dried tuber of *B. striata*, also known as Baiji, firstly recorded in Shennong's Classic of Materia Medica (Simplified Chinese: 白芷), has been widely used as a traditional Chinese medicine (TCM) for thousands of years in China. Chinese pharmacopeias states that it possesses the capability of astringency upon hemostasis and analgesis, therefore, it was widely used for the treatment of hematemesis,

hemoptysis, traumatic bleeding, ulcers, swelling and chapped skin [2, 3]. Pharmacological studies demonstrated that *B. striata* has a wide spectrum of biological activities, such as wound healing [4, 5], anti-ulcer [6, 7], hemostatic [8], anti-inflammation [9], antioxidant [10], antibacterial [11], anti-influenza viral [12], and antiaging [13]. At the same time, *B. striata* contains various classes of chemical compositions, including polysaccharides [14], bibenzyls, phenanthrenes, anthraquinones, flavonoids, and 2-isobutylmalates [3, 15], etc.

B. striata was the main medicine of many skin whitening classic formulas in TCM [16] and it was widely used in cosmetic industry recently [17]. However, its active ingredients were still unclear and even some research results were contrary. For instance, the research results given by Chen et al indicated that 95% ethanol extract of *B. striata* possessed higher anti-tyrosinase activity than those of water extract with inhibition rate of 68.36% *in vitro* [18], while the result of Huang et al showed that the inhibitory activity of *B. striata* water extract on tyrosinase was stronger than that of 95% ethanol extract with inhibition rate of 62% *in vitro* [19]. The research results by Lu et al [20] and Linghu et al [21] demonstrated that both water and 95% ethanol extract of *B. striata*, especially chloroform fractionation, can inhibit B16 cell growth and induce its apoptosis in a concentration-dependent manner. As *in vitro* experiments does not reflect the real and complex metabolic conditions in living organisms, many of the samples showed significant inhibitory activity against tyrosinase and melanoma cell lines *in vitro*, nevertheless, exhibited weakly effective or even ineffective *in vivo* [22].

Meanwhile, *B. striata* fibrous roots (FB) were the by-products generated during the processing of *B. striata* tuber (TB). Modern research results indicated that the FB contains similar compounds with TB, in addition, has higher phenolic content [23], and antibacterial [24], antioxidant and anti-tyrosinase activities [23]. However, the resources of FB were not effectively used and were abandoned in the farmland, which lead to the waste of resources and environmental pollution [25].

Zebrafish (*Danio rerio*), a small tropical freshwater fish, is an emerging animal model for pharmacology and toxicology research *in vivo*, with many advantages including low cost, short life cycle, high transparency, easy to maintain, high fertility, and fewer test samples required [26, 27]. In addition, zebrafish has melanin pigments on the surface, allowing simple observation of the pigmentation process, and was widely used in the anti-melanogenic study [26, 28].

In the present study, we compared the antioxidant and tyrosinase inhibitory activities of crude polysaccharide and 95% ethanol extract of TB and FB *in vitro*, and anti-melanogenic activities in zebrafish model *in vivo*. Meanwhile, the potential anti-melanogenic active constituents were temporary identified by molecular docking. We discovered that 95% ethanol extract of TB (ETB) and FB (EFB) possesses the significantly antioxidant and anti-tyrosinase activities, and can reduce melanin synthesis of zebrafish embryos in dose-dependent manner. Thus, ETB and EFB can be used as natural skin-whitening agents in pharmaceutical and cosmetic industries.

Methods

Chemicals and reagents

Tyrosinase, 3-(3,4-Dihydroxyphenyl)-L-alanine (L-DOPA) and arbutin were purchased from Aladdin reagent company (Shanghai, China). 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino-bis-(3-ethylbenthiazoline-6-sulphonic acid) (ABTS), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), and 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile and methanol were of HPLC grade and purchased from Tedia (Fairfield, OH, USA). Deionized water was prepared with the Milli-Q water system (18.2 MΩ, Millipore, USA).

Plant collection and extraction procedure

B. striata were collected from Quzhou YiNianTang Agriculture and Forestry Technology Co., Ltd, Quzhou, Zhejiang, in China. Voucher specimens were deposited at Herbarium of Zhejiang Pharmaceutical College (Accession no. 190615). The whole plant was divided into tubers and fibrous roots. Subsequently, they were cut into small pieces and dried by vacuum freeze-drying, respectively.

