

Chemical Components of a Submerged Mycelial Extract of *Calocybe indica* and its Anti-inflammatory and Antiaging Properties

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Abstract

Calocybe indica or milky mushrooms, grown in submerged culture, is a potential source of antiaging substances. This study investigated the chemical components, cytotoxicity to RAW 264.7 macrophage cells, and anti-inflammatory activity to human skin keratinocytes (HaCaT) of the ethanolic extract prepared from *C. indica* mycelia grown in submerged culture. Gas chromatography-mass spectrometry (GC-MS) analysis revealed the presence of 50 bioactive compounds, which were primarily categorized into the following major groups: fatty acids (81.97%), esters (4.25%), ketones (1.68%), aldehydes (1.62%), sterols (0.76%), phenols (0.45%), terpenes (0.36%), and others (8.91%). These act as major contributors to its anti-inflammatory and cosmeceutical properties. The *C. indica* mycelial extract was non-toxic to RAW 264.7 macrophage cells. Comparable and significant levels of anti-inflammatory activity were observed in both the mycelial extract and control barley β -glucan. The results show a stimulation of the anti-inflammatory cytokine interleukin-10 (IL-10) at 250.7 pg/mL or 63.4% and an inhibition of the pro-inflammatory cytokine IL-6 at 88.3 pg/mL or 69.8%. The anti-inflammatory effect of prostaglandin E₂ (PGE₂) was observed with a release of PGE₂ at 20.31 pg/mL or 70.8% inhibition. Cosmeceutical formulations made with the mycelial extract, particularly in essence form, followed by gel forms, maintained the extract's bioactivities. The mycelial extract served as an effective antioxidant agent in cosmeceutical formulations that can inhibit collagenase, inflammation, and acne-causing bacteria, providing a dual approach to simultaneously treating both skin aging and acne. This research offers novel insights into *C. indica* mycelium as a valuable source of nutraceutical and pharmaceutical compounds that contribute to various human health benefits. The mycelial extract of *C. indica* possesses no toxic effects and has multiple cosmeceutical functionalities. The results suggest that the mycelial extracts of *C. indica* could be further developed to produce safe natural ingredients for inclusion in nutraceutical and cosmeceutical products.

Keywords: Antioxidant, Anti-inflammation, *Calocybe indica*, Cosmeceutical, Mushroom, Nutraceutical, Submerged culture

Introduction

Mushrooms have long been used in nutraceuticals, pharmaceuticals, and as food. More recently, they have gained popularity in nutricosmetic and cosmeceutical applications due to their abundance of bioactive compounds and their potential to reduce reliance on synthetic chemicals. The mushroom's reproductive structure is known as the fruiting body, while its

vegetative form is called the mycelium. To date, around 80% of the bioactive compounds found in mushrooms have been extracted from their fruiting bodies. These compounds span a variety of chemical classes, including polysaccharides, polyketides, phenolics, triterpenoids, steroids, proteins, fatty acids, and antioxidants, which serve therapeutic purposes and can be utilized in

creating functional food products. In addition to their nutritional benefits, the fruiting bodies of mushrooms are recognized for their valuable anti-inflammatory, antioxidant, antitumor, immune-regulating, anti-aging, and antimicrobial effects [1-4]. However, cultivating fruiting bodies is time-consuming, requiring several months as well as substantial space and substrate volume. Furthermore, the quality of the final products is not always reliable. This makes commercial development extremely difficult for high-quality standardized health foods and medicines [5,6]. As a result, producing mushroom bioactive compounds through submerged mycelial cultivation has attracted significant attention. The main advantage of submerged mycelial cultivation is its independence from environmental conditions, controlling culture conditions in bioreactors, and its ability to continuously produce high-quality material, making submerged culture more reliable than extraction from fruiting bodies [7-10]. However, there may be differences in the chemical composition of the mycelium and fruiting bodies [11].

Inflammation is a natural and essential bodily response triggered by tissue damage or pathogenesis. It typically begins with a rapid initiation phase that stimulates a pro-inflammatory reaction, followed by a resolution phase [12]. While inflammation plays a crucial role in wound healing, disruption of this carefully regulated process can lead to uncontrolled or chronic inflammation, contributing to various disorders including cancer, sepsis, obesity, cardiovascular and neurological diseases, autoimmune conditions, and, in severe cases, death [13]. Inflammatory skin diseases are commonly encountered in dermatology. They present in a variety of forms, ranging from temporary rashes with itching and redness to long-term conditions like atopic dermatitis, rosacea, seborrheic dermatitis, and psoriasis. Cutaneous inflammation has been linked to many diseases, including cancer and visible skin aging [14]. However, visible skin aging can be reduced and prevented by daily use of antioxidants or anti-inflammatory cosmeceuticals, coupled with a diet rich in anti-inflammatory and antioxidant supplements [15]. Traditional treatments for skin wounds, including nonsteroidal anti-inflammatory drugs (NSAIDs), immunomodulatory drugs, and topical corticosteroids, focus on reducing inflammation [16]. However, these

treatments can hinder wound healing and lead to side effects such as skin thinning, osteoporosis, weight gain, and glaucoma [17]. Although it is essential to discover new anti-inflammatory agents with fewer side effects, this is a challenging goal due to the complexity of inflammation and its essential function in immune defense.

Calocybe indica, commonly referred to as the milky mushroom, is a tropical edible mushroom that grows at 25 - 38 °C. It ranks as the third most significant commercially cultivated mushroom in India, after button and oyster mushrooms [18]. Fruiting bodies of *C. indica* are a rich source of proteins, amino acids, vitamins, and minerals. Secondary metabolites such as polysaccharides, polyphenols, flavonoids, alkaloids, and triterpenoids are found in the fruiting bodies. They may have a role in antioxidation, anti-lipid peroxidation, anti-diabetic, and anticancer activities [18-21]. Most previous studies on *C. indica* investigated the bioactive substances and the biological activities of fruiting body extracts, with scant information on extracts derived from mycelia grown in submerged culture. Thus far, there are no studies on the chemical components and anti-inflammatory properties of biomass extracts from submerged *C. indica* mycelium. Following recent studies conducted by our group Vajrobol *et al.* [10], ethanolic extracts derived from optimal submerged growth of *C. indica* mycelia demonstrated cosmeceutical potential as a new source of multifunctional ingredients for anti-aging products and against acne-causing bacteria, which are nontoxic to human skin keratinocytes (HaCaT). Therefore, more research should be done to identify the biologically active components from *C. indica* mycelial extracts. This will yield a better understanding of the bioactive properties and their possible side effects.

The present study is the first systematic evaluation of both chemical composition and anti-inflammatory activity of ethanolic extracts derived from *C. indica* mycelia grown in submerged culture. For the first time, the mycelial extract was evaluated for *in vitro* cytotoxic effects on the macrophage cell line RAW 264.7 and anti-inflammatory properties on the HaCaT cell line. A potential mycelial extract for cosmeceutical applications was developed, potentially creating value-added

products by incorporation into cosmeceutical base products.

