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Research article

Metabolite Profiling and Morphological Screening of *C. militaris* Fruiting Bodies Extracts using UHPLC-QTOF-IMS and GC-MS Analysis

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Abstract: The medicinal mushroom *C. militaris* has several health advantages and has been utilized for many years throughout Asia as a component of traditional medicine systems. It can be used as a functional food and in nutraceutical products. This study investigated the morphological characteristics of *C. militaris* during the large-scale cultivation and metabolic profiling of the ethanolic and aqueous extracts of their fruiting bodies. The cultural and morphological characteristics of *C. militaris* were studied during the growth of this mushroom in terms of production of mycelial growth and fruiting bodies by conventional microbiological techniques. Cordycepin content in the aqueous and ethanol extracts of fruiting bodies was evaluated using UHPLC-QTOF-IMS analysis. The detection of metabolites in the ethanol extract was done by GC-MS analysis. The cordycepin content in the ethanol and aqueous extracts of the fruiting bodies was found to be 16.92 mg/g and 10.88 mg/g, respectively. GC-MS spectra analysis of the *C. militaris* fruiting bodies ethanolic extracts indicated the existence of eighteen metabolites such as 3,4-Dihydroxymandelic acid-terms, n-Hexadecanoic acid, Ethyl pentadecanoate, 1, E-11, Z-13-Octadecatriene, 9,12-Octadecadienoic acid (Z, Z)-, I-9-Octadecenoic acid ethyl ester, 9,12-Octadecadienoic acid (Z, Z)-, Trimethyls, 9(11)-Dehydroergosterol tosylate, Ergosterol, Silane, (phenyloxiranylidene) bis[trimethy, Neophytadiene, 1-Octadecyne, n-Hexadecanoic acid, Ethyl 9-hexadecenoate, 2,5-Diiodo-9-oxabicyclo [4.2.1] nonane, i-Propyl 9,12,15-octadecatrienoate, Ergosta-4,7,22-trien-3.beta.-ol, and TMS Palmitic acid. Evaluating cordycepin content and other bio components of C. militaris will help exploit this mushroom for potential medicinal benefits and develop reasonable quality pharmaceutical, nutraceutical, and functional food products.

Keywords: *Cordyceps militaris*; Caterpillar Fungus; Cordycepin; Metabolite; GC-MS study

1. Introduction

The medicinal mushroom *C. militaris* is an entomopathogenic fungus and has been a part of traditional medicine systems in Asian countries for many years. This crucial medicinal fungus offers the body several vital health advantages [1]. This fungus produces various bioactive compounds as secondary metabolites known for their potential health benefits, including

immunomodulatory, hepatoprotective, anti-inflammatory, anti-diabetic, antimicrobial effects [2] and anticancer effects [3]. Recently, there has been a global increase in the interest of individuals towards traditional and alternative medicine systems [4]. *C. militaris* contains many bioactive components, including cordycepin, polysaccharide, and ergosterol, and is widely used in various medical applications [5]. Due to its many bioactive components, this mushroom has potential health benefits, making it a suitable functional food ingredient. Cordycepin is a nucleoside derivative found in *C. militaris* and is one of the main bioactive metabolites found in this mushroom. This is a low molecular weight compound with several medicinal effects [5, 6]. Cordycepin is also known to suppress the growth of cancer cells by targeting cancer stem cells, inducing cell cycle arrest and upregulation apoptosis of cancer cells [4]. The studies revealed that *C. militaris* might be a promising mushroom for the neuroprotection of the hippocampus and recovery of neuroinflammation [7]. Several studies demonstrate that the bioactive compound cordycepin helps improve antioxidant enzyme levels and has anti-neuroinflammatory effects. Due to the significant anti-neuroinflammatory effect of cordycepin, this compound could help develop promising novel treatment strategies against neurological disorders like Alzheimer disease [8]. *C. militaris* harbors several beneficial biometabolites that can be used to develop herbal medicines [9]. These compounds have several beneficial biological activities [10]. UHPLC-QToF-IMS analysis is a rapid, reliable, and reproducible method developed and validated for simultaneously determining various nucleosides, including cordycepin [11]. The metabolite profiling using various techniques such as NMR, GC-MS and LC-MS analysis may be very useful for the identification of bio components [12]. In the present study, the morphological characteristics of *C. militaris* cultivated on rice medium were studied along with the evaluation of the metabolites detected in the ethanol and aqueous fruiting body extracts.

