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Antioxidant, anti-inflammatory, and cytotoxic properties of fruiting bodies and their mycelia as sources of *Cordyceps*

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ABSTRACT: Mushrooms play crucial roles as reservoirs of naturally occurring bioactive compounds. Among these, *Cordyceps militaris* is significant because of its well-established reputation for organoleptic excellence and positive health effects, which have led to its widespread commercialization. In contrast, the key properties of *Paecilomyces variotii*, an ectomycorrhizal symbiont, has received limited attention. In alignment with current research trends, the study of the mycelia and culture media of these mushrooms hold promise in identifying potential sources of valuable bioactive compounds. In the present study, we investigated *C. militaris* and *P. variotii* for their phenolic acids and sterols, assessing antioxidant capacity, anti-inflammatory effects, and anti-proliferative activity. Interestingly, *P. variotii* mycelia exhibited higher concentrations of ergosterol and phenolic compounds, with comparable levels observed in the fruiting bodies, along with superior antioxidant activity compared to that of *C. militaris*. In contrast, *C. militaris* mycelia demonstrated anti-inflammatory effects (which were absent in *P. variotii* mycelia) and cytotoxicity comparable to, and at times exceeding, that of its fruiting bodies (in contrast to *P. variotii*). In addition, the species analyzed in this study displayed variations in growth rates and mycelial production, which merit consideration for potential future applications and further study.

KEYWORDS: Anti-inflammatory, Bioactive compound, *Cordyceps militaris*, Mycelium, *Paecilomyces tenuipes*

INTRODUCTION

There has been considerable interest in the utilization of edible mushrooms as functional foods or as sources of natural medicinal components (Reis et al., 2017). *Cordyceps militaris* is widely recognized as one of the most favored and extensively consumed mushroom species. Beyond its organoleptic qualities, *C. militaris* is acknowledged as a valuable dietary source of bioactive compounds, with documented effects on reducing hyperlipidemia (Yu et al., 2021), and inhibiting tumor growth (Jin et al., 2018). Most of the available previous paper focuses on the polysaccharide fraction of *C. militaris*, which has been emphasized for its antioxidant, anti-aging, and anti-tumor (Zhang et al., 2019; He et al., 2020).

Paecilomyces variotii, functioning as an ectomycorrhizal symbiont, has the potential to form associations with a diverse range of plant hosts (Moreno-Gavíra et al., 2020). Nevertheless, there is limited data on the chemical composition and bioactivity of this mushroom, with antimicrobial activity having been reported (Herrera Bravo de Laguna et al., 2015).

In addition to the fruiting bodies, exploration of the mycelia and culture media employed in mushroom cultivation has been undertaken as potential sources of bioactive compounds (Antunes et al., 2020). Cultured mycelia are emerging as a promising alternative for obtaining fungal bioactive compounds, primarily due to shorter incubation times and more straightforward culture conditions (Oppong-Danquah et al., 2020). The fruiting bodies of *C. militaris* have previously been examined for their chemical composition and activities related to antioxidant and anti-inflammatory properties, with studies conducted at various harvesting periods (Won and Park, 2005). However, to the best of our knowledge, the anti-inflammatory activity of *P. variotii* has not been reported. Furthermore, neither the culture media nor the mycelia of both species have been investigated for their anti-tumoral and anti-inflammatory activities.

Thus, we conducted evaluations of their phenolic acid and sterol compositions, antioxidant capacity (including

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scavenging DPPH radicals and reducing power), along with assessments of their anti-proliferative activity (against MCF-7, HeLa, and HepG2 cell lines) and anti-inflammatory effects (down-regulating LPS-stimulated NO in RAW264.7 cells). The primary objective of this study was to assess the impact of varied culture conditions on the phenolic acid and ergosterol profiles, as well as the overall bioactivity of both species, aiming to comprehensively characterize their potential utility in food or pharmaceutical applications.