Materials and methods

Reagents and chemicals

Fetal bovine serum, antibiotics-antimycotics, and trypsin-EDTA were purchased from Gibco, USA. Dulbecco's Modified Eagle Medium (DMEM) was obtained from Biowest, USA. A prostaglandin E₂ high-sensitivity ELISA kit, IL-6 human ELISA kit, and IL-10 human ELISA kit were acquired from Abcam, USA. Tricine buffer was purchased from BIO-RAD, USA. Potassium persulfate, FeCl₃·6H₂O, and kojic acid were obtained from Merck, Germany. Phosphate-buffered saline (PBS), sodium dodecyl sulfate (SDS), epigallocatechin gallate (EGCG), 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS), 2,4,6-Tris 2-pyridyls-triazine, mushroom tyrosinase (EC 1.14.18.1), collagenase from *Clostridium histolyticum* (EC 3.4.24.3), 3-(3,4-dihydroxyphenyl)-L-alanine (L-DOPA), N-[3-(2-furyl)acryloyl]-Leu-Gly-Pro-Ala (FALGPA), and barley β -glucan were acquired from Sigma-Aldrich Chemical Co., USA. Mueller Hinton agar (MHA), Mueller Hinton broth (MHB), Tryptic Soy agar (TSA), Tryptic Soy broth (TSB), nutrient broth (NB), nutrient agar (NA), peptone, beef extract, and agar were purchased from Hi-media, India. All chemicals were of analytical grade.

Production of *C. indica* mycelia and preparation of mycelial extract

A pure culture of *C. indica* mycelium was received from the Thailand Mushroom Culture Collection Centre, Department of Agriculture, Ministry of Agriculture and Cooperatives. The *C. indica* mycelium was cultivated under the optimal conditions previously described by Vajrobol *et al.* [10]. An active inoculum was prepared by growing the fungus on potato dextrose agar (PDA) plates at room temperature (RT, 30 $\pm$ 2 °C) for 6 days. 15 5-mm diameter plugs taken from the mycelial growing edge were used as an inoculum in a 250 mL Erlenmeyer flask containing 50 mL of potato dextrose broth (PDB), initial pH 5.9, with pH uncontrolled during cultivation. The cultures were

incubated at 30 °C under static conditions for 14 days. The well-grown mycelia were filtered through Whatman No. 4 filter paper, followed by washing with large amounts of doubly distilled water, and drying at 60 °C.

Dried *C. indica* mycelia were ground into a fine powder, and an ethanolic extraction process was performed [10]. The obtained mycelial powder was macerated with 99% ethanol (1:1.5 w/v) under dark conditions at RT for 3 days and then filtered through Whatman No. 4 filter paper. Ethanol was then removed using a rotary evaporator (BÜCHI Rotavapor R-200) at 45 - 55 °C to obtain a dried ethanolic extract. The extracts were resuspended in 10% DMSO before determining their biological activities.

Biomolecular analysis by GC-MS

Gas chromatography-mass spectrometry (GC-MS) analysis of bioactive molecules present in the ethanolic extract of *C. indica* mycelia was performed using the method described by Shashikant *et al.* [22], with some adjustments. The mycelial extract (0.1 g/mL) was dissolved in GC-grade hexane in a 10:50 ratio. GC-MS analysis was performed using a Shimadzu QP2020 system (Shimadzu, Tokyo, Japan), which was equipped with a fused silica capillary column packed with an Rxi-5Sil MS GC capillary column (30 m length, 0.25 mm inner diameter, 0.25 μ m film thickness). The GC-MS program operated in split injection mode, with the injector temperature held at 250 °C. The GC program was initiated at 60 °C and ramped up at a rate of 10 °C per min until reaching 180 °C, where it was held for 2 min. Subsequently, the temperature was increased to 250 °C at a rate of 5 °C per min over 20 min. The helium gas flow rate was set to 1 mL/min, and the electron energy was maintained at 70 eV. The mass filter was configured to scan within the range of m/z 45 - 450. The resulting m/z spectra were analyzed using the NIST Mass Spectral database.

Cytotoxicity determination

Cell culture

Cytotoxicity was evaluated using the mouse macrophage-like cell line RAW 264.7 obtained from BioVision Cell Line Service, USA. It was maintained in DMEM supplemented with 10% fetal bovine serum (FBS), 100 Units/mL penicillin, and 100 μ g/mL

streptomycin under a 5% CO₂ humidified atmosphere at 37 °C. Then, the cell lines were washed with Dulbecco's Phosphate Buffered Saline (DPBS), and the cells were gently detached from the vessel using a cell scraper. The resuspended cells were centrifuged at 1,500 rpm at 4 °C for 5 min and then resuspended in DMEM.

WST-1 cytotoxicity assay

Cytotoxicity was determined using a WST-1 assay kit (Sigma-Aldrich Chemical Co., USA) following the method of Yin *et al.* [23]. Extract solutions in DMEM at different concentrations (0.15 - 20 mg/mL) were added to separate wells with 200 µL of RAW 264.7 cells (2×10^6 cells/mL) and then incubated under a 5% CO₂ humidified atmosphere at 37 °C for 24 h. Next, the treated cells were washed with DPBS before adding 10 µL of a WST-1 solution and 100 µL DMEM in each well and further incubating at 37 °C for 30 min. Absorbance was measured at 450 nm using a microplate reader (Thermo Scientific Multiskan Go, Thermo Fisher Scientific). The cell viability (%) was calculated as (absorbance of sample/absorbance of control) × 100. The morphology of RAW 264.7 cells was examined with an inverted microscope (TMS-F, Nikon, Japan).

Lactase dehydrogenase cytotoxicity assay

Cell membrane toxicity was assessed following the protocol of an LDH assay kit (Abcam, USA). Extract solutions were added to separate wells with RAW 264.7 cells (2×10^6 cells/mL) and then incubated under a 5% CO₂ humidified atmosphere at 37 °C for 24 h. Lysis solution was used as a positive control, and cells in DPBS served as a negative control. After incubation, the supernatant was collected, and the treated cells were washed with DPBS before examining the cell morphology with an inverted microscope. A 10 µL aliquot of supernatant was then mixed with 200 µL of substrate or WST solution and further incubated at 25 °C for 30 min. Absorbance was measured at 450 nm using a microplate reader. The cell membrane toxicity (%) was calculated as [(absorbance of samples – absorbance of negative control) / (absorbance of positive control – absorbance of negative control)] × 100.

Evaluation of anti-inflammatory activity

Human skin cell culture

Anti-inflammatory activity was assessed in HaCaT cells received from CLS Cell Line Service, Germany, and kept in DMEM with 10% FBS and 1% penicillin-streptomycin, under a 5% CO₂ humidified atmosphere at 37 °C. Then, the HaCaT cells were washed with DPBS before resuspending them in 3 mL of 0.25% trypsin/EDTA. The resuspended cells were centrifuged at 1,500 rpm at 4 °C for 5 min and then resuspended in DMEM [10].

