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Cosmetic and Skincare Benefits of Cultivated Mycelia from the Chinese Caterpillar Mushroom, *Ophiocordyceps sinensis* (Ascomycetes)

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Short running title: **Cosmetic benefits of *Cordyceps sinensis* mycelia**

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ABSTRACT

Fungi or mushrooms are potential sources for novel natural cosmeceutical ingredients. This study was conducted to evaluate the cosmetic (skincare) benefits of the valuable medicinal fungus *Ophiocordyceps sinensis* (=*Cordyceps sinensis*). The mycelial extracts of two *O. sinensis* strains, Cs-HK1 and Cs-4 prepared sequentially with ethyl acetate (EtOAc), ethanol (EtOH) and hot water (HW) were tested by *in vitro* assays for tyrosinase, collagenase and elastase inhibition activity. The EtOAc extracts of both fungi showed potent anti-tyrosinase and anti-elastase activity with low IC_{50} values (0.14-0.47 mg/ml) comparable with the respective references (arbutin and epigallocatechin gallate). All mycelial extracts exhibited moderate or significant anti-collagenase activity; most extracts showed significant photoprotective effect with sun protection factor (SPF) value up to 25. Nevertheless, the results from the present study have shown the potential use of *O. sinensis* fungus as a source of cosmetic ingredients for skincare applications.

KEYWORDS: anticollagenase, antielastase, antioxidant, antityrosinase, *Cordyceps sinensis*, medicinal mushrooms and fungi, mycelial extract, *Ophiocordyceps sinensis*.

ABBREVIATIONS: EGCG, epigallocatechin gallate; **EtOAc,** ethyl acetate; **EtOH,** ethanol; **HW,** hot water; **IC50,** half-maximal inhibitory concentration; **SPF,** sun protection factor; **TEAC,** Trolox-equivalent antioxidant capacity; **UVR,** ultraviolet radiation

I. INTRODUCTION

Today, “natural” is a hot selling point for many cosmetic products on the market because of public concerns with the potential harmful effects of synthetic chemicals on the human body and a general belief that natural products are safe and healthy. Interest is growing worldwide in the cosmetic benefits of bioactive natural products extracted from medicinal plants and herbs. These medicinal natural products can offer protective and therapeutic functions such as anti-inflammatory, antimicrobial, and antioxidant/antiaging effects, but they also produce direct skincare effects when applied topically, such as antiwrinkle, skin-whitening/lightening, skin-moisturizing, and skin-toning benefits and protection against UV rays.[1,2]

Skin aging is generally classified as intrinsic aging, caused by natural physiological processes in the human body, and extrinsic aging such as photoaging, which is mainly attributed to exposure to ultraviolet radiation (UVR).[3] Oxidative stress caused by reactive oxygen species from endogenous and exogenous sources is regarded as the primary cause for both intrinsic and extrinsic aging.4 Therefore, the use of synthetic or natural antioxidants is considered to be the most effective strategy to retard skin-aging processes.[4] Sunscreens can protect skin from UVR and delay the onset and progression of skin aging.[5] Synthetic sunscreens may, however, cause adverse effects such as skin irritation, allergy, and endocrine disruption.[6,7] Natural products including flavonoids, anthraquinones, and tannins, which have a structure similar to that of synthetic sunscreens, have been shown to be capable of absorbing UVR.[8–10]

UVR causes melanocytes in the basal layer of the epidermis to produce melanin, a natural pigment that determines skin color and protects skin against UVR.[11,12] Tyrosinase is the enzyme that catalyzes the first 2 rate-limiting steps of melanin synthesis, namely the hydroxylation of l-tyrosine and the oxidation of l-dopa to dopaquinone.[13] However, abnormal accumulation of melanin leads to skin hyperpigmentation problems such as freckles, senile lentigines, and melisma.[14] To meet the cosmetic need for skin whitening, tyrosinase inhibitors such as arbutin, hydroquinone, and kojic acid are now widely applied in skin-whitening products in the cosmetic industry.[14] Collagenase is a metalloproteinase responsible for the

degradation of collagen, an important protein for maintaining skin strength and elasticity.[15] Elastase is a protease responsible for the breakdown of elastin, which is an important protein found within the extracellular matrix and is vital for giving elasticity to skin.[16–19] In addition to elastin, elastase can cleave collagen, fibronectin, and other proteins in the extracellular matrix.[18,19] Because collagen and elastin are the main structural components of skin, inhibitors of collagenase and elastase can prevent or slow the formation of skin wrinkles and sagging.

