The methanolic extract of *Cordyceps militaris* (L.) Link fruiting body shows antioxidant, antibacterial, antifungal and anti human tumor cell lines properties

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ABSTRACT

Being *Cordyceps militaris* (L.) Link recognized as a medicinal and edible mushroom, this work intends to reveal new interesting bioactive molecules that could be isolated from this species. Hydrophilic and lipophilic compounds were analysed by chromatographic techniques coupled to different detectors. The methanolic extract of *C. militaris* was tested for its antioxidant, antibacterial, antifungal and anti-proliferative properties in different human tumor cell lines. Mannitol (2.01 g/100 g dw) and trehalose (24.71 g/100 g) were the free sugars found in *C. militaris*. Polyunsaturated fatty acids (68.87%) predominated over saturated fatty acids (23.40%) and δ-tocopherol was the only isoform of vitamin E detected (55.86 μg/100 g). The organic acids found in this mushroom were oxalic, citric and fumaric acids (0.33, 7.97 and 0.13 g/100g, respectively). *p*-Hydroxybenzoic acid was the only phenolic acid quantified in this species (0.02 mg/100 g); although cinnamic acid was also found (0.11 mg/100 g). The methanolic extract of *C. militaris* proved to inhibit lipid peroxidation, have reducing power and scavenge free radicals. This extract also revealed strong antibacterial and antifungal properties. Finally, the *C. militaris* extract was able to inhibit the proliferation of MCF-7 (breast), NCI-H460 (non-small lung), HCT-15 (colon) and HeLa (cervical) human carcinoma cell lines.

Keywords: Medicinal/edible mushroom; *Cordyceps militaris*; Chemical characterization; Antioxidant potential; Antimicrobial activity; Anti-proliferative properties.

1. Introduction

The *Cordyceps* genus is well known for its medicinal properties. A number of bioactive constituents from *Cordyceps* species have been reported such as antimicrobial, anti-inflammatory, antioxidant/antiaging, immunomodulatory or antitumor agents (Das et al., 2010). *Cordyceps militaris* (L.) Link is an entomopathogenic fungus (an obligatory parasite that grows on insects or insects larvae) that has been broadly used as a crude drug and a folk tonic food in East Asia (Ying et al., 1987).

The main active constituent of *C. militaris* fruiting bodies is cordycepin, a derivative of the nucleoside adenosine. This molecule was first isolated from *C. militaris* (Cunningham et al., 1950) and it is now produced synthetically since it has insecticidal, antibacterial and antitumor properties (Paterson and Russell, 2008). Other compounds with pharmacological activity have been isolated from *C. militaris* such as ergosterol (a sterol present in fungi) and polysaccharides that provide anti-inflammatory, antioxidant, antitumor, anti-metastatic, immunomodulatory, hypoglycaemic, steroidogenic and hypolipidaemic activities (Ng and Wang, 2005). In fact, *Cordyceps* polysaccharides have been extensively studied. Zhong et al. (2008) have tested polysaccharides from *C. militaris* in tumor models (S180-bearing mice) and verified that they inhibited the tumor growth and decreased toxic effects of chemotherapy. Lee and Hong (2011) demonstrated that the polysaccharides extracted from the fruiting bodies of this species have a potent immunostimulating activity and also suppressed the *in vivo* growth of a solid tumor (melanoma) in an experimental mouse model.

The anti-inflammatory activity and anti-proliferative properties of other extracts or compounds from *C. militaris* (including cultivated strains and mycelium) were studied

(Park et al., 2009a; Rao et al., 2010) as also their anti-angiogenic activity (Won and Park, 2005). The water extracts of *C. militaris* induced apoptosis in human lung carcinoma cells (Park et al., 2009b).

The *in vitro* antioxidant activity of *C. militaris* has been reported especially with regard to its polysaccharidic extracts (Yu et al., 2007; Wang et al., 2012; Chen et al., 2013). Another bioactive compound from *C. militaris* is cordymin. This peptide was studied for its antifungal properties, and found to inhibit mycelial growth of *Bipolaris maydis*, *Mycosphaerella arachidicola*, *Rhizoctonia solani* and *Candida albicans*. Cordymin also displayed anti-proliferative activity toward breast cancer cells (MCF-7) (Wong et al., 2011). The anti-cancer agent cordycepin obtained from *C. militaris* have more than 21 clinically approved beneficial effects for human health (Mizuno, 1999).