Anti-inflammatory effects of cytokines

Anti-inflammatory testing was performed following a modified method of Kim *et al.* [24] using an enzyme-linked immunosorbent assay (ELISA), where cells were exposed to UVA and UVB radiation and analyzed for cytokine production. HaCaT cells were seeded into 24-well plates at a density of 2×10^6 cells/mL. Cells were pretreated with 10 mg/mL of the extract solution in DMEM and then incubated under a 5% CO₂ humidified atmosphere at 37 °C for 24 h. After this time, the treated cells were washed and suspended in 500 µL of PBS before UVA and UVB stimulation in a sun simulator chamber for 22 s (0.32 J/cm²). Next, PBS was removed and the cells resuspended in 600 µL DMEM before further incubating at 37 °C for 24 h. The culture supernatants were analyzed for IL-6 and IL-10 levels using ELISA assay kits. After 24 h of incubation, the supernatant from each well was collected and mixed with 50 µL of biotinylated antibody reagent. The mixture was incubated for 2 h. Subsequently, the cells were washed 3 times with a buffer. Then, 100 µL of streptavidin-horseradish peroxidase reagent was added, and the mixture was incubated for 1 h. After washing the cells again, 100 µL of tetramethylbenzidine (TMB) substrate solution was added to the wells and incubated for 30 min in the dark before the reaction was stopped by adding 100 µL of 1 M H₃PO₄. The absorbance was measured at 450 nm using a microplate reader. Barley β-glucan was used as a reference.

Anti-inflammatory effects of prostaglandin E₂

The anti-inflammatory effect of prostaglandin E₂ (PGE₂) was assessed following the protocol of a PGE₂ ELISA assay kit. The HaCaT cells (2×10^6 cells/mL) were treated with 10 mg/mL of the extract in DMEM.

Preparation of the cell lines and the UV stimulation were conducted as described in the previous section, discussing the anti-inflammatory effects of cytokines. After 24 h of incubation at 37 °C, 100 µL of the supernatant from each well was collected and mixed with 50 µL each of PGE₂-AP conjugate and PGE₂-AP antibody. The mixture was incubated under shaking at 500 rpm at ambient temperature for 2 h. Subsequently, the cells were washed 3 times with a washing buffer, and then 100 µL of pNpp substrate was added to the wells and incubated for 1 h before the reaction was stopped by adding 50 µL of stop solution. The absorbance was measured at 450 nm using a microplate reader. Barley β-glucan and Diclofenac were used as references.

Development of cosmeceutical products with mycelial extracts added

Preparation of cosmeceutical formulations

Ethanollic mycelial extracts of *C. indica* as an active ingredient were formulated into 3 cosmeceutical products in the form of an essence, gel, and cream. Commercial cosmetic bases were kindly supplied by the Asoke Skin Hospital (Thailand) for use in this study. The base cream presented a white color, whereas the base gel and the base essence were free of colorant. All cosmetic base formulations were fragrance-free with a pH of 6.0. Furthermore, all cosmetic base formulations are certified as safe by the Thai Food and Drug Administration (FDA). Base formulations were supplemented separately with the mycelial extracts at 1.0% concentrations to cover all the bioactivities evaluated. The base formulations and the mycelial extracts were thoroughly mixed to ensure sample uniformity and analyzed immediately after incorporation to assess the mycelial extract's capability to retain its bioactivities in the cosmeceutical formulations. Biological activities of these cosmeceuticals were examined in terms of antioxidant, anti-collagenase, and the inhibitory effects against acne-causing bacteria. Base formulations with 10% DMSO instead of mycelial extract were used as a negative control.

Analysis of antioxidant and anti-collagenase properties

DPPH free radical scavenging ability was used to evaluate antioxidant activity as described previously

[10]. The reactions were conducted in 96-well plates, and the absorbance was measured using a microplate reader. DPPH scavenging activity of samples was expressed as a percentage of radical scavenging or inhibition. Anti-collagenase activity was evaluated following the method of Vajrobol *et al.* [10]. The resulting values were applied for determination of the percent collagenase inhibition.

Inhibitory effects against acne-causing bacteria

Two acne-causing bacteria, *Cutibacterium acnes* (DMST 14916) and *S. aureus* (TISTR 1466), were used. *C. acnes* (DMST 14916) was purchased from the Department of Medical Science Thailand Culture Collection, while *S. aureus* (TISTR 1466) was obtained from the Thailand Institute of Scientific and Technological Research Culture Collection. These are 2 inflammatory acne-causing bacterial strains.

C. acnes was inoculated onto MHA and incubated at 37 °C under anaerobic conditions for 72 h. An anaerobic gas pack (Mitsubishi, Japan) and jar were employed. One loop-full of *C. acnes* was inoculated into MHB and incubated at 37 °C for 3 - 4 days under an anaerobic static condition until the visible turbidity was equal to the 0.5 McFarland standard, approximately 1.5×10^8 CFU/mL. *S. aureus* was activated on NA and incubated at 37 °C for 24 h. One loop-full of this bacterium was inoculated into 10 mL of NB, followed by incubation with shaking at 200 rpm for 1 - 2 h at room temperature until the visible turbidity was equal to the 0.5 McFarland standard.

Antibacterial activity against acne-causing bacteria was assessed using a microdilution method with a 1:1 ratio of sample to bacterial inoculum. A 100 µL aliquot of bacterial inoculum was mixed with 100 µL of mycelial extract, or cosmeceutical formulation with mycelial extracts added. *C. acnes* was then incubated at 37 °C for 72 h under anaerobic conditions, while *S. aureus* was incubated at 37 °C for 24 h. Subsequently, the turbidity of the broth cultures after incubation was measured at 600 nm as an indication of growth inhibition. Bacterial inhibition (%) was calculated as $[(A_{600} \text{ of control} - A_{600} \text{ of sample}) / A_{600} \text{ of control}] \times 100$, where A_{600} of control represents the turbidity before incubation and A_{600} of sample represents the turbidity after incubation.

Statistical analysis

All experiments were conducted in triplicate, with results shown as mean $\pm$ standard deviation (SD) values. One-way analysis of variance (ANOVA) was used to analyze the experimental data with the Statistica 10.0 software package (StatSoft Inc., Tulsa, OK, USA). Differences between means for each treatment at the 5% ($p < 0.05$) level were considered statistically significant.