The dried and powdered samples (TB and FB) were reflux extracted with 95% ethanol for three times (1.5 h for each time). The extract was filtered using Whatman filter paper (No.1) and the filtrate was concentrated to dryness under vacuum in a rotary evaporator (Hei-VAP, Heidolph, Germany) at 50 °C and vacuum freeze dryer. The ethanol extraction of TB (ETB) and FB (EFB) yield were 5.21% and 6.53%, respectively. The dregs were used to extract the crude polysaccharide by dispersed in 80 °C water for 4 h and precipitation with ethanol [29]. The polysaccharides yield of TB (PTB) and FB (PFB) were 14.75% and 6.45%, respectively.

Chemical analysis

The chemical analysis was performed on an ultra-high-performance liquid chromatography combined with quadrupole time-of-flight tandem mass spectrometry (UPLC-Q-TOF-MS/MS). The chromatographic separation was performed using a Waters Acquity UPLC™ system (Waters Corp., Milford, MA, US) on a Waters BEH Shield RP C18 column (100×2.1 mm, 1.7 μm) at 30 °C. The mobile phase was consisted of 0.1% formic acid in water (A) and acetonitrile (B), with a linear gradient: 0-3 min, 5-16% B; 3-8 min, 16-30% B; 8-10 min, 30-35% B; 10-15 min, 35-55% B; 15-18 min, 55-80% B; 18-19 min, 80-5% B; 19-20 min, 5% B. The flow rate was 0.3 mL/min, and injection volume was 2 μL.

The TOF-MS/MS experiments were performed by using an AB SCIEX Triple TOF 5600 mass spectrometry (AB SCIEX, Foster City, CA, USA) equipped with an electrospray ionization (ESI). The experiment was performed using MS detection conducted in negative ionization method. The parameters were set as follows: source and desolvation temperature were 100 °C and 450 °C respectively; desolvation gas flow rate was 900 L/h; capillary voltage was 2 kV; cone voltage was 40 V; collision energy was 22 eV; and the full scan spectra was from 100 to 2000 Da.

Antioxidant assays

DPPH radical scavenging activity

The DPPH radical scavenging activity was determined in accordance with Ali et al with slightly modifications [30]. Briefly, 50 μL test sample solution was mixed with 150 μL DPPH ethanol solution (0.2

mM) in 96-well plates and kept in the dark for 30 min. The absorbance of the mixture at 517 nm (A_1) was assessed by microplate spectrophotometer (Thermo Fisher Scientific, America). The blank solution without test sample mixed with DPPH ethanol solution was set as the positive group (A_0). The blank solution without DPPH mixed with test sample solution was set as the blank group (A_2). All experiments were executed in triplicate. The DPPH radical scavenging rate was calculated using the following formula:

$$\text{DPPH radical scavenging rate (\%)} = [A_0 - (A_1 - A_2)] / A_0 \times 100\%$$

ABTS radical scavenging activity

The scavenging capacity to ABTS radical was measured using the method described by Ali et al [30]. Briefly, 7 mM ABST aqueous solution was mixed with 2.45 mM potassium persulfate at a ratio of 1:1 (v/v), and incubating at room temperature in the dark for 16 h to obtain the ABST⁺ stock solution. The stock solution was diluted with phosphate buffered saline (PBS) to adjust the absorbance of (0.72 ± 0.2) at 734 nm. 20 μ L test sample at different concentrations was mixed thoroughly with 180 μ L ABTS⁺ solution. The reactive mixtures were kept in the dark for 5 min and subsequently measured the absorbance (B_1) at 734 nm. The absorbance of the mixtures without test sample and ABST⁺ were set as B_0 and B_2 , respectively. All experiments were executed in triplicate. The scavenging rate to ABTS radical was calculated according to the following formula:

$$\text{ABTS radical scavenging rate (\%)} = [B_0 - (B_1 - B_2)] / B_0 \times 100\%$$

Ferric reducing antioxidant power assay (FRAP)

The ferric iron reducing activity was determined according the procedures by Kosakowska et al [31]. 300 mM acetate buffer, 10 mM TPTZ (2,4,6-tris(2-pyridyl)-s-triazine) and 20 mM FeCl₃ were mixed at a (v/v/v) ratio of 10:1:1 to prepared the working reagent. 100 μ L of each test sample solution was mixed with 100 μ L TPTZ working reagent, incubated at 37°C for 20 min and the absorbance was determined at 593 nm. Meanwhile, a series of FeSO₄ standard solutions in the concentration ranges of 0–1000 μ g/mL were used to prepare the calibration curve. The ferric reducing capacity was calculated in the formation of an intense Fe²⁺-TPTZ blue complex. The results were expressed as Fe²⁺ antioxidant capacity (mmol FeSO₄/g of extract).