2. Materials and Methods

2.1 Characterization of the *C. militaris*

C. militaris (KBRC-1163) was cultured in rice medium from KBRC Laboratory, Bilaspur (H.P.). Large-scale cultivation was carried out using brown rice cultivation medium in glass containers. In addition to cultural and morphological studies, *C. militaris* was investigated to learn more about how this fungus produced mycelia and fruiting bodies as it grows.

2.2 Preparation of extracts

The aqueous and ethanolic extracts of fruiting bodies were prepared per the methodology described elsewhere [13] with certain modifications. The fruiting bodies of *C. militaris* were cleaned and shade-dried, grounded using a mechanical grinder into a coarse powder. Coarse powder (50 g) was filled in two different soxhlet apparatuses containing 150 ml of solvents, water, and ethanol. The extraction was performed for 8 hrs at 80 ^{0} C for ethanol and 100 ^{0} C for water [13].

2.3 Evaluation of cordycepin content by UHPLC-QToF-IMS

A precise and reliable estimation of bioactive components such as cordycepin content in the *C. militaris* extracts was carried out using UHPLC-QToF-IMS analysis as per the methodology [12]. At IHBT, Palampur, Himachal Pradesh, samples were analyzed to assess cordycepin content. Hydrophilic interaction chromatography was used to achieve LC separations using an Eclipse plus C18 RRHD column (2.1 x 50 mm). Elution was monitored at 280 nm using a PDA detector at 30 °C. Mass spectrometry was performed on a high-resolution 6560 ion mobility Q-TOF LC/MS equipped with an Agilent 1290 Infinity II UHPLC system. The mass spectrometer was scanned from 100 to 1700 m/z in full scan mode with a scan rate of 1.50 spectra/s. The 121.050873 m/z ion was chosen for mass calibration to eliminate systematic errors in the reference solution. Internal standard (NMAE = N6-methyladenosine) concentrations were used to normalize metabolite concentrations. Unknown metabolites other than the target were identified from the collected data and matched against the METLIN database [14].

2.4 GC-MS study

Plant material was dissolved in ethanol to prepare the extract for GC-MS analysis, which was carried out according to standard technique [12-14]. Filtration and potential concentration were then carried out. The

extract is separated by raising the gas chromatograph temperature after introducing a little aliquot. The mass-to-charge ratios of the chemicals are then measured when they are ionized in the mass spectrometer. The resultant mass spectra are compared to standard libraries like NIST for identification. This technique successfully identified the extract's volatile constituents.

3. Results and Discussion

Research has shown that the well-known traditional Chinese medicine *C. militaris* possesses anticancer, immunostimulatory, and neuroprotective properties [15]. The application of herbal biotechnology in drug development, employing the medicinal fungus *C. militaris*, is steadily increasing. Technological developments have expanded the potential use of this fungus in goods made with herbs [16]. Highly pleomorphic morphologies are displayed by the medicinal mushroom Cordyceps militaris when cultivated in different culture conditions. As seen in Fig. 1(a) and (b), when Cordyceps was cultivated on nutrient-rich Sabouraud dextrose agar (SDA) for three days, the culture turned turbid as spiny blastospores emerged from the tips of the branching mycelia. Fungal cultures grew flaky mycelia, colored yellow-orange, and generated round to ovoid conidia when cultivated on rice medium for up to three weeks before stroma development (Fig. 1b and Fig. 2a). In rice medium, stromal development happens up to 4 weeks following growth (Fig. 2b). After 5–6 weeks, *C. militaris* complete fruit body formation happens on rice medium with perithecia (Fig. 3a and b). Using light and scanning electron microscopy, the morphological traits of *C. militaris* were studied based on the production of conidial from ascospores that germinated from vegetative hyphae. It has been noted that the physical characteristics of *C. militaris* sexual stage can be used to differentiate it from other species [17]. Numerous bioactive substances are the cause of these possible health advantages. One of the primary ingredients in this mushroom that supports the biological activity and the therapeutic effect is cordycepin. This could be the reason for the potential use of *C. militaris* mushrooms as functional foods and medicinal use [17]. Recent studies have also demonstrated that this mushroom has tremendous health benefits, including COVID-19 [15, 18]. In the present study, the cultivation of *C. militaris* mycelium using artificial media has lately been developed in this mushroom. Numerous investigations have shown that *C. militaris* is the species that produces the most cordycepin and can be grown in artificial environments. This is the primary cause of the mushroom's widespread usage in functional meals and herbal remedies [19].

