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The Melanin Biosynthesis Stimulating Compounds Isolated from the Fruiting Bodies of *Pleurotus citrinopileatus*

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ABSTRACT

In the course to find a stimulating compound for melanin biosynthesis, which should be useful for a gray and a white hair-preventive agent or tanning agent, we evaluated the effects of the methanol extract from mushroom of *Pleurotus citrinopileatus* on melanin production in B16 melanoma cells without theophylline. Activity-guided fractionation led to isolate *myo*-inositol (**3**) and D-mannitol (**4**) as the stimulating compounds on melanin production in B16 melanoma cells. Also, ergosterol (**1**), uracil (**2**), and D-glucose (**5**) have been isolated from the methanol extract of *P. citrinopileatus* and showed no effect on melanin production in B16 melanoma cells. These results indicated that *myo*-inositol (**3**) and D-mannitol (**4**) are potential candidates that could be useful such as a gray and a white hair-preventive agent or tanning agent.

Keywords: *Pleurotus citrinopileatus*; *myo*-Inositol; D-Mannitol; Melanin Stimulating Activity; White Hair-Preventive Agent; Tanning Agent

1. Introduction

Mushrooms are a nutritionally functional food and a source of physiologically beneficial medicines. Fruiting bodies of some wild and cultivatable mushrooms contain medicinal compounds that are used in traditional medicines and cosmetics. There are numerous potential medicinal products from mushrooms that could be used in cosmeceuticals (products applied topically, such as creams, lotions, and ointments) or nutricosmetics (products that are ingested orally). But, there are numerous mushroom species that are untested, undescribed, or not yet cultivatable and that have huge potential for use in the cosmetic industry. Some fungi are also used in bio-transformation, and products such as lactic acid and ceramides could potentially be used in cosmetics [1,2].

Pleurotus citrinopileatus is an edible mushroom (Synonymy: *P. cornucopiae*, *P. cornucopiae* var. *citrinopileatus*) belonging to the genus *Pleurotus*, Pleurotaceae family. The name of this mushroom in English is golden oyster mushroom, tamogitake in Japanese, yuhuangmo in Chinese, goldenseed in Korean, and weishenga limonaya in Russian. A half dozen recent studies have focused on the cultivation of *P. citrinopileatus* for its numerous multifunctional biological activities, such as melanin biosynthesis inhibitory activity, antioxidant, antibacterial, and antihyaluronidase activities [2,3]. However, there are a limited number of previous studies on the chemical composition, and there have been a few of reports identifying the lectin, peptide and protein from water extracts of *P. citrinopileatus* [4,5].

Skin pigmentation results from melanin synthesis by melanocytes and is caused by exposure to UV radiation. Tyrosinase is a key enzyme of melanin synthesis that catalyzes three different reactions: the hydroxylation of tyrosine to 3,4-dihydroxyphenylalanine (DOPA), the oxidation of DOPA to DOPA-quinone, and the oxidation of 5,6-dihydroxyindole (DHI) to indole-quinone [6]. In the absence of thiols DOPA-quinone changes to DOPA chrome and then to DHI or indole 5,6-quinone 2-carboxy-lic acid (DHICA). Broadly, there are two further steps in this melanogenic pathway, one involves tyrosinase related protein-2 (TRP-2; DOPA chrome tautomerase) which catalyzes the conversion of DOPA chrome to DHICA, and the other involves TRP-1 (DHICA oxidase) which catalyzes the oxidation of DHICA [7,8]. There are several signal pathways for enhancing melanin production.

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The cAMP-mediated pathway is a well known melanin synthesis cascade. α-melanocyte stimulating hormone (α-MSH), prostgland-in E2 (PGE2), and adrenocorticotropic hormone (ACTH) activate the cAMP-mediated pathway [9,10].