Although most of the natural ingredients in cosmetic and cosmeceutical products are from plants and animals, edible and medicinal mushrooms have been recognized as a new and promising source of natural cosmetic ingredients.[20,21] Edible and medicinal mushrooms provide a diverse array of bioactive compounds, including polysaccharides and secondary metabolites, with many health benefits such as antitumor, immunomodulatory, antioxidative, antimicrobial, and anti-inflammatory effects.[22,23] These fungi or their constituents have found wide application in functional foods and nutraceutical products. To date, however, only a relatively small number of medicinal fungi have been applied in commercial cosmetic products for topical skincare and haircare. Much more research is needed to explore and assess the cosmetic functions of fungi and their active components for better use of valuable healthful and cosmetic products.

Ophiocordyceps sinensis (Berk.) Sung et al. (=*Cordyceps sinensis*; Ophiocordicypetaceae, Ascomycetes), generally known as the Chinese caterpillar mushroom, or *Dong-Chong-Xia-Cao* in Chinese, is a medicinal species with numerous of health-promoting and therapeutic effects such as anticancer, immunomodulatory, antioxidative, antiaging, neuroprotective, and hepatoprotective actions.24 Because the natural *O. sinensis* caterpillar mushroom, which is formed of a fruiting body on an insect larva, is rare and very expensive, mycelial fermentation is widely applied for commercial production of the fungal components. Although *O. sinensis* has been applied in some commercial cosmetic products,[20,21] the cosmetic benefits of *O. sinensis* are still not well documented in the literature. Cs-HK1 is a *Tolypocladium* fungus isolated from the wild *C. sinensis* fruiting body. Its mycelial

biomass and exopolysaccharides from mycelial fermentation have shown significant health benefits.[25] Cs-4 (*Paecilomyces hepiali*) is a fungus officially approved by Chinese authorities for use in the commercial production of *O. sinensis* health foods.

This study was conducted to assess the cosmetic potential of Cs-HK1 and Cs-4 fungi by measuring their antityrosinase, anticollagenase, antielastase, antioxidant, and sunscreen properties. The mycelial biomass produced by liquid fermentation was extracted with solvents of different polarities (ethyl acetate, ethanol, and water) in order to compare the activities of the different extracts.

II. MATERIALS AND METHODS

A. Materials

Cs-4 mycelium powder was provided by Jiangxi Guoyao Ltd. (Nanchang, Jiangxi, China). Mushroom tyrosinase, L-tyrosine, 4-Hydroxyphenyl-β-D-glucopyranoside (arbutin), porcine pancreatic elastase, N-succinyl-Ala-Ala-Ala-p-nitroanilide (AAAPVN), collagenase, N-[3-(2-furyl)acryloyl]-Leu-Gly-Pro-Ala (FALGPA), epigallocatechin gallate (EGCG) and edetate disodium were purchased from Sigma-Aldrich, homosalate from Macklin Biochemical (Shanghai), catechin from Yuanye Biology Ltd. (Shanghai).

The Cs-HK1 mycelial biomass was prepared by mycelial liquid fermentation in our lab as reported previously.[25] In brief, Cs-HK1 mycelial biomass maintained on potato dextrose agar medium was inoculated into 50 ml of liquid medium in a 250 ml Erlenmeyer flask and incubated as the starter culture for liquid fermentation. The starter culture broth was transferred into 1 L Erlenmeyer flasks each filled with 250 ml liquid medium (at 4% v/v inoculation ratio) to start the mycelial fermentation. The liquid medium was composed of 40 g/L glucose, 10 g/L yeast extract, 5 g/L peptone, 1 g/L KH_2PO_4 and 0.5 g/L $MgSO_4 \cdot 7H_2O$. The liquid culture or mycelial fermentation was operated at 20 °C and 150 rpm on a shaker for 7 days. The mycelial biomass was

recovered from the liquid broth by centrifugation (6000 rpm, 15 min), and freeze-dried.

B. Preparation of mycelial extracts

The dry mycelium powder of Cs-HK1 and Cs-4 was sequentially extracted with three solvents from low to high polarity, absolute ethyl acetate (EtOAc), absolute ethanol (EtOH) and water. The solid and solvent ratio was fixed at 1:10 for EtOAc and EtOH extraction and 1:15 for water extraction, and added into Erlenmeyer flasks. For EtOAc and EtOH extraction, the solid-liquid mixture was stirred magnetically for 24 hours at 150 rpm and 30 °C. The liquid extract was separated from the solid residues by vacuum filtration, then concentrated by vacuum evaporation, and finally dried completely in an oven at 55°C, yielding the EtOAc and EtOH extracts. For hot water extraction, the mixture was stirred on a hot plate at 97 °C for 3 hours and the liquid extract collected after centrifugation was freeze-dried, yielding the HW extract.[26,27]