MATERIALS AND METHODS

Fruiting body and in vitro production of mycelia

Two types of fruiting bodies, *C. militaris* and *P. variotii*, were acquired from Hwasan farm (8, Gajwa 1-ro, Ilsanseo-gu, Goyang-si, Gyeonggi-do, Republic of Korea). *C. militaris* and *P. variotii* were grown based on brown rice and silkworm pupae, respectively. The two species of *Cordyceps* were germinated at 23°C and placed at 22°C for 40 days. Mycelium was isolated from sporocarps of each sample on potato dextrose agar medium (PDA) and potato dextrose broth (PDB) culture media. Mycelia were cultivated in Petri dishes with 15 mL of solid media and flasks with 50 mL of liquid media. These containers were incubated at 24 °C in the dark until mycelium covered most of the medium, approximately 14 days for *C. militaris* and 21 days for *P. variotii*. Radial growth measurements were recorded weekly from the time of inoculation until the complete growth of the mycelium, covering the entire available area. The mycelia were subsequently harvested from the medium. Fruiting bodies, mycelia, and culture media were lyophilized, ground into a 40-mesh fine powder, and weighed to determine the dry biomass.

Preparation of the extracts

The preparation of extract was determined according to the method of Liu *et al*. (2013) with some modifications. Extraction was conducted by shaking the samples (5 g) with methanol (50 mL) at 30 °C and 120 rpm for 6 hours. The resulting extract was separated from the residue through filtration using Whatman No. 2 paper into a round flask. The residue underwent a second extraction under identical conditions, and the filtrates from both extractions were amalgamated and then concentrated under vacuum.

Analysis of phenolic acids

The extracts were prepared in methanol (10 mg/mL), filtered through a 0.2 μm syringe filter, and subsequently subjected to analysis using an HPLC system (YL9100 Plus; YOUNG IN Chromass, Gyeonggi-do, South Korea). Separation was achieved on a Spherisorb (Phenomenex, Torrance, CA) reverse phase C18 column (3 μm, 150 mm × 4.6 mm i.d.) thermostatted at 25 °C. The solvents used were: (A) 2.5% acetic acid in water, (B) acetic acid 2.5%/acetonitrile (90:10), and (C) 100% HPLC-grade acetonitrile. The gradient employed was: isocratic 100% A for 10 min, 50% A and 50% B for 10 min, isocratic 100% B for 15 min, 90% B and 10% C for 10 min, 70% B and 30% C for 10 min, 50% B and 50% C for 5 min, 20% B and 80% C for 5 min, 100% A for 5 min, at a flow rate of 0.5 mL/min. Injection volume was 20 μl. Quantification (μg/g of extract) was performed by comparing the chromatographic peak areas (at 280 and 320 nm) with calibration curves (5–100 mg/mL) obtained from the corresponding commercial standards: protocatechuic acid, *p*-hydroxybenzoic acid, *p*-coumaric acid, and cinnamic acid, ranging from 5 to 80 μg/mL.

Table 1. HPLC calibration curves of phenolics standards

Phenolic compounds	Linear equation	R^2
Protocatechuic acid	y=40.26x	0.995
p-hydroxybenzoic acid	y=36.06x	0.994
p-coumaric acid	y=110.3x	0.997
Cinnamic acid	y=2.73x	0.992

Analysis of ergosterol

The extracts were dissolved in methanol (10 mg/mL), filtered through a 0.2 μm syringe filter and characterized by high performance liquid chromatography coupled to an ultraviolet detector. The mobile phase was acetonitrile/methanol (70:30, v/v) and detection was performed at 280 nm. The solvent flow rate was 1.5 mL/min and the injection volume was 20 μL. Ergosterol was quantified using a calibration curve obtained from a commercial standard. The calibration curve showed a high degree of linearity ($R^2 > 0.999$; y=35000x+13,273).