Human skin is repeatedly exposed to ultraviolet radiation (UVR) that influences the function and survival of many cell types and is regarded as the main causative factor in the induction of skin cancer. It has been traditionally believed that skin pigmentation is the most important photo-protective factor, since melanin, besides functioning as a broadband UV absorbent, has anti-oxidant and radical scavenging properties. Besides, many epidemiological studies have shown a lower incidence for skin cancer in individuals with darker skin compared to those with fair skin. Skin pigmentation is of great cultural and cosmetic importance. In light of the increasing incidence for UV induced skin cancer and the progressive depletion of the ozone layer, which contrasts to public perception of a tan as being healthy, a better understanding of the role of melanin in preventing UV induced DNA damage and malignant transformation of skin cells would be more than desirable [11]. Melanin containing tissues have been located in various parts of the human body outside the skin complex, including in the heart, lungs, liver, brain [12], lymphocytes [13], and inner ear [14]. Melanin is a pigment that gives color to the skin, eyes, and hair. Lack of melanin pigmentation occurs principally due to regional lack of melanocytes (e.g. piebaldism or vitiligo) or to the genetic transmission of mutations in pigment related genes that give rise to hypopigmentation (e.g. albinism) when inherited in a homozygous form. There are several forms of oculocutaneous albinism [15]. Skin lightening or whitening (leukoderma, hypopigmentation) is most commonly the result of decreased melanin content in the skin (hypomelanosis) [16]. Increase of epidermal turnover can also induce hypomelanosis. Hypomelanosis may affect hair color. Canities means a generalized loss of hair color, whereas poliosis refers to localized hypomelanosis involving a tuft of hair or a few hairs in the eyebrows or eyelashes [16].

In our preliminary screening, we have found that the methanol extract of *P. citrinopileatus* showed the stimulating effects on melanin formation in B16 melanoma cells. We investigate the melanin biosynthesis stimulatory effect of the methanol extract from the mushroom of *P. citrinopileatus* on B16 melanoma cells in order to identify potential melanin producing candidates, which are useful such as skin-tanning and white hair-preventive cos metics.

2. Materials and Methods

2.1. General Experimental Procedure

Column chromatography was performed by silica gel (Wakogel C-200 particle size 75 - 150 μm; Wako Pure Chemical Industries, Co., Ltd., Japan). Thin layer chromatography (TLC) was carried out using Merck precoated silica gel 60 F_{254} plates (0.25 mm, Merck & Co., Inc., Darmstadt, Germany) and spots were detected with I_2 detection and under UV light. The compound **1** was isolated by preparative high performance liquid chromatography (HPLC) using a Waters TM 600 Controller, Waters TM 486 Tunable Absorbance Detector and Waters 600 Multi-solvent Delivery System (Japan Water Co., Ltd., Japan). The absorbance was measured by Tecan Spectra microplate reader (Tecan Japan Co., Ltd., Japan) and UV/VIS Spectrometer V-530 (JASCO Co., Japan). Preparative column using Inertsil preparative ODS column (20 mm i.d. × 250 mm) from GL Sciences (GL Sciences Inc., USA).

2.2. Chemicals

Dimethylsulfoxide (DMSO), potassium hydroxide solution (NaOH), hydrochloric acid (HCl) and sodium hydrogen carbonate ($NaHCO_3$) were purchased from Wako (Wako Pure Chemical Industries, Ltd., Japan). Thiazolyl blue tetrazolium bromide (MTT) was obtained from Sigma (Sigma-Aldrich Co., USA). Qualified fetal bovine serum (FBS) was obtained from Gibco® (Life Technologies Co., USA). Ethylene diamine tetraacetic acid (EDTA) was obtained from Dojindo (Dojindo Molecular Technologies, Inc., Japan). Trypsin was obtained from Nihon Pharmaceutical (Nihon Pharmaceutical Co., Ltd., Japan). Eagle's minimal essential media (EMEM) and Glutamine were purchased from Nissui (Nissui Pharmaceutical Co., Ltd., Japan). Theophylline was obtained from Sigma (Sigma Chemical Co., USA).

2.3. Mushroom Materials

Fresh fruiting bodies of *P. citrinopileatus* were obtained from Tamogitake Pharmaceutics Co., Ltd. (Nagano, Japan). The fruiting bodies were cleaned to remove any residual materials and then freeze-dried. The milled freeze-dried *P. citrinopileatus* (900.0 g) were extracted with methanol (2 × 9.0 L) at room temperature for one week and then filtered. The methanol extract was concentrated by a rotary evaporator. The yield of the methanol extract was 114.0 g (12.7%).