C. Analysis of chemical constituents of mycelium extracts

The HW extracts of Cs-HK1 and Cs-4 mycelia were dissolved in distilled water for all analyses; the EtOAc and EtOH extracts were dissolved in ethanol in order to analyze total phenolics and flavonoids, and in distilled water to analyze total carbohydrate and (water-soluble) protein content. All the mixtures were stirred overnight to achieve complete dissolution. Total carbohydrate content was determined with the anthrone test, using glucose as the standard,[28] and the protein content was determined with the Lowry method, using bovine serum albumin as the standard.[29] Total phenolic content was determined with the Folin-Ciocalteu assay using gallic acid as the standard.[26] Total flavonoid content was determined with the aluminium chloride colorimetric assay using catechin as the standard.[30]

D. Enzyme activity assays of mycelium extracts

For the enzymatic inhibition assays, the HW extract samples were dissolved in the buffer solutions; the EtOAc and EtOH extracts were dissolved in dimethyl sulfoxide at a 2% (w/v) final concentration for the tyrosinase inhibition assay and at 1% for both the anticollagenase and the antielastase assays. All assays were performed in triplicate, and the results were averaged. The absorbance of the enzyme-substrate reaction solution with the mycelial extracts was recorded at room temperature with a UV-visible spectrophotometer. The enzyme inhibition activity for all assays was calculated with Eq. (1) for tyrosinase and elastase, or Eq. (2) for collagenase:

$$\frac{A_S - A_C}{A_C} \times 100\% \quad (1)$$

$$\frac{A_C - A_S}{A_C} \times 100\% \quad (2)$$

1. Anti-tyrosinase activity assay

The antityrosinase assay was evaluated on the basis of the inhibition of mushroom tyrosinase activity by the sample, with l-tyrosine as the substrate, according to a protocol described previously.[31] Tyrosinase solution (700 units/mL, 50 µL) was mixed with 250 µL extract solution and 200 µL 0.2 M phosphate buffer (pH 6.5), and maintained at room temperature under gentle agitation for 90 minutes. Immediately after 500 µL 0.03% l-tyrosine was added, the absorbance was recorded at 475 nm with a spectrophotometer. Arbutin was included as an antityrosinase reference for the positive control.

2. Anti-collagenase activity assay

As described previously by Van Wart and Steinbrink[32], the assay was performed using collagenase from *Clostridium histolyticum* (ChC – EC.3.4.23.3) and the synthetic substrate, FALGPA. Eighty microliter of collagenase solution (0.83 U/mL) was mixed with equal volume of sample solution and 50 mM Tricine buffer (pH 7.5, 10 mM $CaCl_2$ and 400 mM NaCl), and maintained at room temperature for 15 min. Immediately after the addition of 160 µl of 2 mM FALGPA, the absorbance was

recorded at 335 nm with a spectrophotometer for 20 min. EGCG was included as a positive control for the anti-collagenase assay.

3. Anti-elastase activity assay

The antielastase assay was conducted as reported by Thring et al.[15]; we used porcine pancreatic elastase (E.C.3.4.21.36) and, as the substrate, *N*-succinyl-Ala-Ala-Ala-p-nitroanilide. Elastase solution (24 µg/mL, 25 µL) was mixed with 250 µL test extracts and 25 µL 0.2 M Tris-HCl buffer (pH 8.0), and maintained at room temperature for 15 minutes. Immediately after 300 µL substrate (1.6 mmol/L) was added, the absorbance was recorded at 410 nm with a spectrophotometer for 20 minutes. EGCG was used as the positive control for the anti–elastase activity assay.

E. Determination of potential UV-sunscreen activities

The extract samples were dissolved to the final concentration of 0.2 mg/ml with absolute ethanol (analytical grade) (for EtOAc and EtOH extracts) or distilled water (for HW extracts). All the sample solutions were prepared by constant stirring overnight at room temperature and then filtered through filter paper. The absorbance of the sample solution was scanned from 200 to 400 nm at 5 nm interval in a 1-cm quartz cuvette on a UV-vis spectrophotometer. The solvent for the particular extract, ethanol or water, was used as a blank for the absorbance measurement. The SPF value was given by the Mansur equation[33],

$$\mathbf{SPF} = \mathbf{CF} \times \sum_{\mathbf{290}}^{\mathbf{320}} \mathbf{EE}(\boldsymbol{\lambda}) \times \mathbf{I}(\boldsymbol{\lambda}) \times \mathbf{A}(\boldsymbol{\lambda}) \qquad (3)$$

where CF is a correction factor, EE(λ) the erythemal efficiency spectrum, I(λ) the solar intensity spectrum and A(λ) is the absorbance of the sample. Homosalate was used as a reference sunscreen and prepared at 8% according to FDA for determining the correction factor so that the formulation has a SPF value of 4. The normalized product function, EE(λ) × I(λ), which represents the relationships between the erythemogenic effect and the solar intensity between 290 and 320 nm in 5 nm

increments, is determined according to Sayre, Agin, LeVee and Marlowe.[34] as shown in Table 1. The SPF values of different extracts can be estimated with the Mansur equation and the normalized product function.