As referred above, the available information about the pharmacological and/or medicinal properties of *C. militaris*, is virtually all related to the polysaccharidic extracts of this mushroom. Therefore, in this study, a chemical characterization of the fruiting bodies of a cultivated species from Korea was performed in order to identify other bioactive molecules that could be isolated for medicinal applications. The composition in hydrophilic (free sugars, organic acids and phenolic acids) and lipophilic (fatty acids and tocopherols) compounds was evaluated, as well as the antioxidant, antimicrobial and anti-proliferative properties of the methanolic extract.

2. Material and Methods

2.1. Mushroom species

The cultivated fruiting bodies of *Cordyceps militaris* (L.) Link (strain: MCI 10304, Meshtech Cordyceps Institute) were a kind gift from Dr. J. M. Sung of Kangwon National University (Chuncheon, Korea). All the samples were lyophilised (FreeZone

4.5 model 7750031, Labconco, Kansas, USA), reduced to a fine dried powder (20 mesh), mixed to obtain homogenous samples and stored in a desiccator, protected from light, until further analysis.

2.2. Standards and Reagents

Acetonitrile 99.9%, *n*-hexane 95% and ethyl acetate 99.8% were of HPLC grade from Fisher Scientific (Lisbon, Portugal). The fatty acids methyl ester (FAME) reference standard mixture 37 (standard 47885-U) was purchased from Sigma (St. Louis, MO, USA), as also other individual fatty acid isomers, sugar standards (D(-)-mannitol, D(+)-raffinose pentahydrate, and D(+)-trehalose) and δ-tocopherol. Racemic tocol, 50 mg/ml, was purchased from Matreya (PA, USA). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was obtained from Alfa Aesar (Ward Hill, MA, USA). Phenolic standards (*p*-hydroxybenzoic and cinnamic acids) and trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) were purchased from Sigma (St. Louis, MO, USA). Mueller–Hinton agar (MH) and malt agar (MA) were obtained from the Institute of Immunology and Virology, Torlak (Belgrade, Serbia). Dimethylsulfoxide (DMSO), (Merck KGaA, Germany) was used as a solvent. Phosphate buffered saline (PBS) was obtained from Sigma Chemical Co. (St. Louis, USA). Foetal bovine serum (FBS), L-glutamine, Hank's balanced salt solution (HBSS), trypsin-EDTA (ethylenediamine tetraacetic acid), nonessential amino acids solution (2 mM), penicillin/streptomycin solution (100 U/ml and 100 mg/ml, respectively), RPMI-1640 and DMEM media were from Hyclone (Logan, USA). Acetic acid, ellipticine, sulphorhodamine B (SRB), trypan blue, trichloroacetic acid (TCA), Tris and all organic acids standards (oxalic acid; citric acid and fumaric acid) were from Sigma Chemical Co. (Saint Louis, USA). Methanol and all other chemicals and solvents were of analytical grade and purchased from

common sources. Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, USA).

2.3. Chemical characterization of the hydrophilic compounds in fruiting bodies.

2.3.1. Free sugars

A lyophilized sample (1 g) was spiked with the Internal Standard, IS (raffinose, 5 mg/ml), and was extracted with 40 ml of 80% aqueous ethanol at 80 ºC for 1h30 min. The resulting suspension was filtered and concentrated under reduced pressure (rotary evaporator Büchi R-210) and defatted three times with 10 ml of ethyl ether, successively. After concentration, the residues were dissolved in water to a final volume of 5 ml, filtered through a 0.22 μm disposable LC filter disk, transferred into an injection vial and analysed by High Performance Liquid Chromatography (HPLC) system consisting of an integrated system with a pump (Knauer, Smartline system 1000), degasser system (Smartline manager 5000) and auto-sampler (AS-2057 Jasco), coupled to a refraction index detector (RI detector Knauer Smartline 2300). The chromatographic separation was achieved with an Eurospher 100-5 NH_2 column (4.6 mm × 250 mm, 5 mm, Knauer) operating at 35 ºC (7971R Grace oven). The mobile phase used was acetonitrile/deionized water, 70:30 (v/v) at a flow rate of 1 ml/min, and the injection volume was 20 μl (Reis et al., 2012a). Identification of sugars was made by comparing the relative retention times of sample peaks with standards. Data were analyzed using Clarity 2.4 Software (DataApex). Quantification was based on the RI signal response of each standard, using the IS method and by using calibration curves obtained from commercial standards of each compound. The recoveries were 92% and 91% for mannitol and trehalose, respectively. The results were expressed in g per 100 g of dry weight.