Tyrosinase inhibitory activity

Tyrosinase inhibitory activities were determined by spectrophotometric method using L-DOPA as substrate [32]. Briefly, 50 μ L sample solution of different concentrations was mixed with 50 μ L tyrosinase (200 units/mL, dissolved in pH 6.8 phosphate buffer) in 96-well plates and incubated for 15 min at 25 °C. The reaction was then initiated with the addition of L-DOPA (50 μ L). After incubating of 30 min at 25°C, the absorbance at 490 nm (A_a) was determined using a multiskan sky microplate spectrophotometer (Thermo Fisher Scientific, America). Similarly, the absorbance of the sample wells without tyrosinase (A_b) and the

control wells with enzyme but without sample (A_b) were detected at the same time. The tyrosinase inhibitory activity was calculated by equation below:

$$\text{Tyrosinase inhibitory rate (\%)} = [1 - (A_a - A_b)/A_c] \times 100\%$$

Melanin inhibitory in zebrafish

Wild-type AB line zebrafish (provided by Hunter Biotechnology, Inc., Hangzhou, Zhejiang Province) were housed in fish water (0.2% instant ocean salt in deionized water, pH 6.9-7.2, conductivity 480-510 mS/cm, and hardness 53.7-71.6 mg/L CaCO_3) in a 14/10 h light/dark photoperiod at a constant temperature 28 ± 0.5 °C, and fed live brine shrimp twice daily. The zebrafish embryos were obtained from natural spawning and collected within 30 min. The zebrafish assay was accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). The present study was approved by the IACUC (Institutional Animal Care and Use Committee) at Hunter Biotechnology, Inc. and the IACUC approval number was 001458.

Embryos at the 6 h post fertilization (hpf) stage were treated each extract at six concentrations (10, 30, 62.5, 125, 250 and 500 mg/L) for 72 h to evaluating the maximum non-lethal concentration (MNLC). As a result, the MNLCs of all the extract were more than 62.50 mg/L. 10 embryos were placed in 6-well and were exposed to tested samples at concentrations of 10 and 30 mg/L from 6 h post fertilization (hpf) to 54 hpf (48 h exposure). DMSO (0.05%, v/v) and arbutin (10 and 30 mg/L) were used as the normal and positive control.

Synchronized embryos were collected and arrayed by pipette were observed under a stereomicroscope (SZX7, Olympus, Japan) equipped with a digital camera (VertA1, China). Melanin accumulation, directly correlated to the zebrafish pigmentation areas, was calculated using the GNU Image Manipulation Program (GIMP version 2.10.2) and expressed as percentage with respect to the negative control (melanin accumulation 100%) [26, 28].

Molecular docking study

158 compounds isolated from *B. striata* in the literature [2], including 19 glycosides, 28 bibenzyls, 19 phenanthrenes, 18 biphenanthrenes, 23 dihydrophenanthrenes, 5 anthocyanins, 11 steroids, 8 triterpenoids, 12 phenolic acids, 5 quinones, and 10 other compounds, were used as the ligands. The 3D structures were drawn by ChemBio3D and were optimized using MM2 and Autodock Tools. The 3D crystal structure of tyrosinase (PDB ID:5M8N) and adenylate cyclase (PDB ID:5IV3) were retrieved from RCSB Protein Data Bank (www.rcsb.org/pdb/home/home.do). All water molecules and hetero atoms were removed from the crystal structures by using Chimera and MGL Tools. Finally, Autodock Vina was used to dock the receptor protein with the small molecule ligands. Parameters of the receptor protein docking site were set -36.700, 7.034, -19.104, and -17.467, -22.807, 2.786, respectively, according to the original ligand mimosine and LRE1 [33].

Results

Chemical composition

The typical base peak chromatogram chromatograms of ETB and EFB were shown in Fig. 1. The PeakView™ softwares were used to identify the constituents. The molecular formula was accurately assigned within mass error of 10 ppm. The exact molecular weight and the fragment ions were used to identify the components according the literatures and the free chemical structure database, such as ChemSpider and Massbank. As a result, 39 chemical compositions, including 24 stilbenoids (bibenzyls, phenanthrenes and their derivatives), 6 glycosides, 4 phenolic acids, 3 quinones, 1 steroid, and 1 other compound were tentatively identified from EFB and ETB. At the same time, their relative semi-quantification was performed by measuring peak areas of each compound in MS mode using the extracted ion chromatograms [34]. The detailed information of the identification and their relative contents (heat map highlights, the darker the color, the higher the contentration) were summarized in Table 1.

Table 1 The compounds identified from the 95% ethanol extracts from *B. striata* tubers and fibrous roots by UPLC-Q-TOF-MS/MS, and their relative peak areas.

NO.	T _R (min)	Formula	Found Mass	Error (ppm)	Identification	Relative peak areas (×10 ⁶)	
						ETB	EFB
1	2.35	C ₁₃ H ₁₈ O ₇	285.0977	-0.8	gastrodin	2.17	1.43
2	4.53	C ₇ H ₆ O ₃	137.0248	3.1	<i>p</i> -hydroxybenzoic acid	0.00	0.77
3	6.70	C ₉ H ₈ O ₃	163.0406	3.2	3-hydroxycinnamic acid	0.00	0.43
4	7.01	C ₃₄ H ₃₂ O ₈	567.2028	0.6	bleochranol D	0.00	2.80
5	7.71	C ₄₀ H ₅₆ O ₂₂	887.3179	-1.3	dactylorhin A	3.93	3.06
6	8.13	C ₂₁ H ₂₂ O ₈	401.1228	-3.6	2,7-dihydroxy-4-methoxyphenanthrene-2-O-glucoside	0.00	0.00
7	8.21	C ₂₇ H ₃₂ O ₁₃	563.1747	-4.1	2,7-dihydroxy-4-methoxyphenanthrene-2,7-O-diglucoside	0.00	1.18
8	9.62	C ₃₄ H ₄₆ O ₁₇	725.2635	-3.8	militarine	30.48	38.39
9	9.76	C ₂₀ H ₂₂ O ₆	357.1332	-3.3	pinoresinol	0.00	0.00
10	10.31	C ₂₁ H ₂₆ O ₈	405.1537	-4.5	3'-hydroxy-5-methoxybibenzyl-3-O-β-D-glucopyranoside	0.72	1.56
11	11.08	C ₁₆ H ₁₂ O ₅	283.0601	-3.8	1,8-dihydroxy-3-methoxy-6-methylantracene-9,10-dione	0.00	1.53
12	12.15	C ₁₅ H ₁₃ O ₄	256.0734	-3.0	7-hydroxy-2-methoxyphenanthrene-3,4-dione	0.00	0.00
13	12.42	C ₁₅ H ₁₂ O ₃	239.0714	0.3	4-methoxyphenanthrene-2,7-diol	0.00	1.92
14	12.99	C ₁₆ H ₁₄ O ₄	269.0820	0.2	2,7-dihydroxy-3,4-dimethoxyphenanthrene	0.00	6.01
15	13.54	C ₅₁ H ₆₄ O ₂₄	1059.3674	-3.8	gymnoside X	1.07	0.89
16	13.64	C ₂₈ H ₂₆ O ₅	441.1691	-3.7	shanciguol	0.00	1.51
17	13.80	C ₁₆ H ₁₈ O ₄	273.1126	-2.2	3,3'-dihydroxy-5,4'-dimethoxybibenzyl	0.00	0.00
18	13.88	C ₁₅ H ₁₆ O ₃	243.1026	-0.4	3,3'-dihydroxy-5-methoxybibenzyl	0.00	1.80
19	14.07	C ₂₂ H ₂₀ O ₄	347.1278	-3.2	4,7-dihydroxy-1-(<i>p</i> -hydroxybenzyl)-2-methoxy-	0.32	0.00