Antioxidant activity

The preparation of extract was determined according to the method of Reis *et al*. (2012) with some modifications. The extracts were dissolved in methanol at suitable concentrations, and various dilutions were prepared from the stock solutions, ranging from 0.005 to 50 mg/mL depending on the specific assay. The concentrations of the samples corresponding to 50% antioxidant activity or 0.5 absorbance (EC50) were determined from the graphs depicting DPPH antioxidant activity percentages or absorbance at 690 nm (for the reducing power assay) plotted against sample concentrations. Trolox prepared as the standard. DPPH radical scavenging activity was assessed using a microplate reader (SpectraMax 190, Molecular Devices LLC, CA, USA). Each well in the 96-well

plate contained a different concentration of the extracts and a methanolic solution containing DPPH radicals. After standing for 60 minutes in the dark, the reduction of the DPPH radical was determined by measuring the absorption at 515 nm. The radical scavenging activity was calculated as the percentage of DPPH discoloration using the equation: % DPPH radical scavenging activity = $[(A_{DPPH} - A_{SAMPLE})/A_{DPPH}] \times 100$, where A_{SAMPLE} is the absorbance of the solution after adding the sample extract at a specific level, and A_{DPPH} is the absorbance of the DPPH solution. This process was performed using the previously mentioned Microplate Reader. The extracts at various concentrations were mixed with sodium phosphate buffer and 1% potassium ferricyanide. After incubating each mixture at 50 °C for 20 minutes, 10% trichloroacetic acid was added. The resulting mixture was transferred to 48-well plates, along with deionized water and 0.1% ferric chloride, and the absorbance was measured at 690 nm.

Anti-inflammatory activity

The preparation of extract was determined according to the method of Samad *et al*. (2014) with some modifications. For the anti-inflammatory activity assay, methanolic extracts were dissolved in water at a concentration of 10 mg/mL. The mouse macrophage-like cell line RAW 264.7 was cultured in DMEM medium supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco, Rockville, MD, USA), glutamine, and antibiotics at 37 °C under 5% CO_2. In the experiment, a cell density of 5×10^5 cells/mL was utilized, with the proportion of dead cells being less than 5% based on the Trypan blue dye exclusion test. Cells were seeded in 96-well plates at 100,000 cells/well and allowed to attach to the plate overnight. Subsequently, cells were treated with various concentrations of each extract for 1 hour. A positive control for the experiment was 50 mM dexamethasone. The next step involved the stimulation with 1 mg/mL LPS for 12 hours. The effect of all the tested samples in the absence of LPS was also assessed to observe potential changes in Nitric oxide (NO) basal levels. In negative controls, no LPS was added. Both extracts and LPS were dissolved in supplemented DMEM.

For the determination of NO, a Griess Reagent System kit containing sulphanilamide, N-(1-naphthyl)ethylenediamine hydrochloride (NED), and nitrite solutions was employed. The cell culture supernatant was transferred to the plate and mixed with sulphanilamide and NED solutions. NO production was determined by measuring the absorbance at 540 nm using a microplate reader (SpectraMax 190, Molecular Devices LLC, CA, USA) and comparing it with the standard calibration curve. The results were expressed in EC50 values (mg/mL), representing the sample concentration providing 50% inhibition of NO production.

Cytotoxic activity

The preparation of extract was determined according to the method of Perez *et al*. (1993) with some modifications. The extracts were dissolved in water at a concentration of 10 mg/mL, and various dilutions were prepared from the stock solutions, ranging from 0.125 to 0.4 mg/mL. Human tumor cell lines MCF-7 (breast adenocarcinoma), HeLa (cervical carcinoma), and HepG2 (hepatocellular carcinoma) were utilized, with cell density determined through a sulforhodamine B assay. RPMI-1640 medium, containing 10% heat-inactivated FBS and 2 mM glutamine, was employed for routine maintenance of adherent cell cultures at 37 °C in a humidified air incubator with 5% CO_2. For the experiments, each cell line was seeded at an appropriate density (1.0×10^4 cells/well) into 96-well plates, and a procedure using the sulforhodamine B assay was conducted. The extract concentration that inhibited 50% of net cell growth was calculated from the graph depicting sample concentration against the percentage of growth inhibition and expressed in µg/mL of extract.

Statistical analysis

Three independent samples were utilized for each culture component, fruiting body, and fungal species. The data were presented as mean ± standard deviation. All statistical tests were conducted at a significance level of 5%. The results underwent comparison through one-way ANOVA (Zhang *et al*., 2020).