2.3.2. Organic acids

A lyophilizedsample (1.5 g) was extracted by stirring with 25 ml of meta-phosphoric acid (25ºC at 150 rpm) for 45 min and subsequently filtered through Whatman No. 4 paper. Before analysis by HPLC (Shimadzu Cooperation, Kyoto, Japan; coupled with a photodiode array detector, PDA), the sample was filtered through 0.2 µm nylon filters. Separation was achieved on a SphereClone (Phenomenex) reverse phase C_{18} column (5 µm, 250 mm × 4.6 mm i.d) thermostatted at 35 ºC. The elution was performed with sulphuric acid 3.6 mM using a flow rate of 0.8 ml/min. Detection was carried out in a PDA, using 215 nm and 245 as preferred wavelengths (Barros et al., 2013). The organic acids were quantified by comparison of the area of their peaks recorded at 215 nm with calibration curves obtained from commercial standards of each compound. The recoveries were 99, 92 and 93% for oxalic, citric and fumaric acid, respectively. The results were expressed in g per 100 g of dry weight.

2.3.3. Phenolic acids and related compounds

The lyophilized samples (1.5 g) were extracted with methanol:water (80:20,v/v; 30 ml) at -20ºC for 1.5 h. After sonication for 15 min, the extract was filtered through Whatman No. 4 paper. The residue was then re-extracted with an additional 30 ml portion of the methanol:water mixture. Combined extracts were evaporated under reduced pressure to remove the methanol. The aqueous phase was submitted to a liquid-liquid extraction with diethyl ether (2 × 20 ml) and ethyl acetate (2 × 20 ml). To the combined organic phases, anhydrous sodium sulphate was added and the extracts were filtrated through Whatman nº 4 paper, evaporated to dryness and then re-dissolved in methanol:water (80:20, v/v). The extracts (1 ml) were filtered through a 0.22 µm disposable LC filter disk for HPLC analysis. The analysis was performed by HPLC

(equipment described in section 2.3.2). Detection was carried out in a PDA using 280 nm as the preferred wavelength (Reis et al., 2012b). The phenolic compounds were characterized according to their UV and mass spectra and retention times, and comparison with authentic standards when available. The identified phenolic acids were quantified by comparison of the area of their peaks recorded at 280 nm with calibration curves obtained from commercial standards of each compound. The recoveries were 94, and 91% for *p*-hydroxybenzoic and cinnamic acid, respectively. The results were expressed in µg per 100 g of dry weight.

2.4. Chemical characterization of the fruiting bodies in lipophilic compounds

2.4.1. Fatty acids

Fatty acids were determined after a transesterification procedure performed with the oil obtained by soxhlet extraction: fatty acids were methylated with 5 ml of methanol:sulphuric acid:toluene 2:1:1 (v:v:v), during at least 12 h in a rotating bath at 50 ºC and 160 rpm; then 3 ml of deionized water were added, to obtain phase separation; the FAME (fatty acids methyl esters) were recovered with 3 ml of diethyl ether by shaking on vortex, and the upper phase was dried with anhydrous sodium sulphate; the sample was recovered in a vial with Teflon stopper, and before injection the sample was filtered with 0.2 µm nylon filter from Millipore. The analysis was performed using a gas chromatographer (DANI 1000) equipped with a split/splitless injector and a flame ionization detector (GC-FID) and a Macherey-Nagel column (30 m×0.32 mm ID×0.25 µm d_f). The oven temperature program was as follows: the initial temperature of the column was 50 ºC, held for 2 min, then a 30ºC/min ramp to 125 ºC, 5ºC/min ramp to 160 ºC, 20ºC/min ramp to 180 ºC, 3ºC/min ramp to 200 ºC, 20ºC/min ramp to 220 ºC and held for 15 min. The carrier gas (hydrogen) flow-rate was 4.0