TABLE 1: The normalized product function used in calculation of SPF data. [34], [a]

Wavelength (nm)	EE x I (normalized)
290	0.0150
295	0.0817
300	0.2874
305	0.3278
310	0.1864
315	0.0839
320	0.0180
Total	= 1

EE, erythemogenic effect; I, solar intensity.
[a] No unit.

F. DETERMINATION OF ANTIOXIDANT ACTIVITIES

1. DPPH radical scavenging activity

The DPPH (1,1-Diphenyl-2-picrylhydrazyl) radical scavenging assay was modified from a procedure reported by Zhan, Dong and Yao.[35] The HW extract was dissolved in 0.05 M acetate buffer (pH 5.5) and the EtOAc and EtOH extracts were dissolved in absolute ethanol. An aliquot of 1 ml of sample solution was mixed with 0.25 ml of 0.50 mM DPPH in ethanol and incubated at room temperature in the dark for 30 min, followed by measurement of absorbance at 517 nm against solvent blank. Trolox in ethanol and 0.05 M acetate buffer (pH 5.5) with 5% ethanol was used as the positive control; the extract solution without DPPH was used as sample blank. The scavenging activity was calculated with Eq. (4):

$$\textbf{Scavenging activity}\ (\%) = \left(1 - \frac{A_s - A_{sb}}{A_b}\right) \times\ 100\% \qquad (4)$$

where A_b, A_s and A_{sb} as the absorbance of the blank, extract or positive control, and sample blank respectively. The half-maximum effective concentration (EC_{50}), the extract concentration at which 50% of DPPH radicals being scavenged, was obtained from the concentration-response curve.

2. Trolox equivalent antioxidant capacity assay (TEAC)

TEAC for scavenging ABTS radical cations was determined as reported previously.[36] Trolox [(S)-(2)-6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid] was used as an antioxidant reference, and the activity was expressed in μmol Trolox/g sample. The radicals $ABTS^{\bullet+}$ were generated by the reaction of ABTS [2,2 Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)] with potassium persulphate at room temperature in the dark. The extract sample solution was mixed with equal volume (500 μl) of the $ABTS^{\bullet+}$ solution for 20 min, followed by measurement of absorbance at 734 nm. The radical scavenging activity was represented by the percentage of radical reduction as for the above DPPH scavenging activity (Eq. 4).

G. Statistical analysis

All enzyme assays and tests were conducted in triplicate and the results were expressed as mean ± standard deviation (SD) using data analysis software SPSS. The statistical significance of treatment effects was determined by one-way analysis of variance (ANOVA) with LSD post hoc test (for differences among the tested samples) or by the Student's *t*-test at $p < 0.05$ (for differences between tested samples and the positive control).

III. RESULTS AND DISCUSSION

A. Yield and chemical composition of Cs-HK1 and Cs-4 mycelial extracts

Table 2 shows the yield and chemical composition of various mycelial extracts from Cs-HK1 and Cs-4. HW extract was the most abundant in both Cs-HK1 (248.4 mg/g) and Cs-4 (144.3 mg/g), with a much higher yield than that of EtOH and EtOAc. For Cs-HK1, the yield of EtOH (43.3 mg/g) was slightly more than that of the EtOAc (26.6 mg/g). The relative yields of EtOAc, EtOH and HW extracts from Cs-HK1 were consistent with those reported by Wu, Zhang and Leung[27]. As for Cs-4, the yield of EtOAc extract (103.4 mg/g) was nearly double of EtOH extract (50.8 mg/g).

TABLE 2: Yield and composition of Cs-HK1 and Cs-4 mycelial extracts with different solvents.

Extracts	Yield (mg/g)	Carbohydrates (wt%)	Proteins (wt%)	Phenolics (mg GAE/g)	Flavonoids (mg CE/g)
Cs-HK1					
EtOAc	26.6	2.9 ± 0.32	9.4 ± 0.21	27.1 ± 1.00	46.8 ± 6.15
EtOH	43.3	2.7 ± 0.09	11.5 ± 0.74	15 ± 0.91	32.9 ± 3.09
HW	248.4	10.5 ± 1.08	40.5 ± 1.42	28.3 ± 1.07	2.87 ± 0.17
Cs-4					
EtOAc	103.4	n.d.	n.d.	58.5 ± 1.49	173.3 ± 6.42
EtOH	50.8	1.4 ± 0.05	20.3 ± 0.10	11.4 ± 0.69	27.2 ± 0.84
HW	144.3	23.5 ± 1.31	33.3 ± 0.81	10.8 ± 0.97	4.48 ± 0.51

Yield and contents: based on total mass of dry mycelium; n.d.: not detected; GAE: gallic acid equivalents; CE: catechin equivalents.

Among the major constituents of Cs-HK1 and Cs-4 extracts (except for the Cs-4 EtOAc), proteins generally accounted for the largest proportion, followed by carbohydrates; the HW extracts had the highest protein and carbohydrate content.

There was no detectable amount of proteins or carbohydrates in the EtOAc extract of Cs-4. EtOAc extracts had relatively high phenolic and flavonoid contents in most cases. The much higher HW extract yields than the organic solvent extracts imply that water-soluble components including total carbohydrate and protein content were most abundant. The extraction yield of a component depends not only on the actual content but also on the polarity and other molecular properties of the component in the raw material.

B. Inhibitory effects of mycelial extracts on tyrosinase, collagenase and elastase

Figure 1 shows the activities of three enzymes, tyrosinase, collagenase and elastase, in the presence of Cs-HK1 and Cs-4 mycelial extracts at various concentrations. The activities of tyrosinase and collagenase were inhibited by all three solvent extracts, EtOAc, EtOH and HW in different degrees. The elastase activity was inhibited by EtOAc and EtOH extracts of Cs-HK1 fungus and EtOAc extract of Cs-4 fungus but not by HW extracts of the two fungi or the EtOH extract of Cs-4. All the inhibiting effects followed the concentration-dependent trend. Table 3 presents the IC_{50} derived from the concentration curves. Overall, the EtOAc extracts of both fungi had the most potent and HW extract had relative weak inhibiting activities on the three enzymes. In most cases, the EtOAc and EtOH extracts of Cs-HK1 showed more significant activities than those of Cs-4 on the three enzymes. In comparison of the IC_{50} values, the EtOAc extract of Cs-HK1 is even more potent than the positive control on the tyrosinase and elastase activity.

As shown in Fig. 1A and Table 3, the potency of both fungal extracts against tyrosinase was in the following rank order: EtOAc > EtOH > HW. The antityrosinase activity of the HW extract of Cs-HK1 was significantly lower than that of the EtOAc and EtOH extracts, whereas the activity of the HW extract of Cs-4 was about 50% less than that of the EtOAc and EtOH extracts. The results are comparable with those in the literature on the antityrosinase effects of mushroom extracts. Chien et al.37 evaluated the antityrosinase activities of several mushrooms including *Agaricus*

brasiliensis, *Antrodia camphorata*, *Cordyceps militaris*, and *Ganoderma lucidum*; *G. lucidum* was the most potent, with an IC50 of 0.32 mg/mL. Their study also showed a similar trend for the different solvent extracts, with the EtOH extract being more potent than the HW extract. Xu et al.31 evaluated the tyrosinase activity of polysaccharides isolated from 14 wild mushrooms, among which the most active