The cordycepin content of the *C. militaris* fruiting body extracts was another objective of our study. The cordycepin content in the ethanolic and aqueous extracts of the fruiting bodies was evaluated using UPLC-QTOF-MS DAD chromatography, and maximum cordycepin content was found in the ethanolic extract (16.92mg/g) while minimum in aqueous extract (10.88mg/g) **(Fig 4 a and b, Table 1**). The UPLC-QTOF-MS DAD chromatograms of cordycepin standard and aqueous and ethanolic extracts of fruiting bodies of *C. militaris* samples are represented in **Fig 4.** The cordycepin content evaluated using UHPLC-QToF-IMS analysis was a fast, reliable, and reproducible method, which was developed and validated for simultaneous determination of various nucleosides such as adenine, adenosine, cordycepin, and guanosine, inosine, thymidine, thymine and uracil in medicinal fungi [12] and evaluated the bioactive components of the *C. militaris* after extraction into digestive juices in the artificial gastrointestinal tract model. The research aided in determining how bioavailable, bioactive ingredients are to the human body. The highest amount of cordycepin was found in fruiting bodies grown on-site (25.8 mg/100 g d.w.) and those purchased commercially (25.9 mg/100 g d.w.). The research aided in determining how bioavailable, bioactive ingredients are to the human body. These researchers showed that C. militaris fruiting bodies and mycelium were suitable for dietary supplements and positively impacted human health [20].

Table 1 Cordycepin content found in extracts of *C. militaris* fruiting bodies.

Sr. No.	*C. militaris* extract	Cordycepin content (mg/g)
1.	Aqueous extract (AE)	10.88
2.	Ethanolic extract (EE)	16.92

In a similar study [21], the cordycepin content of *C. militaris* was determined using reversed-phase HPLC. These workers demonstrated that the HPLC method is fast, simple, accurate, and reproducible for determining cordycepin content. In another study, [22] an improved HPLC method detected the major bioactive ingredients of mycelial and fruiting bodies of C. sinensis and C. militaris. These workers observed that cordycepin content was higher in *C. sinensis* than in *C. militaris*, whereas adenosine content was higher in *C. militaris* [23]. Nonetheless, this investigation assessed *C. militaris* fruiting body extracts. Also, it used reversed-phase HPLC to quantitatively assess the *C. miliatris* extracts and discovered that the fungal mycelium's 60% ethanol extract and 100% ethanolic extract had the highest cordycepin levels. According to their research, this is a valuable analytical technique for determining the best extraction conditions [24]. Several other methods such as Near-infrared (NIRS) spectroscopy in combination with partial least squares regression were also employed to determine cordycepin level in fruiting bodies of *C. militaris* [25]. Another study used the HPLC-based method combined with hierarchical cluster analysis and quantitative analysis of multi-components by single marker (QAMS) to evaluate bioactive components in the fermented *Cordyceps* products. There was no significant difference in the results; therefore, they were found valid for systematic quality control assessment [25]. The development of novel *C. militaris* strains with increased cordycepin content through genetic recombination or increasing cordycepin content by application of growth supplements during submerged cultivation may help to exploit the pharmaceutical and nutraceutical potential of this mushroom [26]. The sample was sent to Jawaharlal Nehru University, New Delhi for analysis. The chemical composition of the ethanolic extract of *C. militaris* was studied by gas chromatography- mass spectroscopy (GC/MS). Eighteen phytoconstituents were identified in the sample representing 27.48% of Octadecatriene, 9,12-Octadecadienoic acid (18.60%), Linoleic acid (18.60%), and isopropyl linolenate (17.59%) were the major constituents as shown in **Table 2.** The identification of various compounds was confirmed by comparing their mass spectra to those in the NIST libraries.

