*Pravara Med Rev; September 2026, 18 (03), 56-66*
*DOI: 10.36848/PMR/2026/9100000009*

***Original article***

# *Phytochemical screening, antioxidant activity, and GC–MS-based secondary metabolite profiling of Cordyceps militaris mycelium*

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**ABSTRACT**

**Background:***Cordyceps militaris* (C. militaris) is an entomopathogenic fungus belonging to Cordyceps genus. It has garnered increasing attention among researchers and pharma sector due to the array of bioactive compounds secreted by them. Mycelium, rootlike structure of C. militaris also contains several bioactive metabolites and can be easily controlled by altering the culture conditions to enhance certain bioactive compounds. Numerous studies on C. militaris primarily focus on evaluation of pharmaceutical potential of fruiting bodies and major bioactive compound Cordycepin. Very few studies have studied the therapeutic potential of mycelium of C. militaris. **Aim and Objectives**: The present study aims to screen the major type of phytochemicals secreted, the DPPH free radical scavenging ability and quantification of secondary metabolites by Gas Chromatography-Mass Spectrophotometry. **Results:** The ethanol extract of C. militaris mycelium were rich in phenols, flavonoids, alkaloids, tannins, terpenes, resin, carbohydrates, proteins, steroids, cardiac glycosides and amino acids. As the concentration of the mycelial extract increases, the free radical scavenging ability also increases. The mycelial extract possessed considerable antioxidant activity as compared with the antioxidant ascorbic acid. About 135 metabolites have been identified in the ethanol extract with majority of the metabolites belonging to the class of fatty acids and sterols. Palmitic acids and linoleic acids are the major bioactive metabolites identified in the extract. **Conclusion:** C. militaris mycelium possesses an array of bioactive compounds which have considerable antioxidant capacity and the bioactive compounds secreted are similar and closely related to the fruiting bodies of the mushroom.

**KEYWORDS**: Cordyceps militaris, Mycelium, Phytochemical screening, Antioxidant activity

## INTRODUCTION

*Cordyceps militaris* (C. militaris) is an entomopathogenic medicinal mushroom which belongs to the Kingdom Fungi, Division Ascomycota, Family Cordycipitaceae, Genus Cordyceps and Species C. militaris. It widely grows in temperate and tropic regions such as Nepal, China, Korea and Japan and in the Himalayas of India. It evades the immune system of the arthropods and adapts to the host systems body mechanism in order to survive and grow

from them (1). In Traditional Chinese Medicine (TCM), Cordyceps species has been mainly used for cardiovascular and respiratory ailments, treatment of hyperlipidemia and hyperglycemia, liver and kidney problems and to cure sexual dysfunctions (2). C. militaris is increasingly becoming important in pharmaceutical industry due to the immense bioactive compounds secreted by them such as cordycepin, D-mannitol, adenosine, carotenoids, polysaccharides, gamma aminobutyric acid (GABA), cordycepic acid and steroids (3). Recently, it has been identified that even mycelium (root like structures) of the C. militaris obtained from the *in-vitro* cultures can be employed as a dietary ingredient and as a supplementation product for humans. It also has been found to have therapeutic ability due to the presence of numerous bioactive compounds just like fruiting bodies (4).

The mycelium of C. militaris showed similar secretion of bioactive compounds as seen in fruiting bodies. Some of the bioactive compounds secreted in the mycelium of C. militaris are Cordycepin, D-mannitol, Ergothioneine, GABA, Lovastatin, Vitamins A, E, B2, B3 and C, Minerals such as Magnesium, Sulfur, Potassium, Selenium, Iron, Calcium and Zinc (2). Numerous pharmacological activities have been demonstrated by C. militaris which include anti-cancer, anti-viral, hypolipidemic, anti-hyperglycemic, anti-oxidant, cardioprotective, neuroprotective, anti-microbial, anti-inflammatory and immunomodulatory properties. Apart from possessing pharmacological actions, several health products in the form of supplements and capsules are already being manufactured and commercially approved in China with the claims of curing respiratory ailments such as cough, cold, asthma and fatigue. They are regularly consumed by the people of China (5).

Phytochemicals are plant based bioactive substances secreted for defence mechanisms against environmental stressors. Usually, these phytochemicals present with beneficial properties such as anti-oxidant, anti-viral, anti-tumour, anti-microbial, anthelmintic, antispasmodic and several other actions and they help in the regulation of gene transcription, immunity and possess therapeutic ability. So, it becomes imperative to screen for the common phytochemicals present to look for potential beneficial properties of the plant (6). Carotenoids, polyphenols, polysaccharides, phytosterols and saponins are phytochemicals possessing beneficial actions to humans. Free radicals are chemical compounds containing unpaired electrons that increase their reactivity rate. Usually, free radicals are formed from element oxygen giving rise to Reactive Oxygen Species (ROS). ROS production in low to moderate amounts are important for many physiological functions but a shift in the balance between production and elimination leads to increase in their levels contributing to oxidative stress (7). The unpaired electrons attack cells, tissues, RNA, DNA and proteins ultimately leading to apoptosis, necrosis and aging of the person. Mushrooms possess potent antioxidants such as glutathione and ergothioneine that are responsible for delaying aging (8). It becomes necessary to study the antioxidant capacity of edible medicinal mushrooms in order to know its therapeutic ability against ailments. A recent study has found out that aqueous and methanol extracts of C. militaris mycelium possess antioxidant capacity since the extracts were rich in phenolic and flavonoid content (9).