Results and discussion

Extraction yield of *C. indica* mycelia grown in submerged culture

Our previous study found that submerged growth of *C. indica* mycelia has potential as a source of multifunctional ingredients for anti-aging products and against acne-causing bacteria. More research is necessary to identify the biologically active components from *C. indica* mycelial extracts for a better understanding of their bioactive properties. Therefore, the present study focused on analysis of the biomolecules of *C. indica* mycelial extracts. *C. indica* mycelia obtained from a 14-day submerged culture was collected and extracted with ethanol to evaluate its biological components and properties. Ethanol was chosen as the extraction solvent due to its capability to effectively and safely extract a wide range of bioactive compounds as well as its environmental friendliness. Additionally, our previous study noted that the ethanolic extract of *C. indica* mycelia displayed greater cosmeceutical activities, including antioxidant, anti-collagenase, and antibacterial activities, than ethyl acetate and water extracts [10]. This technique obtained mycelial biomass of 7.12 g/L and an extraction yield of 8.3% of the dry mycelia used. The mycelial extracts were viscous semisolids with a light brown appearance.

Biomolecules of *C. indica* mycelia by GC-MS analysis

GC-MS analysis, a highly effective method for detecting and identifying a wide range of substances, was used to identify the biomolecules present in *C. indica* mycelia cultivated in submerged culture. The results revealed a variety of bioactive compounds in the mycelial extract, with fifty biologically active compounds identified and primarily classified into the following major groups: fatty acids (81.97%), esters

(4.25%), ketones (1.68%), aldehydes (1.62%), sterols (0.76%), phenols (0.45%), terpenes (0.36%), and the rest of the constituents representing 8.91% of the total. **Table 1** displays the chemical composition along with the corresponding retention times and molecular formulas. *C. indica* mycelium is a promising source of biologically active substances, mainly fatty acids, which correlates well with anti-inflammatory, antioxidant, anti-cancer, and antimicrobial properties [22,25].

The ethanolic extract of *C. indica* mycelia was composed of 14 fatty acids, representing more than half (81.97%) of the constituents identified in the mycelial extract (**Table 2**). Most of the detected fatty acids were unsaturated, while the rest were saturated. Eight unsaturated fatty acids were detected: 9,12-Octadecadienoic acid (Z,Z)- or linoleic acid; oleic acid; ethyl oleate; n-Propyl 9,12-octadecadienoate; 13-Docosnamide, (Z)-; 15-Tetracosenoic acid, (Z)-, TMS derivative; 9,12-Octadecadienoic acid (Z,Z)-, TMS derivative; and 9-Octadecenamide, (Z)-. Six saturated fatty acids were present: n-hexadecanoic acid or palmitic acid; octadecanoic acid or stearic acid; hexadecanoic acid ethyl ester; octadecanoic acid ethyl ester; tetradecanoic acid or myristic acid; and pentadecanoic acid. *C. indica* mycelia was predominantly composed of linoleic acid (20.21%), followed by palmitic acid (18.53%), oleic acid (16.35%), and stearic acid (12.35%). Numerous studies have identified fatty acids as the primary component in fruiting bodies of edible mushrooms such as *Agaricus bisporus*, *Auricularia polytricha*, *Ganoderma lucidum*, *Hericium erinaceus*, *Pleurotus eryngii*, and *P. ostreatus* [26,27]. Consistent with our findings, earlier research reported that linoleic acid is the predominant fatty acid found in the fruiting bodies of *C. indica* and *G. lucidum* [25,28]. Furthermore, the predominant fatty acids found in the fruiting bodies of *G. lucidum* were linoleic, palmitic, oleic, and stearic acids [25], which agrees with our results. Linoleic acid is classified as an essential fatty acid and has been previously reported to exhibit antioxidant and anti-inflammatory properties [29]. It also plays a role in regulating blood pressure, particularly in the context of cardiovascular diseases and arthritis, and helps reduce triglyceride levels. As a result, it reduces the risk of cardiovascular diseases [30].

Table 1 Bioactive compounds identified in an ethanolic extract of *C. indica* mycelia by GC-MS analysis.

RT (min)	PA (%)	Molecular formula	MW	Compounds	Nature of compounds
10.53	0.24	C ₉ H ₁₉ BO ₃	186	2-Isobutoxy-5,5-dimethyl-[1,3,2]dioxaborinane	Others
11.04	0.24	C ₇ H ₁₁ NO ₃	157	2-Pyrrolidinecarboxylic acid-5-oxo-, ethyl ester	Esters
11.88	0.45	C ₁₄ H ₂₂ O	206	2,4-Di-tert-butylphenol	Phenol
13.11	0.66	C ₁₄ H ₂₉ Br	276	2-Bromotetradecane	Alkanes
15.05	0.23	C ₁₀ H ₁₄ N ₂ O ₃	210	3-Methyl-1,4-diazabicyclo[4.3.0]nonan-2,5-dione, N-acetyl-	Ketones
15.25	0.41	C ₁₀ H ₁₃ NO ₂	179	1-Propanone, 1-(2-aminophenyl)-3-methoxy-	Ketones
15.76	0.41	C ₁₄ H ₂₈ O ₂	228	Tetradecanoic acid	Fatty acids
16.51	0.66	C ₉ H ₁₅ N ₃	165	Tetrydamine	Others
16.57	0.32	C ₂₁ H ₄₄	296	Heneicosane	Alkanes
16.92	0.75	C ₁₃ H ₂₁ N ₃ O ₂	251	3-Acetaminoacetamide, N-methyl-N-[4-(1-pyrrolidinyl)-2-butyryl]-	Amides
17.61	0.14	C ₁₅ H ₃₀ O ₂	242	Pentadecanoic acid	Fatty acids
18.05	0.36	C ₁₅ H ₂₄	204	Tricyclo[5.4.0.0(2,8)]undec-9-ene, 2,6,6,9-tetramethyl-, (1R,2S,7R,8R)-	Terpenes
18.63	0.40	C ₁₁ H ₁₈ N ₂ O ₂	210	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-	Ketones
18.79	0.41	C ₂₀ H ₄₀ O	296	Eicosanal-	Aldehydes
18.95	0.64	C ₁₁ H ₁₈ N ₂ O ₂	210	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-	Ketones
19.18	0.22	C ₁₄ H ₂₂ N ₂ O ₂	250	5,10-Diethoxy-2,3,7,8-tetrahydro-1H,6H-dipyrrolo[1,2-a:1',2'-d] pyrazine	Others
19.42	0.14	C ₂₂ H ₃₄ O ₄	362	1,2-Benzenedicarboxylic acid, butyl 8-methylnonyl ester	Esters
19.55	18.35	C ₁₆ H ₃₂ O ₂	256	n-Hexadecanoic acid	Fatty acids
20.13	3.03	C ₁₈ H ₃₆ O ₂	284	Hexadecanoic acid, ethyl ester	Fatty acid ethyl esters
20.29	0.22	C ₂₀ H ₄₂	282	Eicosane	Alkanes
20.77	0.17	C ₂₀ H ₃₆	276	Cyclohexene, 4-pentyl-1-(4-propylcyclohexyl)-	Cycloalkenes
21.34	0.28	C ₃₈ H ₆₈ O ₈	652	l-(+)-Ascorbic acid 2,6-dihexadecanoate	Esters
22.14	0.13	C ₁₆ H ₃₀ O ₂	254	2(3H)-Furanone, 5-dodecyldihydro-	Furanones
22.67	20.21	C ₁₈ H ₃₂ O ₂	280	9,12-Octadecadienoic acid (Z,Z)-	Fatty acids
22.78	16.35	C ₁₈ H ₃₄ O ₂	282	Oleic Acid	Fatty acids