Table 2. IUPAC names, common names, RT, Area%, and chemical structures of compounds identified in ethanolic extracts of *C. militaris* fruiting bodies by GC-MS analysis.

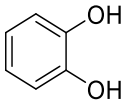
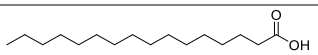
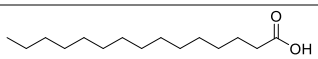
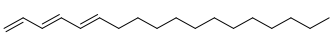
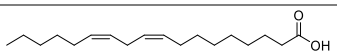
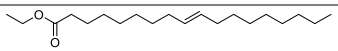
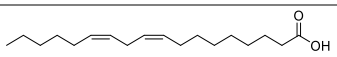
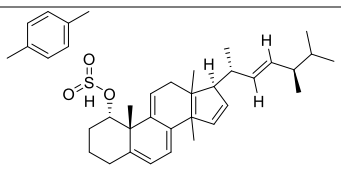
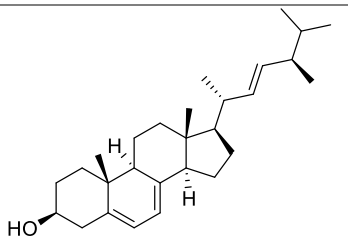
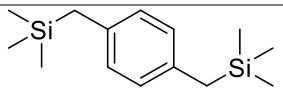
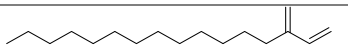
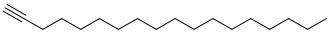
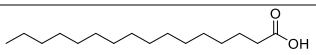
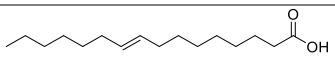
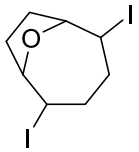
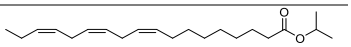
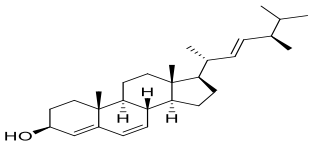
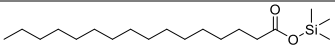
IUPAC Names	Common Names	RT	Area%	Chemical Structures
3,4-Dihydroxymandelic acid-tetratms	**Catechol, mandelic acid**	**12.330**	**1.24**	
n-Hexadecanoic acid	Palmitic Acid	16.136	15.04	
Ethyl pentadecanoate	Pentadecanoic acid	16.341	12.76	
1,E-11,Z-13-Octadecatriene	Octadecatriene	17.768	27.48	
9,12-Octadecadienoic acid (Z,Z)-	9,12-Octadecadienoic acid	17.899	18.60	
I-9-Octadecenoic acid ethyl ester	9-Octadecenoic acid, ethyl ester is a fatty acid ester.	17.950	2.62	
9,12-Octadecadienoic acid (Z,Z)-, Trimethyls	Linoleic acid	18.317	18.60	
9(11)-Dehydroergosterol tosylate	Dehydroergosterol tosylate	25.009	1.64	

Table 2. IUPAC names, common names, RT, Area%, and chemical structures of compounds identified in ethanolic extracts of *C. militaris* fruiting bodies by GC-MS analysis. (Contiune)

IUPAC Names	Common Names	RT	Area%	Chemical Structures
Ergosterol	**Ergosterol**	**28.709**	**11.60**	
Silane, (phenyloxiranylidene)bis[trimethy	1,4-Bis[(trimethylsilyl)methyl]benzene	11.375	1.14	
Neophytadiene	3-methylidenehexadec-1-ene	15.775	4.61	
1-Octadecyne	Octadecyne	16.222	1.34	
n-Hexadecanoic acid	Palmitic acid	17.142	12.49	
Ethyl 9-hexadecenoate	Palmitelaidic acid	18.959	2.42	
2,5-Diiodo-9-oxabicyclo[4.2.1]nonane	9-Oxabicyclo	26.022	1.51	
i-Propyl 9,12,15-octadecatrienoate	isopropyl linolenate	27.415	17.59	
Ergosta-4,7,22-trien-3.beta.-ol	Ergosta-4,6,22-triene-3beta-ol	29.706	12.81	
TMS Palmitic acid	Hexadecnoic acid trimethylsilyl ester	16.791	2.36	