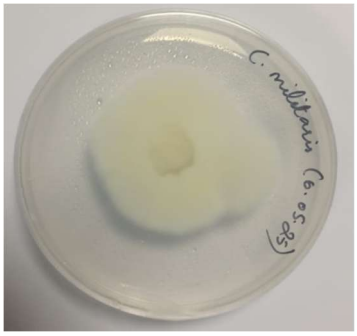



**Fig 1: Cordyceps militaris mother culture grown on Potato Dextrose Agar (PDA) medium**

Gas chromatography-mass spectrometry (GC-MS) based metabolomics is a chromatographic technique used for the identification and quantification of volatile, semi-volatile and even non-volatile metabolites especially small molecules such as sugars, amino acids, drugs, catecholamines, acids, alcohols in order to understand the complex mechanisms behind the many biochemical processes (10). Metabolomics analysis of C. militaris has been employed to study major classes of detected metabolites, to optimise the culture conditions for maximising bioactive compound synthesis, to analyse the differences in secretion of bioactive compounds at various stages of development and also used in identification of strain degradation. One such study revealed that the bioactive compounds Cordycepin, D-mannitol and xylitol are secreted in greater quantities at aging or late development phase in the fruiting bodies of C. militaris (11).

Majority of the works on C. militaris are either optimisation of the culture conditions to enhance the bioactive compounds secretion, evaluation of the therapeutic potential of Cordycepin in C. militaris under various pathological disorders, examination of the pharmacological potential of fruiting bodies of C. militaris. Very few studieshave worked on the mycelium of C. militaris which are mostly related to optimisation for bioactive compound production. This study extensively focuses on exploration of bioactive compounds produced by the mycelium via preliminary phytochemical screening, GCMS metabolite profiling and further evaluation of its antioxidant capability against free radicals. Differences in the secretion of bioactive compounds arise according to the culture conditions. Thus, it is important to study the secondary metabolites produced by the mycelium in order to determine the therapeutic ability.Therefore, the present study aimed to evaluate the phytochemical composition and antioxidant potential of *Cordyceps militaris* mycelium and to characterize its secondary metabolites using gas chromatography–mass spectrometry (GC–MS). The findings are expected to provide comprehensive information on the bioactive constituents of cultured mycelium and support its potential applications in functional foods, nutraceuticals, and pharmaceutical development.



**Fig 2: Cordyceps militaris mycelium cultivated by liquid fermentation approach**

## MATERIALS AND METHODS

### Fungal Material

The C. militaris CM-3 was purchased from a private company named GrowtheFunguy,in Kerala. The culture was stored at 4°C and they were sub cultured regularly on PotatoDextrose Agar (PDA) medium and stored at 4°C until further analysis.

### Chemicals and Reagents

Potato Dextrose Agar from HiMedia (Maharashtra, India), Sucrose, Peptone, Yeast extract, $KH_2PO_4$, $MgSO_4.7H_2O$, $(NH_4)_2SO_4$, Vitamin B1, Ethanol, Chloroform,2,2-Diphenyl-1-Picrylhydrazyl (DPPH) extrapure, 95%, Dimethyl Sulphoxide (DMSO), Ascorbic acid (Sisco Research Laboratories Pvt, Ltd, Maharashtra, India.)

### Cultivation of *Cordyceps militaris* Mycelium

The mother culture of C. militaris mycelium was grown on PDA agar medium and stored at 4°C and they were sub-cultured regularly and stored at 20°C until further use. C. militaris mycelium was cultured using liquid fermentation method. The fermentation medium consists of the following chemical constituents: Sucrose:25g/L, Peptone:10g/L, Yeast extract:20g/L, $KH_2PO_4$:3g/L, $MgSO_4.7H_2O$:3g/L, $(NH_4)_2SO_4$:10g/L, Vitamin B1:0.24g/L. The nutrient medium was prepared for 100 ml and sterilized. The fermentation medium was transferred to sterile flasks and the C. militaris mycelial plugs were cut using a sterile scalpel (1cmx1cm) and three pieces each were transferred to every flask containing nutrient medium. The contents were then incubated at $20^0$C for 7 to 10 days (12).

### Harvesting and Solvent extraction of Mycelium

The mycelium grown in the fermentation medium were carefully taken out using sterilized scalpel and gently cleaned using sterile distilled water and the residual water was removed. The wet weight of the mycelium was weighed. The mycelium was then cutin to very fine particlesand was added to the solvent ethanol.The amount of solvent added was 10 times that of the wet weight of the mycelium and the mixture was allowed to stand for 3 days with frequent shaking (maceration). At the end of the third day, the mixture was filtered through Whatman filter paper and the crude extract was stored at 4°C until further analysis.