RT (min)	PA (%)	Molecular formula	MW	Compounds	Nature of compounds
22.98	1.64	C ₂₃ H ₂₂ N ₂ O ₄	390	Phenylmethyl N-[1-[(3-hydroxyphenyl)aminocarbonyl]-2-phenylethyl]carbamate	Others
23.19	12.35	C ₁₈ H ₃₆ O ₂	284	Octadecanoic acid	Fatty acids
23.26	3.87	C ₂₀ H ₃₈ O ₂	310	Ethyl Oleate	Fatty acids
23.74	2.11	C ₂₀ H ₄₀ O ₂	312	Octadecanoic acid, ethyl ester	Fatty acids ethyl esters
23.81	0.34	C ₂₀ H ₃₆	276	Cyclohexene, 4-pentyl-1-(4-propylcyclohexyl)-	Cycloalkenes
23.88	0.18	C ₂₀ H ₄₂	282	Eicosane	Alkanes
25.28	0.65	C ₂₆ H ₄₉ NO ₄	439	Fumaric acid, 2-dimethylaminoethyl octadecyl ester	Esters
25.47	0.26	C ₁₉ H ₃₆ O ₃	312	Glycidyl palmitate	Esters
26.52	0.47	C ₁₈ H ₃₅ NO	281	9-Octadecenamide, (Z)-	Fatty acids
27.70	0.45	C ₂₇ H ₅₄ O	394	Heptacosanal	Aldehydes
28.04	1.35	C ₁₂ H ₂₃ NO ₂	213	3-Cyclopentylpropionic acid, 2-dimethylaminoethyl ester	Esters
28.13	0.64	C ₈ H ₂₀ N ₂ O	160	Bis(2-(Dimethylamino)ethyl) ether	Ethers
28.29	0.79	C ₁₃ H ₂₂	178	Bicyclo[10.1.0]tridec-1-ene	Others
28.38	0.77	C ₂₇ H ₅₄ O ₂ Si	438	15-Tetracosenoic acid, (Z)-, TMS derivative	Fatty acids
28.58	0.45	C ₂₆ H ₄₉ NO ₄	439	Fumaric acid, 2-dimethylaminoethyl octadecyl ester	Esters
28.69	0.69	C ₂₅ H ₄₂	342	1H-Indene, 1-hexadecyl-2,3-dihydro-	Others
28.83	0.19	C ₁₉ H ₃₆ O ₃	312	Glycidyl palmitate	Esters
29.02	0.69	C ₁₉ H ₃₈ O ₄	330	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester	Esters
29.20	0.32	C ₁₆ H ₃₄ S	258	tert-Hexadecanethiol	Thiol
29.42	0.76	C ₂₁ H ₄₂ O	310	Henicosanal	Aldehydes
30.46	0.95	C ₃₀ H ₅₀ O	426	Lanosterol	Triterpenoids
32.14	0.75	C ₂₁ H ₄₀ O ₂ Si	352	9,12-Octadecadienoic acid (Z,Z)-, TMS derivative	Fatty acid derivatives
32.77	2.37	C ₂₁ H ₃₈ O ₂	322	n-Propyl 9,12-octadecadienoate	Fatty acid esters
35.26	0.79	C ₂₂ H ₄₃ NO	337	13-Docosenamide, (Z)-	Fatty acid amides
41.55	0.76	C ₂₈ H ₄₂ O	394	Ergosta-5,7,9(11),22-tetraen-3-ol, (3β,22E)-	Sterols

Table 2 Groups of biomolecules identified in an ethanolic extract of *C. indica* mycelia using GC-MS analysis.

Biomolecule groups	Area (%)	Number of compounds
Fatty acids	81.97	14
Esters	4.25	9
Ketones	1.68	4
Alkanes	1.38	4
Aldehydes	1.62	3
Cycloalkenes	0.51	2
Triterpenoids	0.95	1
Thiol	0.32	1
Terpenes	0.36	1
Sterols	0.76	1
Phenols	0.45	1
Furanones	0.13	1
Amides	0.75	1
Ethers	0.64	1
Others	4.23	6
Total	100.00	50

The mycelial extract contained 9 ester molecules, representing 4.25% of the total compounds present, identifying a second group of biomolecules. These biomolecules play a significant role in enhancing the aroma and flavor of mushrooms, lending them their distinctive scent and taste, as observed in various natural products [31]. The 3-cyclopentylpropionic acid, 2-dimethylaminoethyl ester, and L-(+)-ascorbic acid 2,6-dihexadecanoate components are noted for their potent antimicrobial, noncytotoxic, and antioxidant properties. 3-cyclopentylpropionic acid and 2-dimethylaminoethyl ester have been identified in the genus *Pleurotus* [32], while L-(+)-ascorbic acid 2,6-dihexadecanoate, also known as a Vitamin C derivative, has been discovered in *G. lucidum* [27, 33]. The mycelial extract contained 7 ketones and aldehydes, representing 3.3% of the total compounds present. Ketones and aldehydes are aroma compounds that play a crucial role in enhancing the flavor of food products. These biomolecules exhibit

high biological activities and low toxicity, making them suitable for use in medicine, food flavoring, cosmetics, and pharmaceuticals [34]. The mycelial extract contained pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-, a pyrrole derivative previously reported to have anticancer and antioxidant properties [35].

GC–MS analysis of *C. indica* mycelial extract identified 2 terpenes, tricyclo[5.4.0.0(2,8)]undec-9-ene, 2,6,6,9-tetramethyl-, (1R,2S,7R,8R)-, and lanosterol. As previously documented, lanosterol has been exclusively identified in *Inonotus obliquus* or Chaga mushroom. It is known for its anticancer, antioxidant, anti-inflammatory, anti-aging, and anti-allergenic properties [36]. Low contents of phenols and sterols were found in the mycelial extract. Phenol and sterol compounds play crucial roles in antioxidant, antimicrobial, and anti-inflammatory activities. The mycelial extract contained an ergosterol derivative, Ergosta-5,7,9(11),22-tetraen-3-

ol, (3 β ,22E)-, previously identified in *G. lucidum* and *P. ostreatus* [27,37]. Ergosterol, found in fungal cell walls, acts as a precursor for the synthesis of Vitamin D₂. Maintaining adequate Vitamin D levels in the body is essential as it reportedly protects against conditions such as osteoporosis, cardiovascular risks, neurodegenerative diseases, and various cancers [38]. Additionally, the presence of 2,4-di-tert-butylphenol in the mycelial extract serves as an antioxidant to inhibit lipid peroxidation [39]. This substance is typically found in *P. ostreatus* and *A. bisporus* [40,41]. The findings suggest that *C. indica* mycelia grown in submerged culture are a significant source of bioactive compounds with considerable health-promoting properties. Notably, the ethanolic extract of these mycelia grown in submerged culture contains bioactive compounds linked to various biological activities, including anti-inflammatory, anti-aging, and antibacterial effects.