GC-MS and Spectra analysis of the *C. militaris* fruiting bodies ethanolic extracts indicated the existence of various biocomponents **(Fig. 5 a and b)**. By comparing their mass spectra to those in the NIST libraries, given in **Table 2**, the 18 compounds were identified and characterized. **Table 3** lists the various biocomponents and their biological activities [27]. A total of eighteen compounds, for instance, 3,4- dihydroxymandelic acid-tetratms, n-hexadecanoic acid,ethyl pentadecanoate, 1,E-11,Z-13-octadecatriene, 9,12-octadecadienoic acid (Z,Z)-, 1-9-octadecenoic acid ethyl ester, 9,12-octadecadienoic acid (Z,Z)-, trimethyls, 9(11)-Dehydroergosterol tosylate, ergosterol, Silane, (phenyloxiranylidene)bis[trimethy, Neophytadiene, 1-Octadecyne, n-Hexadecanoic acid, ethyl 9-hexadecenoate, 2,5-Diiodo-9-oxabicyclo[4.2.1]nonane, i-Propyl 9,12,15-octadecatrienoate, Ergosta-4,7,22-trien-3.beta.-ol, and TMS Palmitic acid were detected. Oh and co-workers 2019, evaluated the metabolites of *C. militaris* fruiting bodies by gas chromatography-mass spectrometry (GC-MS) analysis

method based on their developmental phases. In this study, 39 components were identified associated with carbohydrates and amino acid metabolism. It has been observed that the bioactive compounds such as cordycepin, mannitol, and xylitol accumulated in stage four of the cultivation of this mushroom. However, our study evaluated the ethanolic extracts of dried fruiting bodies and identified eighteen compounds along with cordycepin level. In a similar study, NMR and GC-MS with multivariate statistical analysis were applied to detect biometabolites in *Cordyceps pruinosa* mycelia. This study identified seventy-one metabolites, such as alcohols, amino acids, purines, pyrimidines, fatty acids, and organic acids. These bio-components may vary at different growth conditions [28]. Studies are still required to predict the optimum amount of these compounds in the mushroom and affect different supplementation substrates on the concentration of these compounds during the cultivation. In a similar study conducted by [8], the metabolic profiling of ethanol extracts of C. militaris fruiting bodies was done using ^{1}H-NMR analysis. A total of 44 metabolites were identified. It has been observed that in the aging period of development of *C. militaris* fruiting bodies, higher levels of biometabolites were present. In another study, a simultaneous distillation-extraction (SDE) and gas chromatography-mass spectrometry (GC-MS) method was developed for the profiling of volatile components in the products prepared from *C. sinensis.* In this work, 64, 39, 56, 52, and 44 components were identified in the five different products. 5,6-dihydro-6-pentyl-2H-pyran-2-one, and fatty acids were mostly present [29]. In our study, noradrenalin, fatty acids, and sterols were the most dominating compounds among the identified bio metabolites in *C. militaris* fruiting bodies of ethanolic extract.

The medicinal mushroom *C. militaris* is a rich source of beneficial biometabolites for developing various herbal drug formulations. Due to the potential health benefits associated with this mushroom, it has gained significant importance in biotechnological and clinical applications [10]. *Cordyceps* species are also of great interest because of the broad spectrum of biological effects associated with its bio metabolites, such as cordycepin [30]. Many studies have demonstrated the therapeutic potential of this compound, and therefore, advancements in the development of techniques to produce products with higher cordycepin content have been observed [11]. Detecting metabolites in various products using NMR, GC-MS, and LC-MS analysis is beneficial for investigating bioactive components and their potential application in drug discovery and food products [11]. The metabolic components and biochemical activity indicate the potential application of *C. militaris* in the pharmaceutical and food industry [31].

Table 3. GC-MS analysis shows the nature and biological activity of ethanolic extracts of C. militaris fruiting bodies.