ml/min (0.61 bar), measured at 50 ºC. Split injection (1:40) was carried out at 250 ºC and the volume of injection was 1 µl. Fatty acid identification was made by comparing the relative retention times of FAME peaks from sample with standards. The results were recorded and processed using CSW 1.7 software (DataApex 1.7) (Reis et al., 2012a). The results were expressed in relative percentage of each fatty acid.

2.4.2. Tocopherols

BHT solution (100 µl) and IS solution (tocol, 250 µl) were added to the sample prior to the extraction procedure. The lyophilized sample (~500 mg) was homogenized with methanol (4 ml) by vortex mixing (1 min). Subsequently, hexane (4 ml) was added and again vortex mixed for 1 min. After that, saturated NaCl aqueous solution (2 ml) was added, the mixture was homogenized (1 min), centrifuged (5 min, 4000*g*) and the clear upper layer was carefully transferred to a vial. The sample was re-extracted twice with hexane. The combined extracts were taken to dryness under a nitrogen stream, re-dissolved in 1 ml of hexane, dehydrated with anhydrous sodium sulphate, filtered through a 0.22 µm disposable LC filter disk, transferred into a dark injection vial and analysed by HPLC (equipment described in section 2.3.1), and a fluorescence detector (FP-2020; Jasco) programmed for excitation at 290 nm and emission at 330 nm. Data were analysed using Clarity 2.4 Software (DataApex). The chromatographic separation was achieved with a Polyamide II (250×4.6 mm) normal-phase column from YMC Waters (Japan) operating at 35ºC. The mobile phase used was a mixture of hexane and ethyl acetate (70:30, v/v) at a flow rate of 1 ml/min, and the injection volume was 20 µl (Heleno et al., 2010). The compounds were identified by chromatographic comparisons with authentic standards. Quantification was based on the fluorescence signal response of each standard, using the IS method and by using calibration curves obtained from

commercial standards of each compound. The recoveries were 95, 89, 92 and 91% for α-, β-, γ- and δ-tocopherol, respectively. The results were expressed in μg per 100 g of dry weight.

2.5. Extract preparation for bioactivity evaluation

The lyophilized sample (1 g) was extracted by stirring with 40 ml of methanol for 1 h and subsequently filtered through Whatman No. 4 paper. The residue was then extracted with 20 ml of methanol for 1 h. The combined methanolic extracts were evaporated at 40ºC (rotary evaporator Büchi R-210) to dryness and re-dissolved in *a)* methanol for antioxidant activity assays (20 mg/ml), *b)* 5% solution of DMSO in distilled water for antimicrobial activity assays (100 mg/ml), and *c)* distillated water for anti-proliferative assays (8 mg/ml).

2.6. Evaluation of the antioxidant activity of C. militaris methanolic extract

Successive dilutions were made from the stock solution and submitted to *in vitro* assays already described by Reis et al. (2012b) to evaluate the antioxidant activity of the samples. The sample concentrations (mg/ml) providing 50% of antioxidant activity or 0.5 of absorbance (EC_{50}) were calculated from the graphs of antioxidant activity percentages (DPPH,β-carotene/linoleate and TBARS assays) or absorbance at 690 nm (ferricyanide/Prussian blue assay) against sample concentrations. Trolox was used as a positive control.

2.6.1. Folin-Ciocalteu assay

One of the extract solutions (5 mg/ml; 1 ml) was mixed with *Folin-Ciocalteu* reagent (5 ml, previously diluted with water 1:10, v/v) and sodium carbonate (75 g/l, 4 ml). The

tubes were vortex mixed for 15 s and allowed to stand for 30 min at 40 ºC for color development. Absorbance was then measured at 765 nm (Analytikjena spectrophotometer; Jena, Germany). Gallic acid was used to obtain the standard curve and the reduction of the *Folin-Ciocalteu* reagent by the samples was expressed as mg of gallic acid equivalents (GAE) per g of extract.

2.6.2. