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DEVELOPMENT OF COSMECEUTICAL FORMULATION FROM MYCELIAL EXTRACT OF *CORDYCEPS GRACILIS*

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ABSTRACT

Cordyceps species are known to possess different pharmacological properties such as antioxidant, immune enhacing and anti aging. Use of the extracts prepared from these medicinal fungi for development of products related to cosmetics will be a novel approach for cosmetic industry. Present invertigations have been carried out to develop co cosmeceutical formulations fro Cordyceps gracilis with antiinflammatory and antioxidant properties. The extracts prepared from this fungus were characterized for phenolic acids and ergosterol composition. The extracts were incorporated in a base cosmetic cream to develop antiaging formulations. There were no significant differences observed between phenolic acids, antioxidant activities and antityrosinase activities as compared to the formulation. Results obtained for cosmeceutical formulations showed significant antioxidant and antityrosinase properties. As evidient, these formulations can further be exploited as natural cosmeceutical ingredients.

KEYWORDS: *Cosmeceutical formulation, Cordyceps gracilis, ethanolic extract, bioactivities*

INTRODUCTION

Cordyceps fungi have long history of use as antiaging aging and posseses other pharmacological properties. The bioactive polysaccharides extracted from many *Cordyceps* species showed significan antioxidant and antibacterial activities (Sharma et al., 2015a; Sharam et al., 2015b) Recent studies have shownt the *Cordyceps* fungi significant pharmacological actions, including modulation of immune response (Wu et al., 2006), inhibition of tumor growth (Yoshida et al., 1989) and antihypertensive and antiarrhythmic effects (Manabe et al., 1996; Chiou et al., 2000). However, little research has been conducted on its antiaging effects. Skin aging is caused by an intrinsic or natural mechanism or hormonal changes occurring with age, and by extrinsic mechanisms associated with ultraviolet radiation exposure, which causes free radical species generation (Papakonstantinou et al., 2012) . Natural ingredients for antioxidant and antiaging products are becoming popular because of their role against generation of free radicals and reduced production of oxidative enzymes which cause various inflammatory diseases. Preparation of the antioxidant and antiaging products from *Cordyceps* species will be benificial interms of human health. Under Present investigations *Cordyceps gracilis* collected from wild and cultured through tissue culture. The mycelium was cultivated on liquid medium and harvested. The ethanolic extracts of mycelia were used to make cosmaceutical formulation.

MATERIALS & METHODS

Collection, culturing and processing of samples

Cordyceps gracilis was collected from Macleodganj (Dharamshala, H.P.). After noting the details pertaining to morphology and habitat, culturing of the samples was performed with the

tissue culture technique. Fresh fruit body was washed thoroughly 4 - 5 times with water, dipped into 70 % ethanol for 30 seconds, and then rinsed 3 - 4 times with sterile distilled water. Small sections of fruitbodies were cut and incubated on potato dextrose agar (SD Fine, India) at 25 °C. The isolated pure cultures were maintained at 25 ºC.The samples were then dried in wooden drier at 45 ºC and preserved in air-tight cellophane bags, with a small amount of 1-4-paradichlorobenzene in porous packets to keep them free of insects. The sample was deposited in the Herbarium Department of Botany, Punjabi University Patiala.

Biochemical composition of other bioactive compounds

Extract Preparation

Mycelial powder (2.0 g) was mixed with 150 mL of ethanol and extracted in a Soxhlet apparatus for 4 h. Finally, the solvent was evaporated under reduced pressure in rotary evaporator to obtain the dried ethanolic extract.