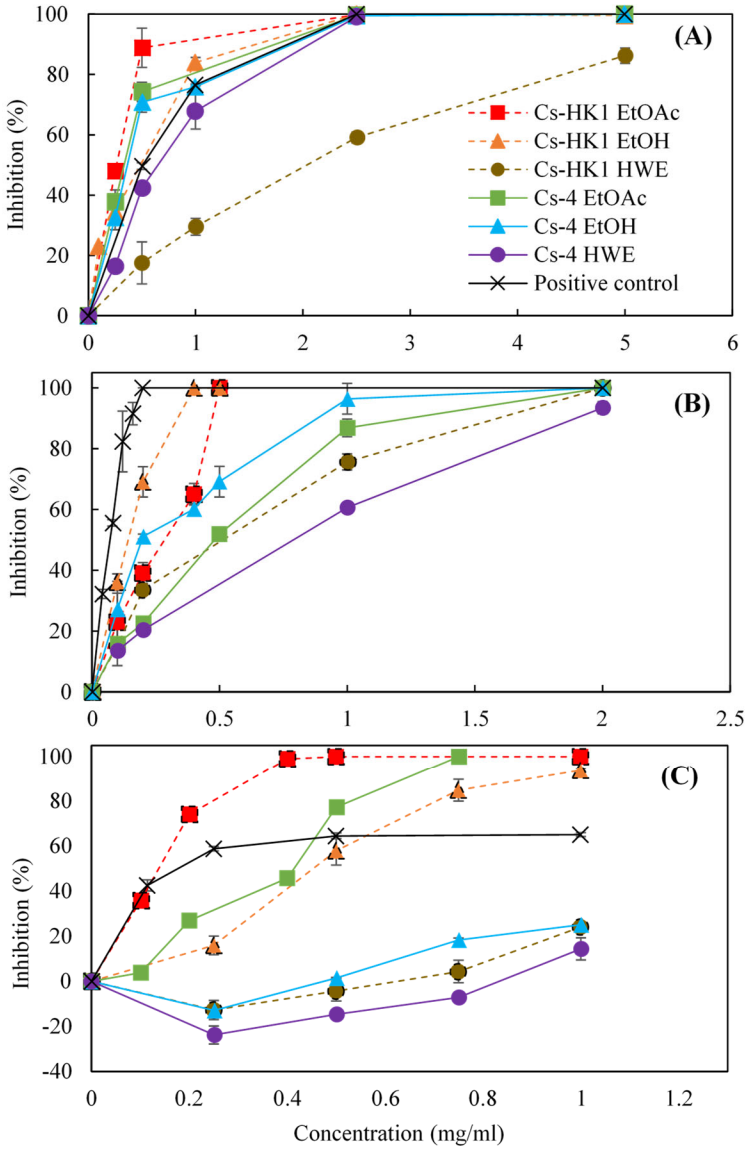

FIG. 1: The inhibitory effects of various concentrations of different mycelial extracts from *Ophiocordyceps sinensis* strains Cs-HK1 and Cs-4 on tyrosinase activity (arbutin as the positive control) (A), collagenase activity (EGCG as the positive control) (B), and elastase activity (EGCG as the positive control) (C). EGCG, epigallocatechin gallate; EtOAc, ethyl acetate; EtOH, ethanol; HWE, hot water extract.

were from *Chroogomphus rutilus* (IC50 = 0.46 mg/mL) and *Handkea utriformis* (IC50 = 0.78 mg/mL); these IC50 were comparable to that of the Cs-4 HW extract.

TABLE 3: The IC_{50} of different mycelial extracts of Cs-HK1 and Cs-4 on the tyrosinase, collagenase and elastase activity and SPF for different mycelial extracts at final concentration of 0.2 mg/ml.

Extracts	IC_{50} value (mg/ml)			SPF
	Tyrosinase	Collagenase	Elastase	
Cs-HK1				
EtOAc	0.28 ± 0.02	0.29±0.03	0.14±0.01	16.2 ± 1.6
EtOH	0.53 ± 0.02	0.14±0.01	0.46±0.04	11.3 ± 2.7
HW	2.49 ± 0.01	0.60±0.01	n.d.	10.2 ± 0.5
Cs-4				
EtOAc	0.33 ± 0.02	0.47±0.01	0.37±0.01	3.1 ± 0.2
EtOH	0.36 ± 0.02	0.20±0.01	n.d.	25.4 ± 0.3
HW	0.71 ± 0.05	0.87±0.12	n.d.	18.5 ± 1.8
Positive control				
Arbutin	0.64 ± 0.03	n.d.	n.d.	n.d.
EGCG	n.d.	0.07±0.01	0.19±0.01	n.d.
Homosalate	n.d.	n.d.	n.d.	4.0 ± 0.3

EGCG, epigallocatechin gallate; EtOAc, ethyl acetate; EtOH, ethanol; HW, hot water; IC_{50}, half-maximal inhibitory concentration; n.d., not determined.

It has been suggested that phenolic compounds including flavonoids, phenolic acids and stilbenes from natural sources are attributable to tyrosinase inhibition.[38-40] In comparison of the results between the flavonoid content (Table 2) and the anti-tyrosinase IC_{50} (Table 3), a rough correlation can be found as both are in the ranking order EtOAc > EtOH >HW. In addition to the individual contributions of constituents, the complex extracts of Cs-HK1 and Cs-4 mycelia can perform biological functions by synergistic or the cooperation action of different compounds.[41]

As shown in Fig. 1B and Table 3, the EtOH extracts of both fungi exhibited the most potent anti-collagenase activity and HW extracts the lowest activity. The EtOH extract of Cs-HK1 showed the highest potency with IC_{50} of 0.14 mg/ml, which was not significantly different from that of the positive control, EGCG (IC_{50} = 0.07 mg/ml) ($p > 0.05$). Similarly, the IC_{50} value of EGCG on collagenase was reported as 0.05 mg/ml by German-Baez and co-workers.[42] Collagenase is a zinc-containing proteinase which requires Ca^{2+} for activity, and its inhibitors are generally chelating compounds like EDTA, carboxylate, hydroxamate, phosphonate/phosphate and thiol that have binding affinity to Zn^{2+}or Ca^{2+}.[32,43] The constituents of *C. sinensis* such as amino acids which are capable of binding with heavy metal ions may be contributable to the anti-collagenase activity.[44,45] Previous studies have suggested that polyphenols and phenolic acids have collagenase inhibiting activity.[46-48] Besides, some polysaccharides may also contribute to anti-collagenase activity.[21]

As shown in Fig. 1C and Table 3, the elastase activity was inhibited by only three of mycelial extracts, EtOAc extracts of both fungi and EtOH extract of Cs-HK1. The EtOAc extract of Cs-HK1 had the most potent inhibitory effect with a low IC_{50} of 0.14 mg/ml, which was similar to positive control, EGCG (IC_{50} = 0.19 mg/ml) ($p > 0.05$, no significant difference). The results are comparable with those in the literature on the anti-elastase effects of medicinal plant extracts. Lee, Kim, Cho and Choi[49] reported the anti-elastase activities of 150 medicinal plants, most of which had IC_{50} values over 0.2 mg/ml except palm *Areca catechu* having an IC_{50} of 0.04 mg/ml. Similarly to the present study, German-Baez and co-workers [42] showed the less polar EtOAc extracts of several plants (tea) had stronger elastase inhibitory activity than the more polar methanol extracts. In another study,[15] 23 plant extracts from 21 plant species were tested against porcine elastase and all of these extracts had a lower inhibition than EGCG. In comparison with the literature results, the anti-elastase activity of Cs-HK1 EtOAc extract was among the highest.