RESULTS AND DISCUSSION

Chemical characterization of the extracts

Table 2 presents the phenolic acids profile and ergosterol contents. Generally, the fruiting body exhibited higher phenolic acid contents. Notably, the mycelium of *P. variotii*, irrespective of culture conditions, displayed higher ergosterol contents (6.7–15.6 mg/g extract) compared to the corresponding fruiting body (4.3 mg/g extract). Limited data exist on the sterol content of *P. variotii*. *C. militaris* showed lower ergosterol contents, both in mycelia (0.7–1.5 mg/g extract) and the fruiting body (3.8 mg/g extract), in comparison to *P. variotii*. A previous report on *C. militaris* mentioned an ergosterol content of 2.5 mg/g dw (Huang et al., 2015), this simillar value was measured in commercial samples, potentially accounting for the parity with the present result.

Among the phenolic acids, p-hydroxybenzoic acid predominated in both mushrooms, with *P. variotii* exhibiting

Table 2. Phenolic acids composition (μg/g extract) in the mycelia and culture media of *C. militaris* and *P. variotii*.

		p-Hydroxybenzoic acid	*p*-Coumaric acid	Cinnamic acid	Ergosterol
C. militaris					
Fruiting body		200.0 ± 9.0	19.6 ± 1.7	82.5 ± 2.7	3.8 ± 0.2
PDB	Mycelium	113.2 ± 2.1b	nd	33.3 ± 1.8a	0.7 ± 0.0b
	Medium	nd	nd	nd	nd
PDA	Mycelium	125.90 ± 1.1a	nd	9.8 ± 1.1b	1.5 ± 0.1a
p-values		<0.001	-	<0.001	<0.001
P. variotii					
Fruiting body		1222.1 ± 1.8	33.8 ± 1.1	118.2 ± 2.1	4.3 ± 0.3
PDB	Mycelium	217.5 ± 1.2b	nd	91.1 ± 1.1a	15.6 ± 0.1a
	Medium	nd	nd	nd	nd
PDA	Mycelium	413.1 ± 2.3a	nd	28.5 ± 1.1b	6.7 ± 0.1b
p-values		<0.001	-	<0.001	<0.001

Values are given as mean ± standard deviation; nd: not detected; $p < 0.05$ indicates that the mean value of at least one component differs from the others (in this case, multiple comparison tests were performed). For each culture component, means within a column with different letters differ significantly ($p < 0.05$).

nearly sixfold higher quantities (1222.1 μg/g extract). Regarding *C. militaris*, the phenolic acids profile concurs with previous studies conducted on samples from the different harvesting years (Xiao et al., 2014). However, a similar study reported syringic acid and vanillic acid in quantities similar to p-hydroxybenzoic acid (Xiao et al., 2017).

Antioxidant activity

The EC50 values obtained for each antioxidant activity assay are summarized in Table 3. Across all evaluated cases, the highest activity was observed in the reducing power assay (*C. militaris*: 1.6 mg/mL extract; *P. variotii*: 0.6 mg/mL extract) and DPPH radical scavenging effects (*C. militaris*: 27.1 mg/mL extract; *P. variotii*: 0.8 mg/mL extract). The fruiting bodies of *P. variotii*, with their higher quantities of phenolic acids, exhibited superior antioxidant activity compared to *C. militaris*, sometimes reaching 15-fold higher values, as evident in the DPPH scavenging activity. However, the antioxidant activity values for *C. militaris* in this study were generally higher (except for the DPPH scavenging activity assay) than those reported previously (Reis et al., 2013). In a similar study of *P. variotii*, high radical scavenging activity and reducing power were reported, although direct comparisons were not feasible as the results were expressed in trolox equivalents (Battestin et al., 2008). Of particular interest in this work is the close (and sometimes superior) antioxidant activity measured in the mycelia and culture media compared to the fruiting bodies, highlighting the high potential of these fungal culture components. This is especially noteworthy for culture media, typically considered by-products of mushroom cultivation. The differences observed among the same culture media after being used to grow each

Table 3. Antioxidant activity (EC50 values, mg/mL extract) of the mycelia and culture media of *C. militaris* and *P. variotii*.