Cytotoxicity determination

The effects of *C. indica* mycelial extract on the cell viability of macrophage RAW 264.7 cells were assessed through a WST-1 assay to evaluate the safety of the

mycelial extract for further applications. This cell line has been previously established as a reliable *in vitro* model. From the results shown in **Figure 1**, the extracts exhibited non-toxicity to cells at concentrations reaching 2.5 mg/mL, and more than 80% cell viability was detected. The maximum tested concentration (50 mg/mL) inhibited cell viability by up to 55%. The IC₅₀ value was 17.01 mg/mL, remaining within the safe range for potential cosmeceutical application [10]. Furthermore, the extract at 0.625 mg/mL promoted remarkable proliferation, up to 10% in RAW 264.7 cells, compared to the control. Additionally, microscopic examination of macrophage cell morphology revealed intact and densely populated surviving cells after exposure to the mycelial extract at concentrations of 0.625, 1.25, and 2.5 mg/mL (**Figures 2(C) - 2(E)**). A similar result was found for the control RAW 264.7 cells in DMEM (**Figure 2(A)**). Based on the results of the WST-1 assay showing 80% cell viability, the *C. indica* mycelial extracts at 0.625, 1.25, and 2.5 mg/mL were chosen to further investigate cell membrane toxicity using the LDH cytotoxicity assay

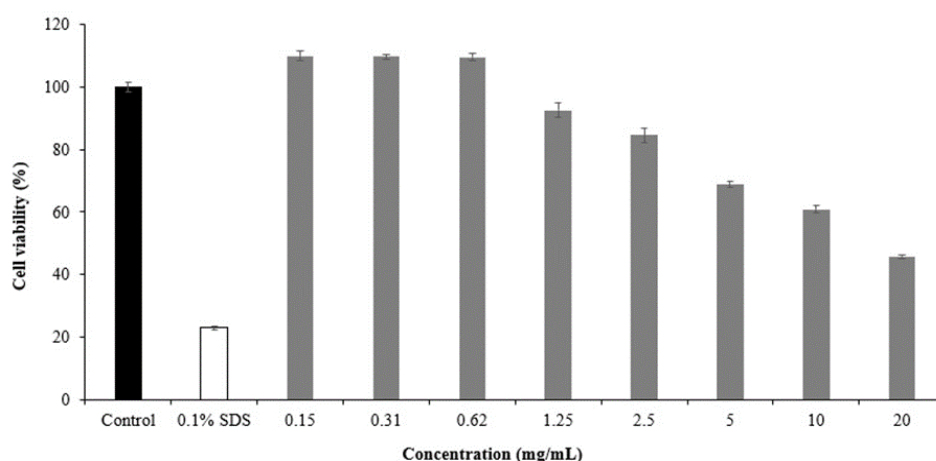


Figure 1 Viability of the macrophage RAW 264.7 cell line after treatment with ethanolic extracts of *C. indica* mycelia at various concentrations. Data are expressed as mean values $\pm$ SD (n = 3).

The LDH cytotoxicity assay is widely used as an effective indicator for evaluating the cytotoxic effects of bioactive compounds. It works by measuring the presence of lactate dehydrogenase (LDH), an enzyme that leaks from the cytoplasm when cell membranes are damaged. LDH catalyzes the reversible conversion of lactate to pyruvate, accompanied by the reduction of NAD⁺ to NADH. Consequently, WST-1 is reduced to

WST-1 formazan. The cell membrane toxicity value is related to the LDH level secreted from damaged cells. In the present work, cell membrane toxicity after treatment with the mycelial extract at various concentrations is illustrated in **Table 3**. All tested concentrations demonstrated non-toxicity to cell membranes compared to control RAW 264.7 cells in DPBS. Likewise, the presence of living RAW 264.7

cells after exposure to the mycelial extract at all concentrations was similar to that of the control in DPBS (**Figure 3**). Cell viability findings from the WST-1 assay align with the results obtained from the LDH

assay. These results indicated, for the first time, the safety of the ethanolic extracts of *C. indica* mycelia against macrophage cells and cell membranes.

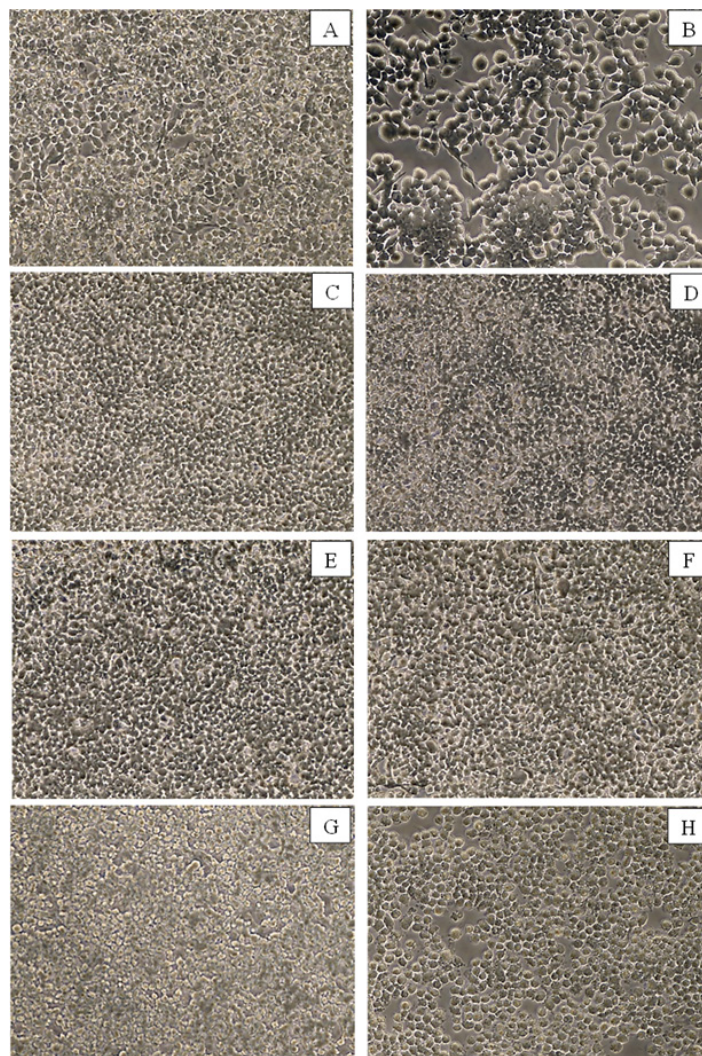


Figure 2 Microscopic images at 40X magnification of the macrophage RAW 264.7 cell line in control (A), treated with 0.1% SDS (B), treated with *C. indica* mycelial extract at 0.625 mg/mL (C), 1.25 mg/mL (D), 2.5 mg/mL (E), 5 mg/mL (F), 10 mg/mL (G), and 20 mg/mL (H).