IUPAC Name	Common Names	Retention Time	Molecular weight	Nature of compound	Biological activity	Reference
3,4-Dihydroxymandelic acid-tetratms	Catechol, mandelic acid	12.330	472	Nor adrenalin metabolite	Higher free radical scavenging activity	[32]
n-Hexadecanoic acid	Palmitic Acid	16.136	256	Fatty acid	Anti-inflammatory compound	[33]
Ethyl pentadecanoate	Pentadecanoic acid	16.341	270	Fatty acid	Antibacterial and antifungal activity	[34]
1,E-11,Z-13-Octadecatriene	Octadecatriene	17.768	248	Fatty acid	Antibacterial property	[35]
9,12-Octadecadienoic acid (Z,Z)- is to be revised to 9,12-Octadecadienoic acid (Z,Z)-Trimethyls.	9,12-Octadecadienoic acid	17.899	280	Lineolic acids and derivatives.	Antimicrobial property	[36]
I-9-Octadecenoic acid ethyl ester	ethyl ester, 9-Octadecenoic acid,	17.950	310	Fatty acid ester	Antibacterial property	[36, 37]
9,12-Octadecadienoic acid (Z,Z)- Trimethyls,	Linoleic acid	18.317	352	Lineolic acids and derivatives.	Antioxidant activities	[37]
9(11)-Dehydroergosterol tosylate	Dehydroergosterol tosylate	25.009	548	Sterol	Immuno modulatory, Anti-inflammatory, activity	**[38]**

Table 3. GC-MS analysis shows the nature and biological activity of ethanolic extracts of C. militaris fruiting bodies. (Contiune)

IUPAC Name	Common Names	Retention Time	Molecular weight	Nature of compound	Biological activity	Reference
Ergosterol	Ergosterol	28.709	396	Sterol	Membrane fluidity, regulation, activity and distribution of integral membrane proteins	[22]
Silane, (phenyloxiranylidene)bis[trimethy	1,4-Bis[(trimethylsilyl)methyl]benzene	11.375	264	Benzene derivatives	Antimicrobial effects	[39]
Neophytadiene	3-methylidenehexadec-1-ene	15.775	278	Diterpenes	Antibacterial activity, treatment of headache, rheumatism and some skin disease	[30]
1-Octadecyne	Octadecyne	16.222	250	Long-chain hydrocarbon and an alkene	Anticancer, antioxidant and antimicrobial activities	[40, 41]
n-Hexadecanoic acid	Palmitic acid	17.142	256	Fatty acid	Balance membrane physical properties	[27]
Ethyl 9-hexadecenoate	Palmitelaidic acid	18.959	282	Fatty acid esters	Antioxidant and Antiandrogenic activity	[34]
2,5-Diiodo-9-oxabicyclo[4.2.1] nonane	9-Oxabicyclo	26.022	378	Lactones	Antibacterial activity	[22]
i-Propyl 9,12,15-octadecatrienoate	isopropyl linolenate	27.415	320	Fatty acid	Antioxidant effects	[34]

Table 3. GC-MS analysis shows the nature and biological activity of ethanolic extracts of C. militaris fruiting bodies. (Contiune)

IUPAC Name	Common Names	Retention Time	Molecular weight	Nature of compound	Biological activity	Reference
Ergosta-4,7,22-trien-3 beta-ol	Ergosta-4,6,22-triene-3beta-ol	29.706	396	Sterol	Anti-fatigue activity, and protection of hepatic and muscle activity	[15]
TMS Palmitic acid	Hexadecanoic acid trimethylsilyl ester	16.791	328	Fatty acid	Anticancer, beneficial for cardiovascular diseases and obesity	Mancini et al. (2015

Figure 1. *C. militaris* (a) yellowish-orange mycelial mat produced two weeks post-inoculation on rice medium (b) stromata formation from the yellowish-orange mycelial mat produced on rice medium.

(a) (b)

Figure 2. *C. militaris* (a) premature fruiting body with perithecia on rice medium (b) mature fruit bodies.