		DPPH scavenging activity	Reducing power
C. militaris			
Fruiting body		17.1 ± 0.2	1.2 ± 0.1
PDB	Mycelium	23.3 ± 0.1b	1.6 ± 0.0a
	Culture medium	27.1 ± 0.1a	0.8 ± 0.0c
PDA	Mycelium	18.3 ± 0.5c	0.9 ± 0.0b
p-values		<0.001	<0.001
P. variotii			
Fruiting body		0.4 ± 0.0	0.3 ± 0.0
PDB	Mycelium	0.5 ± 0.1b	0.5 ± 0.0b
	Culture medium	0.8 ± 0.1a	0.6 ± 0.0a
PDA	Mycelium	0.3 ± 0.1c	0.2 ± 0.0c
p-values		<0.001	<0.001

Values are given as mean ± standard deviation; $p < 0.05$ indicates that the mean value of at least one component differs from the others (in this case, multiple comparison tests were performed). For each culture component, means within a column with different letters differ significantly ($p < 0.05$).

mushroom suggest that the measured antioxidant activity is attributed to the mycelium, not the culture media components. Certain antioxidant compounds remain retained in the mycelium and are not released into the culture medium. Without exception, both the mycelia and culture media of *P. variotii* outperformed their counterparts in *C. militaris*. In line with observations from fruiting bodies, the results from *C. militaris* mycelia surpassed those reported previously, except for DPPH scavenging activity (Liu et al., 2020). However, it is crucial to consider that the results presented here were obtained with raw extracts, not purified fractions. The absence of studies on the mycelia of *P. variotii* limits direct comparisons.

Anti-inflammatory activity

Macrophages, key components of the innate immune system, play crucial regulatory roles in various immunopathological conditions during the inflammatory process (Wu and Lu, 2019). However, the excessive production of inflammatory mediators in uncontrolled inflammation processes can lead to adverse consequences in the pathogenesis of numerous inflammatory diseases, including cancer (Ansar et al., 2016). Hence, macrophages stimulated by LPS have been widely employed for in vitro evaluation of anti-inflammatory activities (Zhong et al., 2012). Moreover, due to the reproducible response of RAW264.7 macrophages to LPS, this cell line has been extensively utilized in inflammatory research (Ho et al., 2020). The anti-inflammatory activity was assessed using a LPS-stimulated RAW264.7 cell line (Table 4). The highest activity was observed in the methanolic extracts prepared from the fruiting bodies of *P. variotii*. The extracts of *C. militaris* also demonstrated the ability to suppress NO production, although to a lesser extent.

Regarding the evaluated culture components, noteworthy results were obtained, particularly for the mycelia of *C. militaris* grown in solid media, exhibiting higher anti-inflammatory activity than the corresponding fruiting bodies. This highlights the potential use of *C. militaris* mycelia in anti-inflammatory applications. Lin et al. (2014) suggested that the phenolic compounds of the fruiting bodies may contribute to their anti-inflammatory activities, inducing inhibition of NO production and iNOS expression in LPS-activated RAW264.7 cells. However, the anti-inflammatory activity of the mycelia of *P. variotii* was not maintained in either of the culture components, suggesting that the anti-inflammatory effects of the extracts might be related to other components besides ergosterol and phenolic acids.

Cytotoxicity

The results for anti-proliferative activity assessed in four human tumor cell lines (MCF-7, HeLa, and HepG2) are presented in Table 5. Extracts prepared from the fruiting bodies of *P. variotii* samples exhibited higher activity against MCF7 and HepG2. However, the same extracts could not inhibit the HeLa cell line, even at the maximum assayed concentration of 400 μg/mL extract. On the contrary, extracts from the fruiting bodies of *C. militaris* samples demonstrated a consistent behavior across all cell lines. The anti-proliferative activity of *C. militaris* extracts, specifically its polysaccharides fraction, was previously reported in HepG2, inducing apoptosis, cell cycle arrest at the S-phase, and intracellular production of reactive oxygen species (Di et al., 2018).