Bioactivities of Extract

Ethanol extract was extracted with 100 mL of methanol at 25 ºC (150 rpm) for 24 hours and filtered through Whatman no. 2 filter paper. The residues were again re-extracted with two additional portions of methanol (100 mL). These extracts were evaporated to dryness at 42 ºC, then redissolved in methanol at a concentration of 50 mg/mL, and stored at 4 ^{0}C. The dried methanolic extracts (100 mg) were shaken vigorously with 10 mL of acetone/hexane mixture (4:6) for 1 min. and filtered. The absorbance of the filtrate was measured at 453, 505, and 663 nm (Nagata & Yamashita, 1992). Lycopene contents were estimated using the following equation: Lycopene (mg/100mL) = (0.0458×$A663$) + (0.372×$A505$) − (0.0806×$A453$) β-carotene (mg/100mL) = (0.216×$A663$) − (0.304×$A505$) + (0.452×$A453$).

Phenolic compounds were quantified using Folin and Ciocalteu's phenol reagent. Extract was mixed with Folin and Ciocalteu's phenol reagent (1 mL). After three min., saturated sodium carbonate solution (1mL) was added to the mixture, and the volume was adjusted to 10 mL with distilled water. The reaction was kept in the dark for 90 min. and the absorbance was read at 725 nm. Gallic acid was used to calculate the standard curve (0.01-0.4 mM; R^2 = 0.9999) and the results were expressed as milligrams of gallic acid equivalents per gram of extract (Singleton & Rossi, 1965).

Anthocyanidins were quantified by using standard protocol (Vamanu & Nita, 2013). Briefly, 0.5 g of dried extract was mixed with the solvent (mixture of 85 : 15 (v/v) of ethyl alcohol and hydrochloric acid 1.5 M) followed by ultrasonication for 15 min. and filtration through Whatman filter paper no. 1. Standard solution was prepared with cyanidin chloride (con. 5-15 μg/mL). The absorption was measured at 546 nm. The total quantity of anthocyanidins (expressed in g of cyaniding chloride/100 g extract) = (Ap × mst × f × 100)/(Ast × mp), where Ap is absorption rate of the sample solution; mp is mass of the processed sample, in g; Ast is absorption rate of the standard solution; mst is mass of the processed standard solution, in g; and f is dilution coefficient.

For tyrosinase activity, ethanolic extract was dissolved in DMSO (50 %) at 10 mg/mL and diluted (10 - 0.62 mg/mL). Tyrosinase inhibition assay was performed using standard method [Yoon et al., 1994). The assays were carried out in a 96-well microplate with each well containing 40 µl of the sample, 80 µl of phosphate buffer (0.1 M, pH 6.8), 40 µl L of tyrosinase enzyme (60 units/mL), and 40 µl L of L-DOPA (3.5 mM). The mixture was incubated for 10 min at 37 ˚C, and the absorbance measured at 475 nm. L-ascorbic acid was used as the positive control and the results were compared with a control comprising 50% DMSO. The percentage of tyrosinase inhibition was calculated as follows: [(Acontrol Asample)/Acontrol] × 100.

Antioxidant activities

DPPH scavenging activity was measured by adding DPPH (200 μL) solution at different concentrations (2-10 mg/mL) to the formulations (0.05 mL) dissolved in ethanol. A control was prepared by adding an equal amount of ethanol to ascorbic acid (Vamanu, 2012).The absorbance was read after 20 min at 517 nm and the inhibition was calculated using the formula DPPH scavenging effect (%) = $A0 - AP$ $A0$ × 100 absorbance in the presence of the sample.

An ABTS radical scavenging activity of ethanolic extracts was measured through spectophotometric method (Li et al., 2011). Briefly, 10 µL of the dried extract was added to 4 mL of the diluted $ABTS^{\cdot+}$ solution (prepared by adding 7 mM of the ABTS stock solution to 2.45 mM potassium persulfate, kept in the dark, at room temperature, for 12-16 h before use). The solution was then diluted with 5 mM phosphate-buffered (pH 7.4) and absorbance was measured at 730 nm after 30 min. The ABTS radical scavenging activity was calculated as S % = (Acontrol−Asample/A control) ×100.