**Preliminary Phytochemical Screening**

Qualitative Phytochemical screening was conducted in order to identify the major classes of bioactive compounds such as alkaloids, saponins, flavonoids, tannins, phenols, steroids, triterpenes, terpenoids, carbohydrates, proteins and amino acids secreted in the C. militaris mycelium.

**Test for alkaloids**

**Mayer's test:** The filtrates are added to Mayer's reagent (potassium mercuriciodide). A yellow precipitate shows that alkaloids are present in the extract (13).

**Wagner's test:** 2ml of the extract is taken and Wagner's reagent (iodine andpotassium iodide) is added to the extract. The formation of reddish-brown precipitateindicates the presence of alkaloids (14).

**Hager's test:** Hager's reagent (saturated picric acid solution) is added to extract.The yellow precipitate indicates the presence of alkaloids (13).

**Test for flavonoids**

**Lead acetate test:** 10% lead acetate solution was prepared and 1ml of the solutionis added to 3ml of aqueous extract. Flavonoids are present if any white or yellowprecipitate is seen (15).

**NaOH test:** Aqueous Sodium Hydroxide was prepared and 2ml was taken. 2ml ofaqueous Sodium Hydroxide and diluted hydrochloric acid were added to 2ml ofextract. A yellow orange colour solution formed should disappear after diluteHydrochloric acid was treated shows the presence of flavonoids (16).

**$H_2SO_4$ test:** Concentrated Sulphuric acid is added to a portion of the crudeextract. Appearance of orange colour solution shows that flavonoids are present(14).

**Ammonium solution test:** The extract is treated with little drops of 1% Ammoniasolution. The formation of a yellow coloration indicates the presence of flavonoids(17).

**Tests for phenols**

**Ferric chloride test (Brayer's test):** 3 to 4 drops of ferric chloride solution isadded dropwise to the extract. Blue-Black solution shows that phenols are present inthe extract (13).

**Folin–Ciocalteu test:** 20% sodium carbonate solution was prepared. 1ml Folin-Ciocalteu and 1 ml of 20% sodium carbonate are added in this order to 1ml of theextract. Dark blue colour formation indicates phenol.

**Test for tannins**

**Ferric chloride test:** 0.1% ferric chloride solution was prepared. A few drops ofthis solution are treated with the extract. A brownish green or bluish black formationindicates that tannins are present in the extract (16).

**Lead acetate test:** 1% lead acetate solution was prepared. Few drops of thissolution were treated to 2 mL of extract. Tannins are present when a yellow precipitateis seen. (18).

**Tests for steroids**

**Salkowski test:** 2 mL of aqueous extract was treated with 2 mL of chloroform and2 mL of Sulphuric acid. Shake the solution well. A reddish chloroform layer plus agreenish-yellow fluorescence indicates the presence of steroids in extract (18).

**$H_2SO_4$ test:** 1 gram of the powder is weighed. To the powder, a few drops ofacetic acid, acetic anhydride are treated. The mixture was warmed and cooled in tapwater. Concentrated Sulphuric acid was added drop by drop along the walls in the testtube. Green colour formation indicates that steroids are present in the solution (19).

**Test for terpenoids**

**Liebermann–Burchard test:** Ethanol, acetic anhydride, and few drops ofsulphuric acid is added to 1ml extract. The transition from pink to violet confirmsthat terpenoids are present in the extract (20).

**Test for terpenes**

**$H_2SO_4$ test:** 0.5 grams of the powder was weighed. 3ml chloroform was addedand shaken. They were then filtered. The filtrate was treated with 10 drops aceticanhydride and 2 drops of concentrated sulfuric acid. A reddish-brown colour formationat the middle of solution indicates that terpenes are present (21).

**Tests for anthraquinone**

**Borntrager's test (for free anthracene derivatives):** 0.5 grams of the powder wasweighed and 5 mL chloroform was added. The mixture was vortexed for 5 minutesand it was filtered. The filtrate was treated with equal volumes of 10% ammoniasolution. After shaking, a pink, violet or red coloration in aqueous layer shows thatfree anthraquinones are present in the extract (20).

**Tests for cardiac glycosides**

**Keller-Killiani test:** A drop of ferric chloride solution was added to glacial aceticacid. 2 ml of this mixture was treated with 5ml extract. The mixture was underlaidwith 1 ml concentrated sulphuric acid. A brown ring between two solutions indicates a deoxy sugar characteristic of cardenolides. In the acetic acid layer, a slow green colour ring formation and beneath the brown ring a violet ring might be seen. This indicatesthe presence of cardiac glycosides (22).

**Tests for anthocyanin**

**Test for anthocyanins:** 2N Hydrochloric acid was prepared. 2ml of this solutionand ammonia were treated with 2ml aqueous extract. The formation of pink-red thatmust change into blue-violet shows that anthocyanin is present in the extract (18).

**Test for anthocyanin and betacyanin:** 2N sodium hydroxide was prepared and 1ml of this solution was treated with 2ml of extract. The mixture was boiled for 5minutes at 100°C. Presence of blue green colour in the solution shows that anthocyaninis present in the extract and yellow colour solution shows that betacyanin is present inthe extract (23).