Despite generally lower activity against tumor cell lines, extracts from *C. militaris* did not exhibit a toxic effect on the primary cell line, in contrast to the observed toxicity for *P. variotii* fruiting bodies. Concerning the tumor cell lines, the mycelia of *C. militaris* displayed similar cytotoxicity to the fruiting bodies, often demonstrating higher activity. In some instances, such as the mycelium grown in PDA medium, the values were comparable to those obtained with purified polysaccharide fractions, representing a noteworthy result. On the other hand, the mycelia of *P. variotii* showed significantly lower anti-proliferative activity compared to the fruiting bodies. The culture media did not exhibit any anti-proliferative activity at the maximum assayed concentrations for both mushroom

Table 4. Anti-inflammatory activity (EC50) of mycelia and culture media of *C. militaris* and *P. variotii*.

		RAW264.7
C. militaris		
Fruiting body		311 ± 17
PDB	Mycelium	196 ± 11b
	Culture medium	>400a
PDA	Mycelium	151 ± 10c
p-values		<0.001
P. variotii		
Fruiting body		103 ± 11
PDB	Mycelium	90 ± 7b
	Culture medium	>400a
PDA	Mycelium	>400a
p-values		-

Values are given as mean ± standard deviation; $p < 0.05$ indicates that the mean value of at least one component differs from the others (in this case, multiple comparison tests were performed). For each culture component, means within a column with different letters differ significantly ($p < 0.05$).

Table 5. Anti-cancer extract (EC50) of mycelia and culture media of *C. militaris* and *P. variotii*.

		MCF-7	HeLa	HepG2
C. militaris				
Fruiting body		212 ± 10	196 ± 11	138 ± 10
PDB	Mycelium	170 ± 11b	218 ± 15b	162 ± 9b
	Culture medium	>400a	>400a	>400a
PDA	Mycelium	152 ± 10c	102 ± 10c	133 ± 11c
p-values		<0.001	<0.001	<0.001
P. variotii				
Fruiting body		93 ± 5	111 ± 7	68 ± 3
PDB	Mycelium	218 ± 10b	155 ± 7	175 ± 5b
	Culture medium	>400a	>400	>400a
PDA	Mycelium	199 ± 17c	>400	103 ± 11c
p-values		<0.001	-	<0.001

Values are given as mean ± standard deviation; $p < 0.05$ indicates that the mean value of at least one component differs from the others (in this case, multiple comparison tests were performed). For each culture component, means within a column with different letters differ significantly ($p < 0.05$).

species.

The primary objective of this study was to assess the potential of mycelia from *C. militaris* and *P. variotii*, along with their culture media, as alternative sources of bioactive compounds or ingredients for applications with antioxidant, anti-inflammatory, or cytotoxic activities. Generally, *P. variotii* mycelia exhibited higher contents of ergosterol and phenolic compounds, which were also more abundant in the fruiting body of this species. Additionally, the antioxidant activity was more pronounced in the components of *P. variotii*. However, these extracts did not display anti-inflammatory activity at the maximum assayed concentrations, in contrast to the observed results for *C. militaris* mycelia. Furthermore, the latter component exhibited cytotoxicity similar to or often superior to its fruiting bodies, unlike *P. variotii*, where mycelia showed a notable decrease in anti-proliferative activity. Overall, each culture component demonstrated distinct activity, which should be considered in conjunction with the growth rate and biomass yield for each mushroom.

Edible mushrooms, in general, are esteemed primarily for their nutritional value and bioactive properties mostly provided by different active substances, such as polysaccharides, lipids, peptides, sterols, or fiber (Gupta *et al*., 2018). The great majority of the studies reporting the previous features are conducted on the fruiting body, but the mycelia, as well as the culture media utilized in different stages of mushroom production, might also represent a good source of valuable compounds. Besides the differences in bioactive compounds and corresponding activities, the growth rate and yielded biomass of mycelia are of paramount importance, since these parameters might define the industrial interest of each species. As it can be seen, *C. militaris* presented a higher growth rate, despite the similarities in the produced biomass (except for the mycelia grown in PDB). In the following sections, the mycelia and culture media are compared by evaluating different bioactive compounds and biological activity indicators. In all tables, results corresponding to fruiting body of each studied species are also presented as reference values.

In our study, only PDA and PDB were used for mycelial culture. Based on this result, future studies on more diverse secondary metabolites using various media are needed.

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