Reducing power estimation was done by mixing the dried extract with sodium phosphate buffer (pH 6.6), 1 mM $FeSO_4$, and 1 % potassium ferricyanide and incubated for 20 min. at 50 ˚C after that trichloroacetic acid was added and the mixtures were centrifuged. Supernatant (2.5 mL) was mixed with an equal volume of water and 0.5 mL $FeCl_3$ (0.1 %). The absorbance was measured at 700 nm (Papuc et al., 2010). Iron chelating activities of the samples were measured by mixing them with 3.7 mL of ultrapure water. The mixture was reacted with ferrous chloride (2 mmol/L, 0.1 mL) and ferrozine (5 mmol/L, 0.2 mL) for 20 min

and the absorbance was read at 562 nm with using EDTA as control.The chelating activities were calculated using the formula: chelating activity (%) = $[(Ab - As)] \times 100$, where Ab is the absorbance of the blank and As is the absorbance in the presence of the extract (Oyetayo et al., 2009).

The scavenging activities of superoxide anion radicals were measured following standard method (Marklund & Marklund, 1974). Dried extracts (0-2.0 mg/mL, 1 mL) and Tris-HCl buffer (50.0 mM, pH 8.2,3mL) were incubated in a water bath at 25 ˚C for 20 min. and to this pyrogallic acid (5.0 mM, 0.4 mL) was added. HCl solution (8.0 M, 0.1 mL) was added to terminate the reaction after 4 min. The absorbance of the mixture was measured at 320 nm. The scavenging ability if a sample was calculated using the following formula: scavenging ability (%) = $(1-A\text{sample}/A\text{control}) \times 100$.Where Acontrol is the absorbance of control without the sample and Asample is the absorbance in the presence of the sample.

Ferric reducing antioxidant power (FRAP) of all the formulations was calculated using TPTZ assay. Briefly, a reagent was prepared by mixing TPTZ (2.5 mL, 10 mM in 40 mM HCl), 25 mL of 300 mM acetate buffer, and 2.5 mL of $FeCl_3.6H_2O$. After this, freshly prepared FRAP reagent (1.8 mL) was taken in a test tube and incubated at 30 ˚C in water bath for 10 min. Then, absorbance was read at 0 min ($t0$). After this, formulaions extract (100 µL) and distilled water (100 μL) was added to the test tube, mixed, and incubated at 30 ˚C for 30 min. Then, the absorbance was taken at 593 nm ($t30$). Ferrous sulphate was used as standard (Benzie & Starin, 1996). FRAP activity was determined against a standard curve of ferrous sulphate. The values were expressed as μM Fe^{2+} equivalents per gram of extract and calculated using the following equation: FRAP value = Absorbance (sample + FRAP reagent) − Absorbance (FRAP reagent).

Development of the Cosmeceutical Formulations Based on Mushroom Extracts

The cosmetic cream used in this study is a commercial base cream purchased from local market. It is described as a white to yellowish color and free from fragrances, colorants, parabens, mineral and ethoxylates; and composed of purified water, vitamin E, chelating agents, silicone, and preservatives. The base-cream and the extract were carefully mixed to guarantee sample homogeneity and analyzed immediately after incorporation to study the ability of the extracts to maintain their bioactivities in the cosmetic base cream. The final cream formulations were extracted with methanol for 30 min, filtered, dried in a rotary evaporator.

RESULTS AND DISCUSSION

Chemical Characterization of the mycelial ethanolic extracts is presented in Table 1. *Cordyceps gracilis* showed the significantaly higher values (62.17 ± 5.2 mg/100g) for phenolic compounds. Cosmeceutical formulation was also analyzed with approximately same contents (56.23 ± 5.8 mg/100g). There was not any significant difference observed in the phenolic compounds mycelial extract and cosmeceutical formulation. The results are consistent with previous reports on extracts of other medicinal mushrooms (Taofiq et al., 2015). There was not any significant difference observed in lycopene content of the ethanolic extracts (0.46 g/100g) and cosmeceutical formulation (0.43 g/100 g). The results indicating that cosmeceutical formulation of *Cordyceps gracilis* contained significant amount of phenolic compounds and lycopene which are directly linked to the antioxidant properties and antiaging properties. EC50 values for antityrosinase activity of *Cordyceps gracilis* cosmoceutical formulations (2.21 ± 0.8 mg/mL) was measuered higher as compared to *Cordyceps gracilis* extract (0.89 ± 0.0 mg/mL).