2.4. Extraction and Isolation

A portion of the methanol extract (100.0 g) was applied to a silica gel column (Wakogel C-200, 4.0 kg; 19 cm i.d. × 50 cm) and eluted with *n*-hexane/chloroform (7:3, 5:5, 3:7, 0:10), ethyl acetate, acetone, ethanol and methanol (each 8.0 L), followed by methanol/water (50:1, 6.0 L; 11:1, 2.0 L; 8:1, 3.0 L), affording eight fractions (Fr.1 to

Fr.8). Based on TLC analysis, using *n*-hexane/chloroform (1:9), Fr.1 (Rf = 0, 0.21, 0.34, 0.41, 0.62, 0.72, 0.82), Fr.2 (Rf = 0, 0.17, 0.28, 0.34, 0.62, 0.69) and Fr.3 (Rf = 0, 0.17, 0.28, 0.34, 0.62, 0.69) were combined to yield Fr.1' (7.0 g). A portion of the Fr.1' (4.6 g) was applied to a silica gel column chromatography (Wakogel C-200, 900 g; 5.5 cm i.d. ×120 cm) and eluted with *n*-hexane/ethyl acetate (20:1, 15:5, 10:10, 0:20, each 1.5 L), ethyl acetate/methanol (20:1, 15:5, 10:10, 0:20, each 1.5 L) to give eight fractions (Fr.1'-1 to Fr.1'-8). Fr.1'-4-2 (494 mg) was recrystallized from methanol to give compound **1** (401 mg). Fr.1'-7 (344 mg) was fractionation by preparative HPLC (Inertsil Prep-ODS column, 20 mm i.d. × 250 mm) and retention time was 16.5 min to give compound **2** (36 mg). Fr.1'-8 (1.0 g) was recrystallized from methanol to give compound **3** (42 mg). Fr.4 and Fr.5 were combined to Fr.4' (4.5 g), which was fractionated by silica gel column chromatography (Wakogel C-200, 4.0 kg; 19 cm i.d. × 50 cm) to obtain six fractions (Fr.4'-1 to Fr.4'-6). Fraction 4'-6 (1.0 g) of by silica gel column chromatography (120 cm × 6.0 cm i.d.; *n*-hexane/chloroform 2:8, 1:9, 0:10; chloroform/methanol 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, 1:9, 0:10, each 1400 mL) to obtain seven fractions (Fr.4'-6-1 to Fr.4'-6-7). Fraction 4'-6-5 (182 mg) was recrystallized from methanol to give compound **4** (54 mg). Fr. 6 (3.0 g) was fractionated by silica gel column chromatography (120 cm × 6.0 cm i.d.; chloroform/methanol 2:8, 1.5:8.5, 1:9, 0.5:9.5, 0:10, each 1400 ml) to obtain five fractions. Fr.6-3 (2.4 g) was recrystallized from methanol to give compound **5** (2.3 g). The nuclear magnetic resonance (NMR, 400MHz, JEOL Ltd., Japan) and gas chromate-graphy mass spectrometry (GC-MS) (GC-17A/QP5050; Shimadzu Corporation Ltd., Japan) data of the isolating compounds (**Figure 1**) were compared with those of authentic samples and reference [17-24].

2.5. Inhibitory Effect on Melanogenesis Using Cultured B16 Melanoma Cells

2.5.1. Determination of Melanin Content

B16 melanoma, a mouse melanoma cell line producing melanin, was obtained from RIKEN Cell Bank. The cells were maintained in EMEM supplemented with 10% (v/v) fetal bovine serum (FBS) and 0.3 mg/ml glutamine. The cells were incubated at 37˚C in a humidified atmosphere of 5% CO_2. Confluent cultures of B16 melanoma cells were rinsed in phosphate buffered saline (PBS) and removed from the plastic using 0.25%trypsin/0.02%EDTA. The cells were placed at a density of 1×10^5 cells/well and incubated for 24 h in media prior to being treated with the samples. After 24 h, the media were replaced with 900 μL (998 μL) of fresh media and 100 μL (2 μL) of water (DMSO) was added with or without (control)

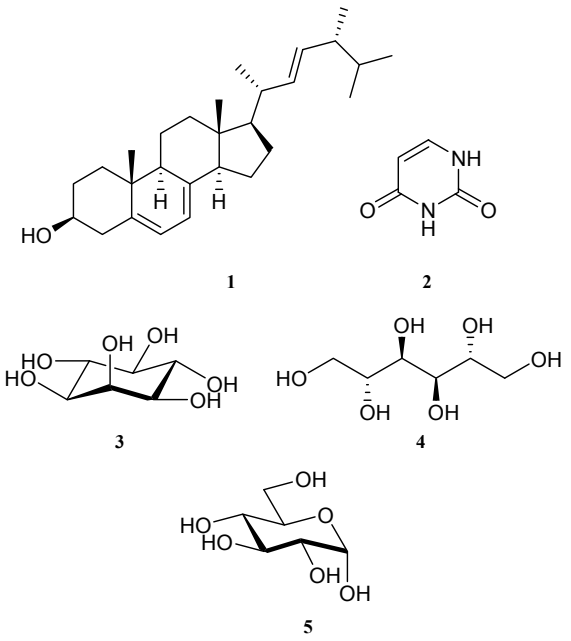

Figure 1. Structures of compounds 1 - 5.

the test sample at various concentrations and its replicates were three times. The cells were incubated for an additional 48 h, and then the medium was replaced with fresh medium containing each sample. After 24 h, removing the medium and washing the cells, the cell pellet was dissolved in 1.0 mL of 1 N NaOH. The crude cell extracts were assayed using a micro plate reader at 405 nm to determine melanin content. The results from the samples were analyzed as a percent of the control culture. Theophylline was used as a positive control.

2.5.2. Cell Viability

Cell viability was determined by use of the microculture tetrazolium technique (MTT assay). A culture was initiated, and after incubation, 50 μL of MTT in phosphate buffered saline (5 mg/mL) was added to each well. The plates were incubated for 4 h. After removing the medium, formazan crystals were dissolved in 1.0 mL of 0.04 N HCl and the absorbance was measured at 570 nm relative to 630 nm.

3. Results

In present study, we evaluate the effect of the methanol extracts of the fruiting bodies of *P. citrinopileatus* on melanin production in B16 melanoma cells without theophylline. To search for melanin production stimulating compounds, we modified the assay using B16 melanoma cells. It should be noted that theophylline is usually added to medium for stimulating melanin production in B16 melanoma cells. Theophylline is known as an analogue of cAMP, which is a second messenger for mela-

nin biosynthesis [25]. So, in our modified assay, theophylline was not added into medium, which is for finding melanin production stimulating compound. An index such as "mean of melanin content (%)/mean of cell viability (%)" called as MC/CV value was applied for evaluating the stimulating activity of melanin production per cell induced by samples.

The methanol extracts were assayed by using B16 melanoma cells in order to evaluate the stimulation of melanin production and cell viability. The stimulating effect of methanol extracts on melanin production in B16 melanoma cells was shown at various concentrations (**Table 1**). At the concentration of 5.0 mg/mL, the methanol extract showed melanin production stimulating activity per cell with MC/CV value of 1.5.

The methanol extract was subjected to a silica gel column (Wakogel C-200, 4.0 kg; 19 cm i.d. × 50 cm), gradient eluted with *n*-hexane/chloroform (7:3, 5:5, 3:7, 0:10), ethyl acetate, acetone, ethanol and methanol (each 8.0 L), followed by methanol/water (50:1, 6.0 L; 11:1, 2.0 L; 8:1, 3.0 L), affording eight fractions (Fr.1 to Fr.8). Fr.3 and Fr.4 showed (**Table 2**) higher melanin production stimulating activity with MC/CV value of 4.0 and 4.4, respectively. Activity-guided fractionation of Fr.3 and Fr.4 led to the isolation of uracil (**2**), *myo*-inositol (**3**) and D-mannitol (**4**) as main components. Activity-guided fractionation of Fr.4 led to the isolation of D-mannitol as dominant component. Both of *myo*-inositol (**3**) (12 mg/ml) and D-manntiol (**4**) (18 mg/ml) showed (**Tables 3 and 4**) potential melanin production stimulating activity with MC/CV value of 1.6. Also, another weak activity-guided fractionation of Fr.2 and Fr.6 led to ergosterol (**1**) and D-glucose (**5**). However, ergosterol (**1**), uracil (**2**), and D-glucose (**5**) showed no effect on melanin production in B16 melanoma cells (data not show). These results indicated that *myo*-inositol (**3**) and D-mannitol (**4**) are potential candidates that could be useful, as a tanning and a white hair-preventive agent.

Table 1. The effect of methanol prepared extract from the fruiting bodies of *P. citrinopileatus* on B16 melanoma cells.

Concentration (mg/mL)	Melanin content (% vs. control)	Cell viability (% vs. control)	MC/CV[b]
0.0	100.0 ± 1.5	100.0 ± 3.2	1.0
0.3	92.6 ± 5.5	92.7 ± 4.8	1.0
0.6	96.5 ± 3.6	97.9 ± 0.5	1.0
1.3	86.0 ± 2.4**	93.6 ± 4.7	0.9
2.5	95.3 ± 0.7*	77.3 ± 6.1*	1.2
5.0	88.3 ± 1.8**	60.4 ± 4.4**	1.5
Theophylline[a] (0.01)	138.6 ± 3.4**	96.1 ± 3.4	1.4

Data presented as means ± R.S.D. (n = 3); *p < 0.05, **p < 0.01, Significantly different from control group. [a]Positive control for melanin stimulating activity. [b]MC/CV indicate "mean of melanin content (%)/mean of cell viability (%)".

Table 2. The effect of each fractions obtained from methanol extract from the fruiting bodies of *P. citrinopileatuson* B16 melanoma cells.

Samples	Concentration (mg/mL)[b]	Melanin content (% vs. control)	Cell viability (% vs. control)	MC/CV[c]
Control	0.0	100.0 ± 2.1	100.0 ± 2.7	1.0
Fr.1	0.4	103.5 ± 7.4	87.9 ± 6.0*	1.2
Fr.2	1.9	86.1 ± 7.5	35.3 ± 1.8**	2.4
Fr.3	1.7	67.2 ± 7.9*	16.8 ± 3.4**	4.0
Fr.4	1.9	73.6 ± 0.9*	16.6 ± 1.5**	4.4
Fr.5	2.6	78.1 ± 5.2*	26.8 ± 0.8**	2.9
Fr.6	3.1	121.4 ± 4.6*	58.5 ± 4.5**	2.1
Fr.7	5.6	50.8 ± 2.2**	40.2 ± 2.9**	1.3
Fr.8	4.9	51.7 ± 0.7**	18.3 ± 1.0**	2.8
Theophylline[a]	0.01	138.6 ± 3.4**	96.1 ± 3.4	1.4

Data presented as means ± R.S.D. (n = 3); *p < 0.05, **p < 0.01, Significantly different from control group. [a]Positive control for melanin stimulating activity. [b]The concentration of each sample with maximum solubility were selected. [c]MC/CV indicate "mean of melanin content (%)/ mean of cell viability (%)".

Table 3. The effect of *myo*-inositol isolated from the fruiting bodies of *P. citrinopileatus* on B16 melanoma cells.

Samples (mg/mL)	Melanin content (% vs. control)	Cell viability (% vs. control)	MC/CV[b]
0	100.0 ± 2.2	100.0 ± 4.0	1.0
1	106.3 ± 5.7	92.8 ± 2.5*	1.2
2	103.8 ± 2.9	91.5 ± 0.9**	1.1
5	101.9 ± 2.9	89.2 ± 1.1**	1.1
9	110.1 ± 3.8*	76.8 ± 0.41**	1.4
18	111.4 ± 5.8*	70.8 ± 4.7**	1.6
Theophylline[a] (0.01)	140.5 ± 5.0**	102.6 ± 6.7	1.4

Data presented as means ± R.S.D. (n = 3); *p < 0.05, **p < 0.01, Significantly different from control group. [a]Positive control for melanin stimulating activity. [b]MC/CV indicate "mean of melanin content (%)/mean of cell viability (%)".

4. Discussion

As described above, skin pigmentation results from melanin synthesis by several enzymes such as tyrosinase in melanocytes and is caused by exposure to UV radiation. Outside of them, there are several pathways for enhanc-

Table 4. The effect of D-mannitol isolated from the fruiting bodies of *P. citrinopileatus* on B16 melanoma cells.

Sampes (mg/mL)	Melanin content (% vs. control)	Cell viability (% vs. control)	MC/CV[b]
0	100.0 ± 2.5	100.0 ± 1.7	1.0
2	101.3 ± 10.1	88.5 ± 7.5	1.1
3	112.9 ± 4.3	104.0 ± 2.0**	1.1
6	111.2 ± 3.5	94.4 ± 5.1**	1.2
12	116.1 ± 6.3*	86.0 ± 2.8**	1.3
25	106.3 ± 2.8*	66.0 ± 3.1**	1.6
Theophylline[a] (0.01)	137.9 ± 1.3**	100.3 ± 0.7	1.4

Data presented as means ± R.S.D. (n = 3); *p < 0.05, **p < 0.01, Significantly different from control group. [a]Positive control for melanin stimulating activity. [b]MC/CV indicate "mean of melanin content(%)/ mean of cell viability (%)".

ing melanin production. The cAMP-mediated pathway is a well-known melanin synthesis cascade and α-MSH, prostaglandin E2 (PGE2), and adrenocorticotropic hormone (ACTH) activate the cAMP-mediated pathway [9,10].