**Test for resins**

A few drops of acetic anhydride are treated with the 1ml ofextract. The 1ml Concentrated Sulphuric acid was added to the mixture. Orange coloursolution turning into yellow colour shows that resin is present in the extract (14).

**Test for oxalate**

A few drops of ethanoic/ glacial acetic acid are treated with 2mlof extract. Green black colour solution shows that oxalates are present in the extract(24).

**Tests for proteins and amino acids**

**Ninhydrin test:** 0.2 % Ninhydrin solution was prepared. The extract was treatedwith this 0.2% ninhydrin solution and boiled. When the solution turns into violet colour,it shows that amino acid and proteins are present (25).

**Xanthoproteic test:** 1 ml of concentrated Sulphuric acid was treated with 1 mL ofextract. White precipitate should be formed that when boiled would change intoyellow colour precipitate. Adding ammonium hydroxide to this yellow precipitate,must change yellow precipitate to orange colour. This shows that proteins are present inthe extract (18).

**Test for Carbohydrates**

**Molisch's test:** A few drops of Molisch's reagent was added to the crude extract followed by the addition of Conc. $H_2SO_4$ along the sides of the test tube. After two minutes, the solution was diluted by adding 5ml of distilled water. Formation of a light violet or red ring at the interphase indicates the presence of carbohydrates (26).

**Barfoed's test:** About I ml of the extract was mixed with 1ml of the Barfoed's reagent and heated in water bath for 2 minutes. The reddish precipitate formation indicates the presence of monosaccharides (26).

**Test for Vitamin C**

2,4-Dinitrophenylhydrazine test: The test sample was treated with dinitrophenylhydrazine dissolved in concentrated sulfuric acid. The formation of yellow precipitate indicated the presence of vitamin C (27).

**Antioxidant Assay**

**Free radical scavenging activity by 2,2-Diphenyl-1-picrylhydrazyl (DPPH) assay:** 0.1 mM DPPH solution was prepared and dissolved in 100 ml of ethanol in a standard flask. About 0.1 g of the mycelium was weighed and dissolved in 100 ml of distilled water. Ascorbic acid is taken as the positive control. The mycelium stock was prepared into varying concentrations ranging from 20-160 µg/ml. Similarly, ascorbic acid was prepared into these concentrations. All of these solutions were made up to final volume of 50 µl with DMSO. 3.4 ml of DPPH solution was added to all the test tubes. DMSO and DPPH were taken as the test control. All the test tubes were incubated at room temperature in dark for 20 minutes. The absorbance of the solution was read at 517 nm in UV-Visible spectrophotometer. The percent Radical Scavenging Ability (RSA) was calculated from the formula given below (28).

% RSA = Absorbance of control - Abs of sample / Absorbance of control X 100

**Secondary metaboliteanalysis by Gas Chromatography-Mass Spectrophotometry (GCMS)**

GCMS analysis of ethanol extract was performed using Shimadzu GCMS-QP2010 Plus system. The sample was introduced via split injection mode with an injection temperature of 250 °C and a split ratio of 30:1. The carrier gas flow was regulated under pressure control mode at 50.5 kPa, with a total flow of 32.8 mL/min, column flow

of 0.96 mL/min, and a purge flow of 3.0 mL/min. The column oven was initially held at 50 °C for 1 minute, ramped at 10 °C/min to 280 °C, and held for 2 minutes. The equilibrium time was set to 0.3 minutes. The mass spectrometer operated in scan mode with a mass range of m/z 40–900 and a scan speed of 3333. The ion source temperature was maintained at 200 °C, and the interface temperature was set at 280 °C. The solvent cut time was 3.00 minutes, and data acquisition was conducted from 3.5 to 26.0 minutes. Detector gain was set relative to tuning results at 1.06 kV + 0.10 kV, with a threshold of 1000. This standardized protocol was applied for ethanol extract to facilitate profiling of phytoconstituents. Almost all of the compounds were identified using mass spectra obtained from Wiley spectral library collection and NIST library. The volatile and non-volatile compounds were quantified based on the metabolites detected in mass spectrometer. The compounds were identified by comparing their retention times and fragmentation pattern of mass with published data as well as with Wiley and NIST mass spectral library.

**Results**

**Preliminary phytochemical screening**

The ethanol extract of C. militaris mycelium showed the presence of alkaloids, flavonoids, phenols, tannins, steroids, terpenes, cardiac glycosides, terpenoids, protein, resin, amino acids and carbohydrates. Alkaloids, flavonoids and terpenoids were present in higher quantities as compared to other phytochemicals. Phenols, proteins, amino acids and carbohydrates were present in very minimal quantities. Anthraquinones, coumarins, anthocyanins, oxalates and vitamin C were absent in the crude extractof the mycelium.