Anthocynadin content of ethanolic extracts of *Cordyceps gracilis* mycelia were slightly higher (11.21 ± 1.2 mg/100 g) as compared to Cosmecutical formulation (10.4 ± 1.3 mg/100g) prepared from ethanolic extracts. There was not any loss observed in the anthocynadin content of ethanolic extract as well as cosmeceutical formulation.

Results obtained for higher antioxidant of *Cordyceps gracils* are in confirmity with previous results on different extracts of *Cordyceps gracilis* (Sharma et al., 2015a).

Table 1:

Bioactivities	C. gracilis Extracts	Cosmeceutical Formulation
Lycopene(g/100 g)	0.46 ± 0.0	0.43 ± 0.0
Phenolic compounds (mg/100 g of gallic acid)	62.17 ± 5.2	56.23 ± 5.8
Antityrosinase (mg/mL)	0.89 ± 0.0	2.21 ± 0.8
Anthocyanidins (mg cyanidin chloride/100 g extract)	11.21 ± 1.2[b]	10.4 ± 1.3[b]

Antioxidant Properties

Antioxidant properties of all the formulations were expressed as EC_{50} values and are presented in table 2. Higher EC_{50} values indicate lower effectiveness and lower values indicate higher effctiveness. EC_{50} values obtained for DPPH radical scavenging activity in the *Cordyceps gracilis* etahnolic extract and cosmeceutical formulation showed slightly differences in effectiveness in antioxidant properties. Mycelial formulation of *Cordyceps gracilis* showed lower EC_{50} values as compared to cosmeceutical formulation. However there was not significant difference observed for DPPH scavenging activities of ethanolic extract and cosmeceutical formulation. Similar results were obtained for ABTS radical scavenging activities. EC50 values of *Cordyceps gracilis* extract were found to be slightly lower (3.23 ± 0.6 mg/mL) as compared to cosmeceutical formulation (3.58 ± 0.7 mg/mL) showing slight lower ABTS radical scavenging activities.

Reducing power of ethanolic extract was measured slight higher as compared to cosmecutical formulation. However, there was not much difference observed in the reducing power properties of both. Similar results were obtained for iron chelating, scavenging activity and ferric reducing antioxidant power. It is evident from the results that there is not much difference in the antioxidant properties of ethanolic extract of *Cordyceps gracilis* mycelia and cosmeceutical formulation.

Table 2: EC50 values of different antioxidant assays

Antioxidant Assays	C. gracilis Extract	Cosmeceutical Formulation
DPPH radical scavenging activity(mg/mL)	2.22 ± 0.3	2.98 ± 0.1
ABTSmg/mL)	3.23 ± 0.6	3.58 ± 0.7
Reducing power(mg/mL)	3.79 ± 0.2	4.06 ± 0.2
Fe^{2+} chelating activity (mg/mL)	2.22 ± 0.1	2.86 ± 0.2
Scavenging on superoxide anion radical (mg/mL)	1.98 ± 0.3	2.41 ±0.1
FRAP (mol Fe^{2+} quivalents/g DW)	1.76± 0.5	2.50 ± 0.1

CONCLUSIONS

Based on the above findings, it can be concluded that ethanolic extracts of *Cordyceps gracilis* mycelia have the ability to cope with oxidative stress promoting and acting as skin anti-aging. The extracts revealed significant bioactivities and antioxidant properties. Incorporating the extracts into the base cosmetic cream still showed significant activities and suppression of tyrosinase activity. These different properties displayed by the Cordyceps gracilis mycelial extracts and after incorporation in the base cream formulations suggest that formulation are capable to antioxidant properties which are directly linked with antiaging properties. However, studies in dermal and epidermal cells should be conducted in order to clarify the role of the compounds responsible for the assessed bioactive properties.

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