It has been shown that flavonoids such as kaempferol, quercetin and myricetin have significant anti-elastase activities.[50] A flavonoid, 3'–hydroxyfarrerol, which has a similar chemical structure to the well-known serine proteinase inhibitors,

isocoumarins, may act as a reversible, non-competitive inhibitor of neutrophil elastase.[51,52] In the present study, the flavonoid content and anti-elastase activity have the similar trend EtOAc > EtOH > HW, which may suggest the possible contribution of flavonoid compounds to the elastase inhibition. However, the relationships requires verification through more vigorous assessment of the flavonoid constituents and their effects on the elastase activity.

C. Sunscreen effects of different mycelial extracts

The SPF of mycelial extracts are shown in Table 3. The Cs-4 EtOH extract exhibited the highest SPF value of 25.4, followed by several other extracts including the EtOAc, EtOH and HW of Cs-HK1 and HW of Cs-4 with the similar SPF values in the range of 10.2 to 18.5. The Cs-4 EtOAc had a relatively low SPF value of 3.1. According to USFDA[53], there are three types of sunscreens, namely minimal sun protection products of SPF2-12, moderate sun protection of SPF12-30, and high sun protection of SPF30 or above. Therefore, most of the mycelium extracts have moderate or minimal sun protection function. However, other components in the formulation such as esters, emulsifiers and emollients, which can interact with the sunscreen active ingredients to increase or lower the actual SPF.[54]

Sunscreen products usually provide photoprotective effects either by reflecting and scattering the UVR or by directly absorbing the light rays, so as to reduce the amount or intensity of harmful UVR reaching our skin.[55] The Cs-HK1 and Cs-4 extracts may contain molecules with similar structures to chemical sunscreens which can act as natural photoprotective agents.[56] Additionally, polysaccharides insides the mycelial extracts may also offer the photoprotective effects.[57] The photoprotective effect may be attributed to combined or cooperative action of different constituents of the extracts. Cs-HK1 and Cs-4 extracts are generally regarded as safe and non-toxic to human.[57,58]

D. Antioxidant activities of Cs-HK1 and Cs-4 mycelial extracts

As shown in Fig. 2, all mycelial extracts of the Cs-HK1 and Cs-4 fungi exhibited a concentration-dependent scavenging effect on DPPH radicals (Fig. 2A) and ABTS radical cations (Fig. 2B) at different levels. Table 4 presents the corresponding half-maximal effective concentrations for scavenging DPPH radicals and the TEAC values for scavenging ABTS radical cations. The EtOH extracts of both fungi showed the highest activity, the lowest half-maximal effective concentration for scavenging DPPH radicals, and the highest TEAC for scavenging ABTS radical cations. Overall, all the Cs-HK1 extracts exhibited stronger radical scavenging activities than the Cs-4 extracts with the same solvents.