Figure 3. Dried fruiting bodies of *C. militaris*

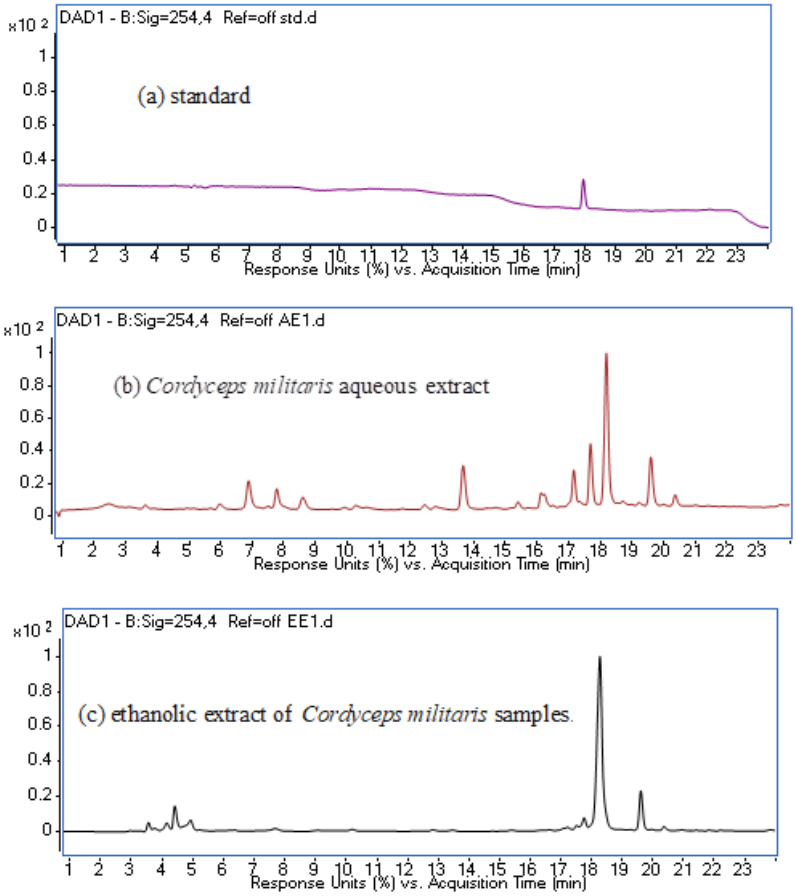


Figure 4. UPLC-QTOF-MS DAD chromatograms of cordycepin (a) standard, (b) *C. militaris* aqueous extract, and (c) *Cordyceps militaris* ethanolic extract.

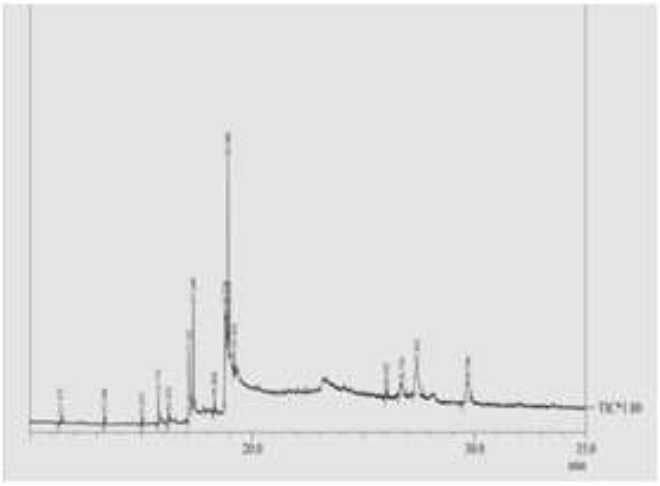


Figure 5. GC-MS chromatogram of ethanol extract of *C. militaris* fruiting bodies.

4. Conclusions

C. militaris have many therapeutic benefits and are commonly used in traditional medicinal systems. Due to various active phytoconstituents, *C. militaris* can be used to produce pharmaceutical and functional food products. The morphological traits change depending on the stage of cultivation. Cordycepin, a main bioactive component found in this mushroom, evaluated using the extracts from fruiting bodies, revealed that the higher cordycepin content was in the ethanolic extract in comparison to the aqueous extract, by UHPLC-QTOF-IMS analysis. Also, the ethanol extract showed the presence of eighteen compounds, and fatty acids and sterols were the most predominant among these compounds by GC-MS analysis. The characterization of *C. militaris* will help exploit this mushroom to develop herbal-based formulations and nutraceuticals. Detecting metabolites using UHPLC-QTOF-IMS and GC-MS analysis is beneficial for detecting bioactive components. These studies may help assess the potential application of these fungi in drug discovery and the food industry.

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