**Table 1: Preliminary qualitative phytochemical screening of the ethanol extract of C. militaris mycelium**

| S.NO | QUALITATIVE TESTS | ETHANOL EXTRACT |
|---|---|---|
| 1 | **TEST FOR ALKALOIDS** | |
| a) | Mayer's test | Present (++) |
| b) | Wagner's test | Present (+) |
| c) | Hager's test | Present (+) |
| 2 | **TEST FOR FLAVONOIDS** | |
| a) | lead acetate test | Present (+++) |
| b) | NaOH test | Present (+++) |
| c) | H2SO4 test | Present (+) |
| d) | Ammonia solution test | Present (+) |
| 3 | **TEST FOR PHENOLS** | |
| a) | Ferric chloride test | Absent (-) |
| b) | folin - ciocalteau test | Present (+) |
| 4 | **TEST FOR TANNINS** | |
| a) | ferric chloride test | Absent (-) |
| b) | lead acetate test | Present (+) |
| 5 | **TEST FOR STEROIDS** | |
| a) | Salkowski test | Present (+) |
| b) | $H_2SO_4$ test | Present (+) |
| 6 | **TEST FOR TERPENOIDS** | |
| b) | Liebermann burchard test | Present (+) |
| 7 | **TEST FOR TERPENES** | Present (+) |
| 8 | **TEST FOR ANTHRAQUINONE** | |
| a) | Borntrager's test | Absent (-) |
| 9 | **TEST FOR CARDIAC GLYCOSIDE** | |
| a) | Keller - killiani test | Present (+) |
| 10 | **TEST FOR COUMARIN** | Absent (-) |
| 11 | **TEST FOR ANTHOCYANIN** | Absent (-) |
| 12 | **TEST FOR RESINS** | Present (++) |

| | | |
|---|---|---|
| 13 | **TEST FOR OXALATE** | Absent (-) |
| 14 | **TEST FOR VITAMIN C** | |
| a) | 2,4- dinitrophenylhydrazine test | Absent (-) |
| 15 | **TEST FOR PROTEINS AND AMINO ACID** | |
| a) | xanthoproteic test | Present (+) |
| b) | ninhydrin test | Absent (-) |
| 16 | **TEST FOR CARBOHYDRATES** | |
| a) | mollisch's test | Present (++) |
| b) | barfoed's test | Absent (-) |
| + = trace presence, ++ = moderate presence, +++ = high presence, - = absence | | |
| | | |

**Free radical scavenging activity by 2,2-Diphenyl-1-picrylhydrazyl (DPPH) assay**

The anti-oxidant capacity of C. militaris mycelium was evaluated by the method of DPPH free radical scavenging assay. Ascorbic acid was taken as the positive control. Various concentrations of the test extract and ascorbic acid dissolved in distilled water were taken ranging from 20, 40, 60, 80, 100, 120, 140 and 160 µg/ml. The absorbance values of the test extract are 0.920, 0.915, 0.908, 0.897, 0.853, 0.850, 0.847 and 0.834. The absorbance values of the positive control ascorbic acid are 0.665, 0.466, 0.247, 0.197, 0.158, 0.152, 0.014 and 0.009. From the graph, it can be seen that as the concentration of the mycelial extract increased, the DPPH free radical scavenging ability also increased. But the scavenging potential is minimal as compared to the positive control ascorbic acid but the free radical scavenging ability is present and increases as the concentration of mycelium increases. This indicates that C. militaris mycelium possesses free radical scavenging activity but as compared to strong antioxidants, the potential of antioxidant action is minimal.

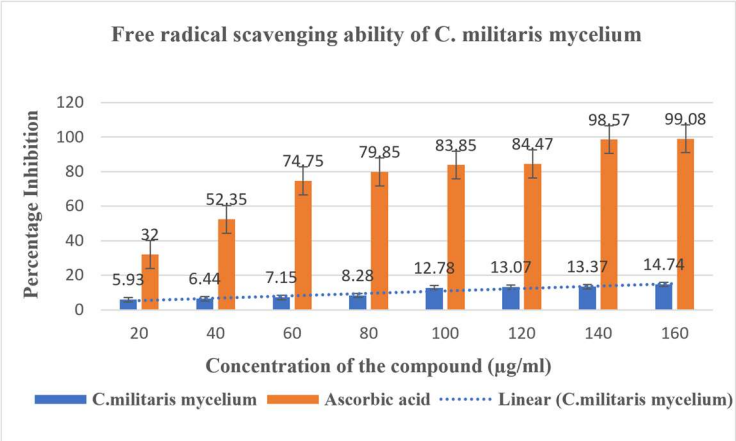



**Fig 3: Bar graph indicating the DPPH free radical scavenging ability of C. militaris mycelium which is compared with the known antioxidant ascorbic acid**