9,10-dihydrophenanthrene								
20	14.26	C ₂₉ H ₂₄ O ₅	451.1507	-9.8	1,8- <i>bis</i> (<i>p</i> -hydroxybenzyl)-4-methoxyphenanthrene-2,7-diol	0.00	8.73	
21	14.31	C ₂₂ H ₁₈ O ₄	345.1124	-2.4	1-(<i>p</i> -hydroxybenzyl)-4-methoxyphenanthrene-2,7-diol	0.25	0.00	
22	14.50	C ₂₅ H ₂₄ O ₆	419.1480	-4.8	bleochranol B	0.21	1.89	
23	14.67	C ₂₃ H ₂₀ O ₅	375.1225	-3.6	bleformin B	0.00	5.08	
24	14.71	C ₃₀ H ₂₆ O ₆	481.1631	-5.3	blestrin A	0.79	5.08	
25	14.89	C ₂₂ H ₂₂ O ₄	349.1434	-3.1	3,3'-dihydroxy-4-(<i>p</i> -hydroxybenzyl)-5-methoxybibenzyl	0.00	0.00	
26	14.94	C ₃₀ H ₂₄ O ₆	479.1478	-4.6	blestrin D	0.46	1.91	
27	15.53	C ₃₂ H ₂₆ O ₈	537.1529	-4.9	4,8,4',8'-tetramethoxy-[1,1'-biphenanthrene]-2,7,2',7'-tetrol	0.01	9.15	
28	15.74	C ₃₇ H ₃₂ O ₇	587.2047	-4.9	bleformin D	0.00	9.63	
29	16.08	C ₁₅ H ₁₄ O ₃	241.0869	-0.7	2,4-dimethoxyphenanthrene-7-ol	0.13	1.48	
30	16.49	C ₂₇ H ₂₆ O ₇	461.1584	-4.7	pleionesin C	0.04	0.36	
31	16.61	C ₁₆ H ₁₄ O ₃	253.0862	-3.1	2-hydroxy-4,7-dimethoxyphenanthrene	0.01	1.42	
32	16.89	C ₁₈ H ₁₈ O ₄	297.1126	-2.1	2,3,4,7-tetramethoxyphenanthrene	0.01	2.28	
33	16.94	C ₂₉ H ₂₆ O ₅	453.1671	-8.2	2,7-dihydroxy-1,6-bis(<i>p</i> -hydroxybenzyl)-4-methoxy-9,10-dihydrophenanthrene	0.10	0.64	
34	17.46	C ₂₃ H ₂₄ O ₄	363.1585	-4.7	bulbocol	0.15	3.85	
35	17.67	C ₂₂ H ₂₂ O ₃	333.1486	-3.2	5-hydroxy-2-(<i>p</i> -hydroxybenzyl)-3-methoxybibenzyl	0.60	3.34	
36	18.27	C ₂₇ H ₃₅ O ₉	502.2211	0.5	(20 <i>S</i> ,22 <i>R</i>)-1 β ,2 β ,3 β ,4 β ,5 β ,7 α -hexahydroxyspirost-25(27)-en-6-one	9.14	1.06	
37	18.30	C ₁₈ H ₃₀ O ₃	293.2117	-1.7	striatolide	0.00	2.49	
38	18.78	C ₁₆ H ₃₂ O ₂	255.2327	-1.0	palmitic acid	0.00	0.95	
39	18.87		522.1329	1.7	3',7',7-trihydroxy-2,2',4'-	7.20	0.76	

	C ₃₁ H ₂₃ O ₈	trimethoxy-[1,8'-biphenanthrene]-3,4-dione	
Total			57.79 123.37

Antioxidant capacity

It is well known that Ultraviolet A (UVA)-irradiation can induce reactive oxygen species (ROS) production and mediate excessive melanogenesis in skin cells. Natural polyphenols was the inhibitors of ROS generation and could be responsible for the anti-melanogenic activity of plant extracts [35, 36]. Thus, in the present study, the antioxidant capacity was assessed through 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity, 2,2'-azino-bis-(3-ethylbenthiazoline-6-sulphonic acid) (ABTS) radical scavenging activity and ferric reducing antioxidant power (FRAP) assay.

The results (Table 2) showed that the EFB (IC₅₀=5.94 mg/L) possesses the strongest DPPH radical scavenging activity *in vitro* compared to the PTB (IC₅₀=548.24 mg/L), PFB (IC₅₀=285.81 mg/L), and ETB (IC₅₀=65.25 mg/L). A similar trend was observed in ABTS and FRAP assay. The 95% ethanolic extracts of TB and PB had stronger antioxidation activity *in vitro* than their crude polysaccharides. In addition, EFB exhibit stronger antioxidation activity than ETB, which was consistent with the previous research results [23].

Table 2 The results of antioxidant activity analyses *in vitro* (DPPH, ABTS and FRAP).

Sample	IC ₅₀ values (mg/L)		FRAP
	DPPH scavenging	ABTS scavenging	mmol FeSO ₄ /g
PTB	548.24±8.93 ^a	626.49±9.75 ^a	0.11±0.01 ^a
PFB	285.81±5.31 ^b	348.62±6.30 ^b	0.42±0.03 ^b
ETB	65.25±1.85 ^c	78.40±2.51 ^c	2.45±0.05 ^c
EFB	5.94±0.46 ^d	11.69±0.64 ^d	6.92±0.10 ^d
Trolox	3.52±0.27 ^e	3.68±0.31 ^e	-

Means marked in columns with different letters differ at $p < 0.05$.