On the other hand, a cGMP-mediated pathway can also increase melanin production. This pathway is activated by nitric oxide (NO), which is released by keratinocytes following UV-B irradiation. Protein kinase C (PKC) can activate tyrosinase. UV light might activate cell membrane bound phospholipase C, and augmented diacylglycerol (DAG) can activate PKC [26].

Skin is a major candidate target of oxidative stress caused by reactive species (RS), including reactive oxygen species and reactive nitrogen species. RS are major and significant contributors to skin hyper pigmentation and skin aging. It is generally believed that agents having antioxidant activity show anti-aging, whitening, and anti-inflammatory activities [27]. If free radicals are inappropriately processed in melanin synthesis, hydrogen peroxide (H_2O_2) is generated, leading to the production of hydroxyl radicals (HO·) and other reactive oxygen species (ROS) [28]. Oxidative stress may be induced by increasing the generation of ROS and other free radicals. UV radiation can induce the formation of ROS in skin such as singlet oxygen and superoxide anions, promoting biological damage in exposed tissues via ironcatalyzed oxidative reactions. These ROS enhance melanin biosynthesis, damage DNA, and may induce proliferation of melanocytes [29]. Yamakoshi *et al*. [29] found evidence for a role of oxidative stress in the pathogenesis of skin disorders. It is known that ROS scavengers or inhibitors such as antioxidants may reduce hyperpigmentation. Additionally, superoxide dismutase (SOD, EC 1.15.1.1), which catalyzes the dismutation of the superoxide anion into hydrogen peroxide and molecular oxygen, is one of the most important antioxidative enzymes.

The *myo*-inositol (**3**) and D-mannitol (**4**) were known as hydroxyl radical scavengers [30], so should be concerned in radical pathway. Further, the two compounds showed no activity against ORAC, SOD like assay and DPPH (data not show). Considering the role of ROS and their effects against ROS, their mechanisms of the melanin production stimulating activity in B16 melanoma cells should be related with other factor such as cAMP signaling rather than their effects on ROS.

According to the increase of the elderly population, many people are afflicted with white hair. Thus, the market for hair-dye and anti-white hair agents are growing. White hair is caused by a genetic predisposition, aging, decrement of melanocytes by environmental stress, and decrement of the biosynthesis of melanin pigment, or melanogenesis [31,32]. Hair-dye agents are used for the treatment of white hair, and some anti-white hair agents are under development. However, there remain some problems with these agents, such as insufficient activity and side effects due to the dyes. Thus, there is a need for safer anti-white hair agents exhibiting satisfactory melanogenesis activity and white hair prevention [33]. Since a melanocyte reservoir exists in the human hair follicle [31], it is considered that stimulation and/or activation of melanocyte in the hair follicle is a prospective means to prevent white hair [33].

In addition, inositol 1,4,5-trisphosphate (IP_3) releases calcium from intracellular stores [34,35], signal transduction, stress, protection, hormonal homeostasis and cell wall biosynthesis in plants [36]. The functions and roles of *myo*-inositol in humans have been linked to bipolar disorder [37], production of L-*chiro*-inositol and D-*chiro*-inositol in insulin action [38], multiple sclerosis [39], Alzheimer's disease [40] and regulation of the sorbitol pathway in diabetic patients [41]. Mannitol is used as a sweet-tasting bodying and texturing agent [42]. The complex of boric acid with mannitol is used in the production of dry electrolytic capacitors. It is an extensively used polyol for production of resins and surfactants [43]. Mannitol is used in medicine as a powerful osmotic diuretic (to increase the formation of urine in order to prevent and treat acute renal failure and also in the removal of toxic substances from the body) and in many types of surgery for the prevention of kidney failure (to alter the osmolarity of the glomerular filtrate) and to reduce dye and brain oedema (increased brain water content). Hypertonic mannitol can enhance the transport of drugs across the blood-brain barrier for the treatment of life-threatening brain diseases [44]. Inhaled mannitol improves the hydration and surface properties of sputum in patients with cystic fibrosis [45]. Mannitol hexanitrate is a well-known vasodilator, used in the treatment of hypertension [46]. Mannitol is also a scavenger of hydroxyl radicals [30].

5. Conclusion

In this study, we found a new facet of biological activity of *myo*-inositol (**3**) and D-mannitol (**4**) isolated from *P. citrinopileatus*, stimulating activity of melanin production. Therefore, these compounds are potential candidates that could be useful as a gray and a white hair-preventive agent or a tanning agent.

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