**Secondary metabolite analysis by Gas Chromatography-Mass Spectrophotometry**

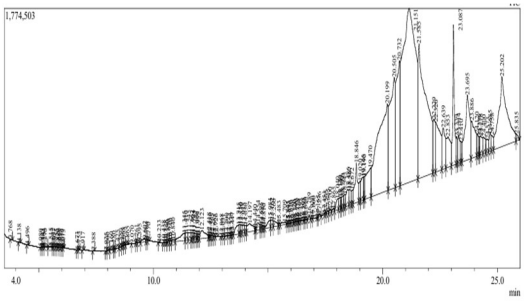


**Fig 4: Chromatogram of the bioactive metabolites identified by GCMS analysis**

The crude ethanol extract of C. militaris mycelium was subjected to GCMS analysis which revealed the different classes of metabolites secreted by the C. militaris mycelium. Overall, about 135 bioactive compounds were identified. Major bioactive compounds were identified by the area% during analysis. Among all the 135 bioactive compounds, 15 compounds showed highest peak area% as shown in the table. Compounds which showed the peak area% of above 5 are as follows- 9,12-OCTADECADIENOIC ACID (Z,Z) (10.47%), Ethyl Oleate (6.78%), ETHYL NONADECANOATE (5.53%), 17-Octadecynoic acid (26.04%), Octanoic acid, 2-dimethylaminoethyl ester (12.75%), 9,12-Octadecadienoic acid (Z,Z)-, 2-hydroxy-1-(hydroxymethyl)ethyl ester (6.76%) and the peak area% between 2-5 include 1,2,4-Trioxolane-2-octanoic acid, 5-octyl-, methyl ester (2.71%), 3-Cyclopentylpropionic acid, 2-dimethylaminoethyl ester (2.67%), Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester (3.66%). These bioactive compounds are recorded to be having many biological properties such as anti-oxidant, anti-bacterial, anti-fungal activity, anti-microbial, anti-inflammatory, anti-tumour and anti-cancer properties. These findings reveal that ethanol extract of C. militaris mycelium contain several bioactive compounds with immense pharmacological actions that will be therapeutic use to humans.

**Table 2: Major bioactive compounds identified by area% in the crude extract of C. militaris mycelium**

| Peak | Retention Time | Area | Area % | Height | Name of the metabolite |
|---|---|---|---|---|---|
| 1 | 18.846 | 2312794 | 1.31 | 270508 | HEXADECANOIC ACID, ETHYL ESTER |
| 2 | 19.47 | 2986321 | 1.69 | 198821 | 2-Diisopropylsilyloxymethyltetrahydrofurane |
| 3 | 20.199 | 18535424 | 10.47 | 592727 | 9,12-OCTADECADIENOIC ACID (Z,Z)- |
| 4 | 20.505 | 12008077 | 6.78 | 756063 | Ethyl Oleate |
| 5 | 20.732 | 9782067 | 5.53 | 855580 | ETHYL NONADECANOATE |
| 6 | 21.151 | 46094767 | 26.04 | 1186791 | 17-Octadecynoic acid |
| 7 | 21.585 | 22563789 | 12.75 | 919289 | Octanoic acid, 2-dimethylaminoethyl ester |
| 8 | 22.229 | 2341914 | 1.32 | 364488 | 2H-PYRAN-2-ONE,5,6-DIHYDRO-3,5,5-TRIMETHYL- |
| 9 | 22.32 | 4797446 | 2.71 | 339971 | 1,2,4-Trioxolane-2-octanoic acid,5-octyl-, methyl ester |
| 10 | 22.639 | 2616281 | 1.48 | 281457 | 9-Octadecenamide, (Z)- |
| 11 | 22.853 | 2671826 | 1.51 | 207674 | 4-Decen-1-ol, (Z)-, |
| 12 | 23.087 | 4721629 | 2.67 | 958052 | 3-Cyclopentylpropionicacid, 2-dimethylaminoethyl ester |
| 13 | 23.695 | 6479049 | 3.66 | 442218 | Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester |
| 14 | 23.886 | 3042702 | 1.72 | 255157 | Di-n-octyl phthalate |
| 15 | 25.202 | 11959941 | 6.76 | 477291 | 9,12-Octadecadienoic acid (Z,Z)-, 2-hydroxy-1-(hydroxymethyl)ethyl ester |

**Discussion**

C. militaris is a long-revered mushroom in Eastern ethnomedicine, famous due to a wealth of bioactive constituents contained in its mycelial cultures, which have prompted significant scientific interest. In vitro cultured mycelia and fruiting body chemical analyses indicate a complicated phytochemical profile containing 5-

aminobutyric acid (GABA), ergothioneine, ergosterol, lovastatin, phenolic acids (i.e. p-hydroxybenzoic acid and cinnamic acid), flavonoids (i.e. quercitrin, rutoside), tocopherols, indole compounds (i.e. 5-hydroxy-L-tryptophan), and, of note, these bioactive make the *C. militaris mycelium* a good candidate as a source of antioxidant and pharmacologically active molecules.

Phytochemical screening across multiple studies has found out that C. militaris is rich in proteins, carbohydrates, phenolics, flavonoids, tannins and saponins (29). This study also showed similar results where the ethanol extract of C. militaris myceliumshowed the presence of alkaloids, flavonoids, phenols, tannins, steroids, terpenes, cardiac glycosides, terpenoids, protein, resin, amino acids and carbohydrates. The phytochemicals secreted by the fruiting bodies and mycelium are heavily influenced by the culture and environmental conditions in which they grow. The mycelial biomass (MB) present in the submerged cultures usually secrete higher amounts of Cordycepin and carbohydrates (30). The phytochemical screening of C. militaris mycelium shows that mycelium secretes almost all the major bioactive compounds similar to the fruiting bodies. Certain major bioactive compounds like Cordycepin are secreted in high amounts as compared to fruiting bodies influenced by culture conditions.