Table 3 Cell membrane toxicity effect of *C. indica* mycelial extract on the macrophage RAW 264.7 cell line as measured by LDH cytotoxicity assays.

	<i>C. indica</i> mycelial extract (mg/mL)			Control	Lysis buffer
	0.625	1.25	2.5		
Cell membrane toxicity (%)	0.00 ± 0.59 ^a	0.03 ± 0.40 ^a	0.11 ± 0.23 ^a	0.00 ± 1.58 ^a	100.00 ± 0.08 ^b

All experiments were performed in triplicate and expressed as mean ± SD ($n = 3$).

^{a-b} Means within the column with different lowercase superscripts are significantly different ($p < 0.05$) from each other, using Tukey's Honestly Significant Test.

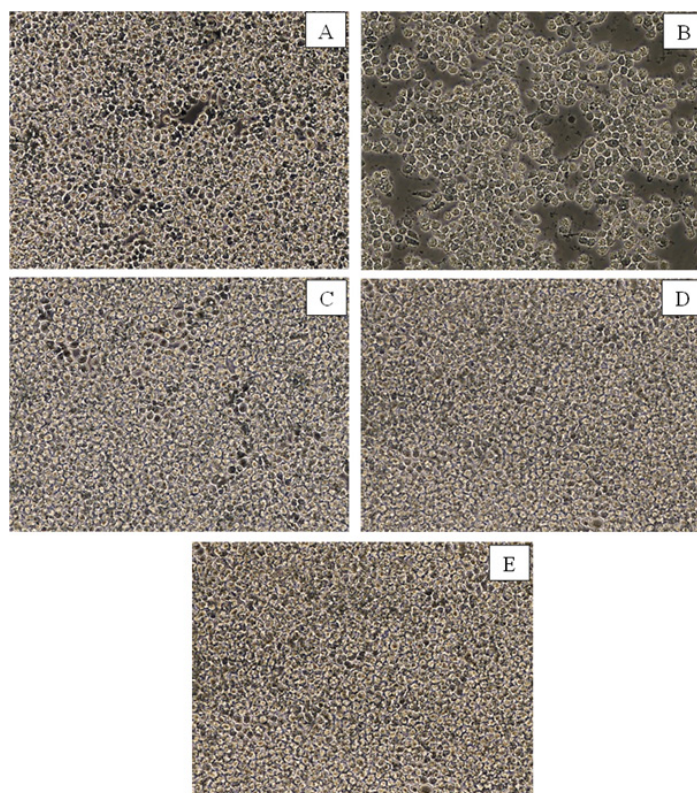


Figure 3 Microscopic images at 40X magnification of the macrophage RAW 264.7 cell line as measured by LDH cytotoxicity assays in control (A), treated with lysis buffer (B), treated with *C. indica* mycelial extract at 0.625 mg/mL (C), 1.25 mg/mL (D), and 2.5 mg/mL (E).

Evaluation of anti-inflammatory activity

Inflammation is a vital physiological process that plays a key role in complex biological functions, usually marked by symptoms like pain, heat, redness, swelling, and loss of function. Overproduction of pro-inflammatory cytokines such as IL-6 constitutes a significant aspect of the initial inflammatory response. Conversely, anti-inflammatory molecules like IL-10 are generated during sustained infections to regulate inflammation and maintain immune homeostasis [42-44]. Our previous studies found that a mycelial extract of *C. indica* was nontoxic to HaCaT cells at concentrations reaching 10 mg/mL [10]. Therefore, evaluation of anti-inflammatory activities was done at 10 mg/mL. The HaCaT cells were induced by UV exposure and subsequently treated with the samples. The *C. indica* mycelial extract at 10 mg/mL exhibited the lowest IL-6 level, 88.25 pg/mL, whereas a control

barley β -glucan exhibited an IL-6 level of 104.58 pg/mL (**Table 4**). This result indicates that the release of IL-6, an interleukin associated with inflammation, after treatment with barley β -glucan, is higher than that of the mycelial extract. The levels of IL-10 detected after treatment with barley β -glucan and the mycelial extract were 261.44 and 250.72 pg/mL, respectively, indicating that barley β -glucan can produce IL-10 to reduce inflammation more effectively than the mycelial extract. However, when comparing the IL-6/IL-10 ratios of the mycelial extract and barley β -glucan, which were 0.35 and 0.45, respectively, the mycelial extract gave a lower ratio than barley β -glucan. This indicates that the mycelial extract induced higher levels of IL-10 than IL-6, thereby significantly enhancing its anti-inflammatory effect.

Table 4 Anti-inflammatory activity of a *C. indica* mycelial extract on HaCaT cells in the presence of IL-6 and IL-10.

	Concentration (pg/mL)		Ratio IL-6/IL-10	% IL-6 inhibition	% IL-10 production
	IL-6	IL-10			
Control	124.26 ± 0.11	133.99 ± 0.04	0.92	-	-
UV	291.97 ± 0.10	187.61 ± 0.19	1.56	-	-
Control barley β-glucan	104.58 ± 0.20	261.44 ± 0.63	0.40	64.18 ± 0.28	59.34 ± 0.63
Mycelial extract	88.25 ± 0.33	250.72 ± 0.90	0.35*	69.77 ± 0.51*	63.40 ± 0.43*

All experiments were performed in triplicate, and the results are expressed as mean ± SD ($n = 3$). Statistical analyses were performed by means of one-way analysis of variance (ANOVA) followed by Tukey's range test at 95% confidence, * $p < 0.05$.

The mycelial extract also presented the anti-inflammatory effects of PGE₂ (**Table 5**). This assay measured the concentration of PGE₂, a lipid mediator derived from arachidonic acid through the action of cyclooxygenase, the rate-limiting enzyme in this pathway. PGE₂ interacts with 4 receptor subtypes (EP₁ to EP₄) to trigger responses such as fever, pain perception, and inflammation [45]. The release of PGE₂ from HaCaT cells after treatment with the mycelial extract at 10 mg/mL was 20.31 pg/mL, resulting in a 70.81% inhibition of PGE₂. It was significantly lower than the control and with exposure to UV. Interestingly, the mycelial extract exhibited effective anti-

inflammatory properties, comparable to a control barley β-glucan, with a percent inhibition of PGE₂ similar to Diclofenac, a common commercial anti-inflammatory drug. The investigations on anti-inflammatory activity across all experiments revealed the potential of the mycelial extract to be utilized as an anti-inflammatory agent. This marks the first report detailing its effects on cytokines and PGE₂ properties. The observed anti-inflammatory effect of the mycelial extract in this study may be attributed to the bioactive compounds present in the extract, linoleic acid, oleic acid, ethyl oleate, n-hexadecanoic acid, hexadecanoic acid ethyl ester, and fatty acid derivatives [22,37,40].