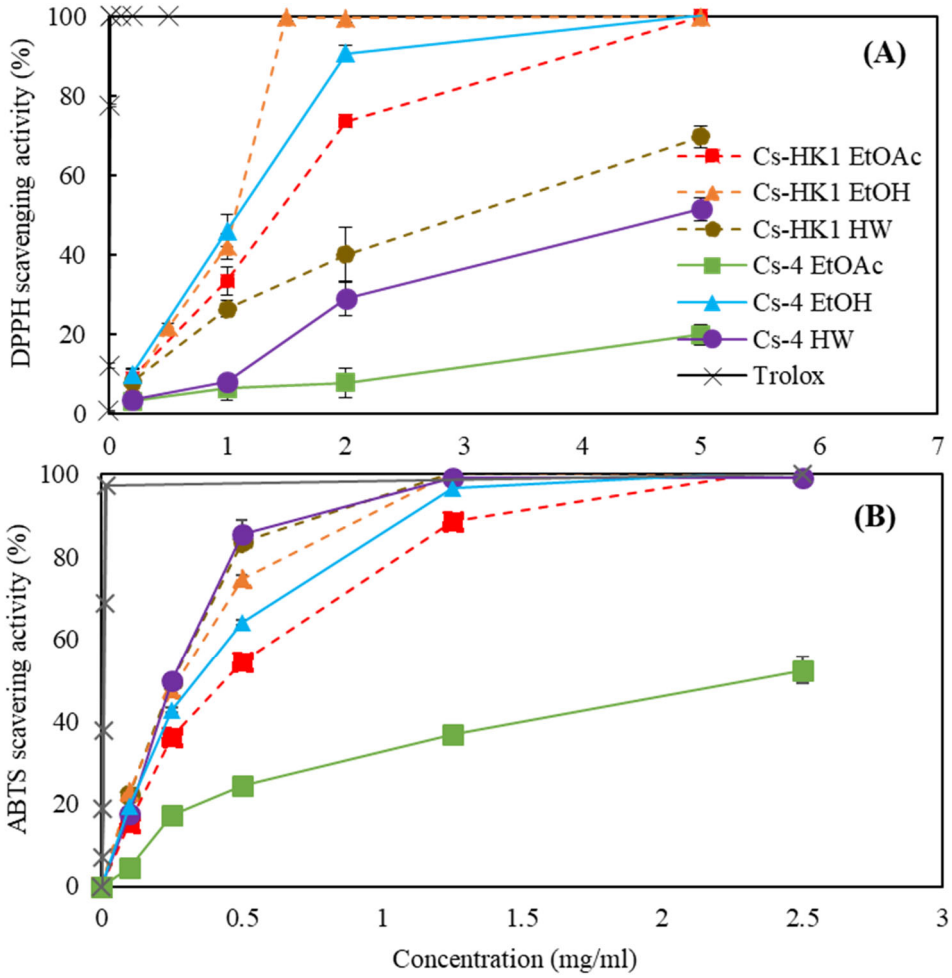

FIG. 2: Antioxidant activities of different mycelial extracts of *Ophiocordyceps sinensis* strains Cs-HK1 and Cs-4 in scavenging DPPH radicals (A) and ABTS radical cations (B) (Trolox-equivalent antioxidant capacity assay). Error bars represent the standard deviation (n = 3). EtOAc, ethyl acetate; EtOH, ethanol; HW, hot water.

TABLE 4: Antioxidant activities of different mycelial extracts from Cs-HK1 and Cs-4 measured by DPPH radical scavenging activities and TEAC assay.

Extracts	DPPH (EC_{50} in mg/ml)	TEAC (μmol Trolox/g)
Cs-HK1		
EtOAc	1.38 ± 0.1	111.0 ± 15.8
EtOH	1.08 ± 0.1	148.4 ± 29.0
HW	3.20 ± 0.2	100.3 ± 11.3
Cs-4		
EtOAc	9.21 ± 0.5	74.5 ± 26.3
EtOH	1.12 ± 0.1	131.8 ± 22.3
HW	5.68 ± 0.3	90.35 ± 3.9
Positive control		
Trolox in EtOH	0.01 ± 0	n.d.
Trolox in buffer	2.06 ± 0.1	n.d.

Differences were significant between data within the same column ($P < 0.05$). EC50, half-maximal effective concentration; EtOAc, ethyl acetate; EtOH, ethanol; HW, hot water; n.d., not determined; TEAC, Trolox-equivalent antioxidant capacity.

Two previous studies[59,60] have also shown that EtOH extracts of various medicinal mushrooms were more active in scavenging free radicals than HW extracts. Although phenolics and flavonoids are generally recognized as major antioxidant constituents, the activity data in Table 4 are not in a consistent trend with the phenolic and flavonoid contents shown in Table 2. Besides the total content, the structure and property of constituents can significantly influence the bioactivity.[61-63] In addition, other constituents such as polysaccharides, peptides and proteins also play a significant role in the radical scavenging.[64-66] Many previous studies have linked the anti-tyrosinase (whitening), anti-collagenase, anti-elastase, photoprotective properties with the antioxidant activities of plant and fungal extracts.[67-69] However, in the present study, a direct and quantitative correlation has not been established

between these skincare benefits and the antioxidant activities or the contents of antioxidants.

IV. CONCLUSIONS

This study systematically assessed the potential cosmetic benefits of 2 strains of *O. sinensis* through *in vitro* enzyme assays of the mycelial extracts. Some of the mycelial extracts showed potent antityrosinase, anticollagenase, and antielastase activity comparable to or more favorable than that of the reference compounds. In most cases, the least polar EtOAc extract was more active than the most polar HW extract. However, no clear or consistent correlation was found between the enzyme-inhibitory activity and the antioxidant activity or the major chemical components. The results substantiate the cosmetic benefits, encouraging the use of *O. sinensis* extracts for skincare and cosmeceutical applications with skin-whitening, antiwrinkle, antioxidative, and sunscreen functions. Further studies should be conducted to identify the active components and to investigate the underlying mechanisms of action and the structure-function relations of active ingredients.

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