Tyrosinase inhibitory activity

Tyrosinase was the key rate-limiting enzyme in melanin biosynthesis pathway and was widely used to identify the potential of natural products with anti-melanogenesis effect [37]. The results (Fig. 2) showed that ETB and EPB had stronger tyrosinase inhibitory activity *in vitro* than PTB and PPB, which was consistent with previous research results [23]. Meanwhile, the IC₅₀ was calculated using an IC₅₀ Calculator (<https://www.aatbio.com/tools/ic50-calculator>). EFB exhibit stronger tyrosinase inhibition activity in a dose

dependent manner with $IC_{50}=58.92$ mg/L than ETB ($IC_{50}=75.44$ mg/L) and the positive compound arbutin ($IC_{50}=66.02$ mg/L).

Melanin inhibitory in zebrafish

Zebrafish are recognized as a highly advantageous vertebrate model system to evaluate anti-melanogenesis activity, as it possesses similar organ systems and gene sequences to human beings [26]. Therefore, zebrafish was used for the anti-melanogenesis activity. As shown in Fig. 3, a large number of melanin (black spots) deposited in the zebrafish embryo in the control and 0.05% DMSO group. The microscopy images showed there was no significant difference in total melanin content between two groups ($p > 0.05$), indicating that the vehicle 0.05% DMSO didn't affect the melanin production in zebrafish embryos. However, after exposure to the tested samples and arbutin for 48 h, zebrafish embryos exhibited varying degrees of melanin deposition (Fig. 2B, 3), excepting PFB 10 mg/L group. It is noticeable that the relative melanin contents in ETB 10 mg/L (78.38%) and 30 mg/L (64.72%) groups were significant lower than that in PTB 10 mg/L (93.06%) and 30 mg/L (82.02%) groups ($p < 0.01$). It demonstrated that the anti-melanogenesis activity of ETB was superior to that of PTB, which was consistent with the tyrosinase inhibitory activity. Whereafter, at the same time, anti-melanogenesis activity of ETB (81.65%, 75.61%) and EFB (78.38%, 64.72%) were stronger than that of arbutin (93.57%, 81.48%) at same dose of 10 mg/L and 30 mg/L ($p < 0.01$ or 0.05).

Molecular docking

Tyrosinase is the rate-limiting enzyme in melanin biosynthesis: hydroxylation of tyrosine to 3,4-dihydroxyphenylalanine (DOPA), and oxidation of DOPA to dopaquinone. Therefore, tyrosinase has been considered a critical target for the development of melanogenesis inhibitors [38]. Additionally, adenylate cyclase is key enzyme of cAMP-induced melanogenesis. The binding of melanotropin alpha polypeptide (α -MSH) to MC1R receptors induced the activation of adenylate cyclase, increase in the cAMP level, up-regulated expression of tyrosinase gene, and subsequently increase the melanin synthesis [39]. Therefore, we performed a molecular docking study of the 158 compounds isolated from *B. striata* previously [2], with tyrosinase and adenylate cyclase. The binding energies of the studied ligands with tyrosinase and adenylate cyclase were summarized in Table S1. There were 83 (including 60 stilbenoids) and 85 (including 70 stilbenoids) compounds exhibit stronger binding affinities toward tyrosinase and adenylate cyclase, comparing to the binding affinity of the original ligand mimosine (-6.4 kcal/mol) and LRE1 (-8.5 kcal/mol). 1,8-bis(p-hydroxybenzyl)-4-methoxyphenanthrene-2,7-diol (**56**), blestrin D (**75**), and 2,7-dihydroxy-1,6-bis(p-hydroxybenzyl)-4-methoxy-9,10-dihydrophenanthrene (**93**) were the top three ligands toward tyrosinase, with binding energy -10.2, 10.0, and 9.7 kcal/mol, respectively. While blestrin D (**75**), blestrin B (**73**), and 3,3'-dihydroxy-5-methoxy-2,5',6-tris(p-hydroxybenzyl) bibenzyl (**42**) were the top three ligands toward adenylate cyclase, with binding energy -12.1, -11.9, and -11.6 kcal/mol, respectively. Their theoretical binding affinities and ligand-amino acid interactions were summarized in Table 3.