The anti-oxidant capacity or the free radical scavenging ability of C. militaris arises due to the presence of flavonoids, polysaccharides and phenolic compounds. These compounds inhibit the free radicals such as DPPH and chelating ferrous ions. The antioxidant capacity increases as the concentration of the extract increases (31). This study also exhibits similar results where the C. militaris mycelium's free radical scavenging ability increases as the concentration of the extract increases although the scavenging ability is not as pronounced as the positive control ascorbic acid. The reason for the low scavenging activity might be due to the aqueous extract which usually contains lower concentration of phenolics and flavonoids as compared to ethanol extract. Studies have highlighted the same result where as compared to the aqueous extract, the ethanol extract showed superior antioxidant activity (32). In vivo study on grower pigs have found out that fermented C. militaris supplementation has led to higher levels of superoxide dismutase (SOD) in the serum (33). All these clearly indicate that C. militaris fruiting bodies as well as mycelium exhibit promising antioxidant activity.

A study on ethanol extract of fruiting bodies and mycelial biomass of C. militaris identified that ethanol extract contains fatty acids and sterols in larger amounts with palmitic and linoleic acids present in predominant levels in the mycelial biomass (30). The study showed very similar findings where the majority of the compounds secreted belonged to palmitic and linoleic acids such as the 9,12-Octadecadienoic acid (Z,Z)-, 2-hydroxy-1-(hydroxymethyl) ethyl ester, 9,12-OCTADECADIENOIC ACID (Z,Z)-, Ethyl Oleate, 17-Octadecynoic acid and HEXADECANOIC ACID, ETHYL ESTER. In conclusion, the study has revealed that C. militaris mycelium is a reservoir of major phytochemicals which is very similar to the phytochemicals secreted by the fruiting bodies. The mycelium also has shown that it has good free radical scavenging ability and the secondary metabolites secreted by the mycelium predominantly are palmitic acid and linoleic acids.

**Conclusion**

C. militaris species remains a promising source of bioactive compounds with immense therapeutic potential to humans. Most of the studies focus on the exploration of the fruiting bodies of the mushroom or the major bioactive compound Cordycepin's pharmacological potential. Very few studies have evaluated the therapeutic potential of mycelium. The study revealed that C. militaris mycelium contains major bioactive phytochemicals such as flavonoids, alkaloids, terpenes, terpenoids, phenols, tannins, steroids, carbohydrates and proteins. The mycelium also had found to have moderate antioxidant capacity which is heavily influenced by the culture and environmental conditions. The metabolomics analysis revealed that the mycelium also is a rich source of small molecules, volatile and non-volatile compounds with majority of the compounds belonging to the class of fatty acids and sterols.

**References**

1) Abdullah S, Kumar A. A brief review on the medicinal uses of Cordyceps militaris. Pharmacological Research-Modern Chinese Medicine. 2023 Jun 1;7:100228.
2) Jedrejko, K.J.; Lazur, J.; Muszynska, B. Cordyceps militaris: An Overview of Its Chemical Constituents in Relation to Biological Activity. Foods **2021**, 10, 2634.
3) Chou YC, Sung TH, Hou SJ, Khumsupan D, Santoso SP, Cheng KC, Lin SP. Current progress regarding Cordyceps militaris, its metabolite function, and its production. Applied Sciences. 2024 May 27;14(11):4610.