Table 5 Anti-inflammatory activity of *C. indica* mycelial extract on HaCaT cells in the presence of PGE₂.

	PGE ₂ (pg/mL)	% PGE ₂ inhibition
Control	44.32 ± 0.77	-
UV	69.57 ± 0.90	-
Control barley β-glucan (100 µg/mL)	23.72 ± 1.55	65.90 ± 1.75
Diclofenac (50 µg/mL)	15.52 ± 1.35	77.69 ± 1.53
Mycelial extract (10 mg/mL)	20.31 ± 1.02*	70.81 ± 1.45*

All experiments were performed in triplicate, and the results are expressed as mean ± SD ($n = 3$). Statistical analyses were performed by means of one-way analysis of variance (ANOVA) followed by Tukey's range test at 95% confidence, * $p < 0.05$.

Development of cosmeceutical products and their cosmeceutical properties

Our previous study [10] showed that an ethanolic extract derived from *C. indica* mycelia demonstrated cosmeceutical potential, as a new source of multifunctional ingredients for anti-aging products and

against acne-causing bacteria. *C. indica* mycelial extracts are promising candidates for creating topical formulations for cosmeceutical applications. The effects of cosmeceutical formulations in the form of an essence, gel, and cream on the cosmeceutical-related bioactivities were investigated. Base formulations of

essence, gel, and cream incorporating 10% DMSO instead of a mycelial extract were used as negative controls that exhibited a lack of antioxidation, anti-collagenase, and antibacterial activities against acne-causing bacteria. The bioactive properties of the produced cosmeceutical formulations were confirmed. All 3 formulated cosmeceutical products, especially the essence, followed by gel form, retained all cosmeceutical-related bioactivities exhibited by the mycelial extracts (Table 6). When the mycelial extracts were incorporated into cream and gel formulations, a decreased percent inhibition was noted. This decline may result from interference by the cream and gel bases, potentially limiting the availability of the mycelial extract to exhibit the same bioactivity. Additionally, the cream formulation exhibited lower cosmeceutical activity than gel formulations, likely due to its emulsion

structure, which consists of both aqueous and oily phases. The oily phase can entrap lipophilic compounds such as fatty acids, esters, ketones, alkanes, and cycloalkenes identified in the mycelial extract by GC-MS analysis, thereby slowing the diffusion of these compounds from the cream base. Moreover, entrapment also depends on the specific affinity and partition behavior of each lipophilic compound between the oil and aqueous phases [46,47]. Furthermore, Torrado *et al.* [48] reported that the skin permeability of melatonin in gel formulations significantly reduced the lag time, which was attributed to their lower viscosity compared to cream formulations. Low viscosity implies better spreadability, which may also explain how a gel containing Pemulen TR-1 exhibited the highest drug release compared to cream formulations [49].

Table 6 Cosmeceutical biological properties in different formulations based on an ethanolic extract of *C. indica* mycelia, 10 mg/mL.

Cosmeceutical formulation	Biological activity (% inhibition)			
	Antioxidation	Anti-collagenase	Anti- <i>C. acnes</i>	Anti- <i>S. aureus</i>
Mycelial extract	76.2 ± 0.7	84.5 ± 2.7	100.0 ± 0.0	100.0 ± 1.7
Essence	74.7 ± 1.4	82.5 ± 1.1	92.0 ± 1.0	90.7 ± 0.4
Gel base	68.6 ± 0.9	78.8 ± 3.5	87.0 ± 0.8	80.0 ± 1.6
Cream base	42.2 ± 0.6	57.5 ± 1.0	54.2 ± 2.2	58.2 ± 1.4

All experiments were performed in triplicate, and the results are expressed as mean ± SD ($n = 3$).

The color attributes of the produced cosmeceutical formulations with mycelial extract showed a light-yellow coloration with a pH of 6.0, which is considered within the suitable pH range for skin contact purposes. These achievements are of interest to the cosmetic industry for minimizing the use of synthetic ingredients. The results suggest that mycelial extracts of *C. indica* can be further developed as natural ingredients for use in cosmeceutical products. Many studies indicate that cosmetic products with a pH between 4.0 and 6.0 are generally preferred because most pathogenic bacteria grow optimally at neutral pH levels. Skin microflora and barrier homeostasis are maintained under these conditions [50, 51]. Based on the antioxidant properties exhibited by the produced formulations, the mycelial

extract can also scavenge free radicals and protect the formulations from auto-oxidation.

The growing demand for multifunctional products is driving innovation in the cosmeceutical industry, as budget-minded consumers seek items that offer both enhanced vitality and effective skin protection. The information obtained in the present study highlights the potential capacity of ethanolic *C. indica* mycelia extract to serve as an effective component in cosmeceuticals for blocking free radicals, potentially enhancing the skin barrier, offering anti-collagenase benefits to improve skin firmness, and providing anti-inflammation as well as anti-acne activities. Moreover, no apparent toxicity was detected in HaCaT keratinocytes and macrophage cells. This paper is the first report regarding a mycelial extract of *C. indica* grown in submerged culture with

potential development of cosmeceutical formulations that delay skin aging and have anti-acne properties.

Conclusions

This study is the first to examine the biochemical composition of *C. indica* mycelium grown in submerged culture, revealing that it is a promising source of bioactive compounds. GC-MS analysis highlighted a significant presence of fatty acids, along with several biologically active molecules categorized primarily into esters, ketones, aldehydes, and sterols. These findings indicate that *C. indica* mycelium holds potential as a source of nutraceutical and pharmaceutical compounds, offering numerous health benefits for humans. *C. indica* mycelial extract is highly valuable as it possesses no toxic effects and has multiple cosmeceutical functionalities. It contains promising antioxidant agents that can inhibit collagenase, inflammation, and acne-causing bacteria, providing a dual approach to simultaneously treating both skin aging and acne. These findings introduce an application of *C. indica* mycelia as a potentially safe natural ingredient, based on current *in vitro* results, for use in cosmeceutical and nutraceutical products. Conducting skin permeation studies using *in vitro* skin models, along with assessing the stability of formulations in terms of their ability to maintain bioactivity over various storage periods, will further confirm the potential of *C. indica* mycelial extract as a topical ingredient. Further investigations are currently in progress.

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Declaration of Generative AI in Scientific Writing

The authors declare that generative AI and AI-assisted technologies were not used in the writing process.

CRedit Author Statement

Natthawadee Vajrobo: Investigation, validation, analysis, data interpretation, and drafted the original manuscript. **Theerachart Leepasert:** Data interpretation. **Weerasak Taengphan:** Conducted some experiments. **Churapa Teerapatsakul:** Designed, conceptualized and conducted experiments, performed data interpretation, manuscript revision, and supervision.

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