Table 3 Summary of binding affinities and ligand-amino acid interactions.

Protease	Ligand	Binding Energy (kcal/mol)	No. of H-Bond	Ligand-amino acid interactions
Tyrosinase	1,8-bis(p-hydroxybenzyl)-4-methoxyphenanthrene-2,7-diol	-10.2	0	Lys233, Leu229, Arg114, Glu451, Arg230, Pro115, Gly107, Pro445, Met452, Tyr226, Ser106, Asn459
	blestrin D	-10.0	2	Pro446, Arg114, Pro445, Ser106, Gly107, Cys113, Lys233, Arg230, Pro115, Tyr226, Leu229, Met452, Glu451
	2,7-dihydroxy-1,6-bis(p-hydroxybenzyl)-4-methoxy-9,10dihydrophenanthrene	-9.7	6	Glu232, Leu229, Ile128, Pro115, Tyr226, Glu451, Val447, Gly107, Thr112, Ser106, Cys113, Lys223, Gln236
Adenylate cyclase	blestrin D	-12.1	1	Val167, Leu166, Lys95, Phe45, Ala97, Asn412, Arg416, Ala415, Val172, Phe336, Phe338, Arg176, Leu102, Met337
	blestrin B	-11.9	3	Met419, Phe338, Phe45, Lys95, Met337, Leu166, Phe165, Phe336, Leu102, Val167, Ala97, Phe296, Ala415, Arg416
	3,3',5-trimethoxybibenzyl	-11.6	2	Asp47, Ala100, Leu345, Ala97, Phe336, Phe296, Met419, Ala415, Met418, Lys95, Leu166, Val335, Phe165, Leu102, Phe45, Phe338, Asn180, Arg176, Gln179

It was worth noting that blestrin D (**75**), a biphenanthrenes compound, insided binding pocket of tyrosinase and adenylate cyclase (Fig. 4) and exhibited significant binding affinities toward both tyrosinase and adenylate cyclase. The Van der Waals interactions contributes to overall energy interaction value, meanwhile hydroxyl group produces hydrogen bonds with amino acid residues Glu 451, Arg114 of tyrosinase, and Val 167 of adenylate cyclase, respectively (Fig. 5). Compound **93** also exhibited significant binding affinities toward tyrosinase through formation of 6 hydrogen bonds with amino acid residues Glu232, Glu451, Ser106, Cys113, Lys223, and Gln236.

Discussion

It was worth noting that the types and contents of the chemical components in EFB were more than that in ETB. The peak areas of all the identified compounds in EFB were approximately two-fold of that in ETB. Militarine was the most abundant compounds in both EFB and ETB, which possessed significant antioxidant, anti-inflammatory and neuroprotection activities, and was chosen as the chemical markers for the quality control of *B. striata* in Chinese Pharmacopoeia 2020 edition [40]. The relative peak area of

militarine in EFB (38.39×10^6) was slightly higher than that in ETB (30.48×10^6). Gastrodin is a well known compound in many Chinese herbal medicines, such as *Gastrodia elata*, which exhibit significant tyrosinase inhibitory and radical scavenging effects [28]. The relative peak area of gastrodin in EFB (1.43×10^6) was slightly lower than that in ETB (2.17×10^6).

The 95% ethanolic extracts of TB and PB had stronger antioxidation activity *in vitro* than their crude polysaccharides. In addition, EFB exhibit stronger antioxidation activity than ETB, which was consistent with the previous research results [23]. This phenomenon may be attributed to that the EFB contains higher level of antioxidant compounds, such as militarine and stilbenoids (natural plant polyphenols). For example, the relative peak area of 4,8,4',8'-tetramethoxy-[1,1'-biphenanthrene]-2,7,2',7'-tetrol, a biphenanthrene with four phenolic hydroxyl groups, was 9.15×10^6 in FB, while it was 0.01×10^6 in TB.

In the present study, PPB and PTB showed slight tyrosinase inhibition activity in a dose dependent manner. However, it demonstrated that no activity was detected in the aqueous extract of PB (contains PPB) [23]. This phenomenon may be due to the different sample preparation. The aqueous extract of PB was used to the evaluate tyrosinase inhibitory activity in Jiang's experiment [23], while in the present study, aqueous extract was precipitation with ethanol to obtain the crude polysaccharide.