4) Jędrejko KJ, Kała K, Sułkowska-Ziaja K, Krakowska A, Zięba P, Marzec K, Szewczyk A, Sękara A, Pytko-Polończyk J, Muszyńska B. Cordyceps militaris-fruiting bodies, mycelium, and supplements: valuable component of daily diet. Antioxidants. 2022;11(10).
5) Phull AR, Ahmed M, Park HJ. Cordyceps militaris as a bio functional food source: pharmacological potential, anti-inflammatory actions and related molecular mechanisms. Microorganisms. 2022 Feb 10;10(2):405.
6) Kumar A, P N, Kumar M, Jose A, Tomer V, Oz E, Proestos C, Zeng M, Elobeid T, K S, Oz F. Major phytochemicals: recent advances in health benefits and extraction method. Molecules. 2023 Jan 16;28(2):887.
7) Jomova K, Raptova R, Alomar SY, Alwasel SH, Nepovimova E, Kuca K, Valko M. Reactive oxygen species, toxicity, oxidative stress, and antioxidants: chronic diseases and aging. Archives of toxicology. 2023 Oct;97(10):2499-574.
8) Mwangi RW, Macharia JM, Wagara IN, Bence RL. The antioxidant potential of different edible and medicinal mushrooms. Biomedicine & Pharmacotherapy. 2022 Mar 1;147:112621.
9) Deshmukh N, Lakshmi B. Antioxidant potential of Cordyceps militaris mycelium: A comparative analysis of methanol and aqueous extracts. Biosciences Biotechnology Research Asia. 2023;20(4):1487-99.
10) Fiehn O. Metabolomics by gas chromatography–mass spectrometry: Combined targeted and untargeted profiling. Current protocols in molecular biology. 2016 Apr;114(1):30-4.
11) Oh J, Yoon DH, Shrestha B, Choi HK, Sung GH. Metabolomic profiling reveals enrichment of cordycepin in senescence process of Cordyceps militaris fruit bodies. Journal of Microbiology. 2019 Jan;57(1):54-63.
12) Yang S, Jin L, Ren X, Lu J, Meng Q. Optimization of fermentation process of Cordyceps militaris and antitumor activities of polysaccharides in vitro. journal of food and drug analysis. 2014 Dec 1;22(4):468-76.
13) Tiwari P, Kumar B, Kaur M, Kaur G, Kaur H. Phytochemical screening and extraction: a review. Internationale pharmaceuticasciencia. 2011 Mar;1(1):98-106.
14) Kumar JK, Prasad AD, Chaturvedi V. Phytochemical screening of five medicinal legumes and their evaluation for in vitro anti-tubercular activity. AYU (An International Quarterly Journal of Research in Ayurveda). 2014 Jan 1;35(1):98-102.
15) Pradeep A, Dinesh M, Govindaraj A, Vinothkumar D, Ramesh Babu NG. Phytochemical analysis of some important medicinal plants. Int J Biol Pharm Res. 2014;5(1):48-50.
16) Evans WC. Trease and evans. Pharmacognosy, 9th Edition published by Saunders Elsevier. 2002;553.
17) Krishnaiah D, Devi T, Bono A, Sarbatly R. Studies on phytochemical constituents of six Malaysian medicinal plants.
18) Harsha, S. N., Anilakumar, K. R., & Mithila, M. V. (2013). Antioxidant properties of Lactuca sativa leaf extract involved in the protection of biomolecules. *Biomedicine & Preventive Nutrition*, *3*(4), 367-373.
19) Salna KP, Sreejith K, Uthiralingam M, Mithu AP, John Milton M, Albin TF. A comparative study of phytochemicals investigation of Andrographis paniculata and Murrayakoenigii. Int J Pharm Pharm Sci. 2011;3(3):291-2.
20) Sofowora A. Recent trends in research into African medicinal plants. Journal of ethnopharmacology. 1993 Mar 1;38(2-3):197-208.
21) Kagbo HD, Ejebe DE. Phytochemistry and preliminary toxicity studies of the methanol extract of the stem bark of Garcinia kola (Heckel). Internet J Toxicol. 2010;7(2).
22) Edeoga HO, Okwu DE, Mbaebie BO. Phytochemical constituents of some Nigerian medicinal plants. African journal of biotechnology. 2005 Aug 19;4(7):685-8.
23) Rajesh KD, Vasantha S, Rajesh NV, Panneerselvam A. Qualitative and quantitative phytochemical analysis in four pteridophytes. International Journal of Pharmaceutical Sciences Review and Research. 2014;27(2):408-12.
24) Domettila C, Joselin J, Jeeva S. Phytochemical analysis on some south Indian seaweeds. Journal of Chemical and Pharmaceutical Research. 2013;5(4):275-8.
25) Yadav RN, Agarwala M. Phytochemical analysis of some medicinal plants. Journal of phytology. 2011 Dec 14;3(12).
26) Usman H, Abdulrahman FI, Usman A. Qualitative phytochemical screening and in vitro antimicrobial effects of methanol stem bark extract of Ficus thonningii (Moraceae). African Journal of Traditional, Complementary and Alternative Medicines. 2009;6(3).

27) Al-Ani M, Opara LU, Al-Bahri D, Al-Rahbi N. Spectrophotometric quantification of ascorbic acid contents of fruit and vegetables using the 2, 4-dinitrophenylhydrazine method. Journal of Food Agriculture and Environment. 2007 Jul 1;5(3/4):165.
28) Gulcin İ, Alwasel SH. DPPH radical scavenging assay. Processes. 2023 Jul 26;11(8):2248.
29) Gupta A, Sharma CK, Sharma M. Evaluation of Phytochemical Composition, Antioxidant Activities and Cytotoxicity on Hep-G2 cancer cell line in vitro from Cordyceps militaris Medicinal Mushroom. Evaluation. 2023 Dec;13(4).
30) Chan JS, Barseghyan GS, Asatiani MD, Wasser SP. Chemical composition and medicinal value of fruiting bodies and submerged cultured mycelia of caterpillar medicinal fungus Cordyceps militaris CBS-132098 (Ascomycetes). International journal of medicinal mushrooms. 2015;17(7).
31) Dang HN, Wang CL, Lay HL. Effect of nutrition, vitamin, grains, and temperature on the mycelium growth and antioxidant capacity of Cordyceps militaris (strains AG-1 and PSJ-1). Journal of Radiation Research and Applied Sciences. 2018 Apr 1;11(2):130-8.
32) Gupta A, Sharma CK, Sharma M. Evaluation of Phytochemical Composition, Antioxidant Activities and Cytotoxicity on Hep-G2 cancer cell line in vitro from Cordyceps militaris Medicinal Mushroom. Evaluation. 2023 Dec;13(4).
33) Pi CC, Cheng YC, Chen CC, Lee JW, Lin CN, Chiou MT, Chen HW, Chiu CH. Synergistic fermentation of Cordyceps militaris and herbal substrates boosts grower pig antioxidant and immune function. BMC Veterinary Research. 2024 Nov 27;20(1):531.