Recently, the zebrafish model widely used in evaluation of anti-melanogenesis effect *in vivo* [41, 42]. Arbutin, a hydroquinone glucoside compound existing in various plants, which has been commercially used in the cosmetic industry, was set as positive control. The anti-melanogenesis activities of ETB and EFB were stronger than that of arbutin, indicating that ETB and EFB can be applied as skin-lightening agents in cosmetic industry.

The relative peak area of blestrin D in EFB (1.91×10^6), is approximately four times of that in ETB (0.46×10^6). The relative peak area of compound **56** (8.73×10^6) and **93** (0.64×10^6) in EFB is also significant higher than that in ETB (0.00×10^6 and 0.10×10^6). This may be one of the reasons why EFB exhibits stronger anti-melanogenic effects than ETB. It is interesting to note that top three ligands toward tyrosinase and adenylate cyclase all belong to stilbenoids (bibenzyls, phenanthrenes and its derivative). Stilbenoids were the natural plant polyphenols, which has attracted great interest in the last years because of their remarkable bioactivities such as anti-inflammatory, antimicrobial and antioxidant activity [43]. Resveratrol is the most common stilbenoid. The previous research indicated that resveratrol and its derivatives can significantly inhibit the catalytic activity, gene expression, and posttranslational modifications of tyrosinase. Thus, they showed potentially useful as skin lightening and antiaging agents in cosmetics [35, 44].

Conclusions

In this study, the antioxidant and anti-melanogenic activity of the crude polysaccharide from the *B. striata* tuber (PTB) and fibrous roots (FPB), and 95% ethanol extract of *B. striata* tuber (ETB) and fibrous roots (EFB) were systematically investigated. The results show that EFB possesses the strongest DPPH, ABTS radical scavenging activity and ferric iron reducing activity. In addition, ETB and EFB can significantly reduce tyrosinase activity *in vitro* and melanin synthesis of zebrafish embryos in dose-dependent manner.

Molecular docking indicated that there were a large number of compounds, mostly belongs to stilbenoids, exhibit stronger binding affinities toward tyrosinase and adenylate cyclase, comparing to the binding affinity of the original ligand. The present findings supported the rationale for the use of ETB and EFB as natural skin-whitening agents in pharmaceutical and cosmetic industries.

Abbreviations

TCM: Traditional Chinese medicine; TB: *Bletilla striata* tuber; FB: *Bletilla striata* fibrous roots; ETB: 95% ethanol extract of *Bletilla striata* tuber; EFB: 95% ethanol extract of *Bletilla striata* fibrous roots; PTB: Crude polysaccharide of *Bletilla striata* tuber; PFB: Crude polysaccharide of *Bletilla striata* fibrous roots; DPPH: 2,2-Diphenyl-1-picrylhydrazyl; TPTZ: 2,4,6-Tris(2-pyridyl)-s-triazine; FRAP: Ferric reducing antioxidant power assay; ABTS: 2,2'-Azino-bis-(3-ethylbenthiazoline-6-sulphonic acid; Trolox: 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid; L-DOPA: 3-(3,4-Dihydroxyphenyl)-L-alanine; UPLC-Q-TOF-MS/MS: Ultra-high-performance liquid chromatography combined with quadrupole time-of-flight tandem mass spectrometry; MNLC: Maximum non-lethal concentration; ROS: Reactive oxygen species.

Declarations

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Data Availability

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Authors' Contribution

YYL, HJC designed the experiments, analyzed the data and drafted the manuscript; YYL performed the UPLC-Q-TOF-MS/MS analysis; SL, YW, ZRW performed the antioxidant analysis; JW, SJC performed the molecular docking analysis; All authors reviewed and approved the manuscript.

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Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Ethics approval and consent to participate

This work was approved by the IACUC (Institutional Animal Care and Use Committee) at Hunter Biotechnology, Inc. and the IACUC approval number was 001458.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

Figure 1

Base peak chromatogram of 95% ethanol extracts from *B. striata* tubers (A) and fibrous roots (B).

Figure 2

Impact of melanogenic inhibitors on relative tyrosinase activity (A) in vitro and melanin synthesis (B) in zebrafish embryos.

Figure 3

The impacts of PTB, PFB, ETB and EFB on the melanin synthesis of zebrafish embryos.

Figure 4

Best calculated poses for blestin D inside binding pocket of homology model of tyrosinase (A) and adenylate cyclase (B).

Figure 5

Two-dimensional representation of interaction between studied ligands and amino acids inside binding pocket of homology model of (A) tyrosinase and (B) adenylate cyclase.

Supplementary Files

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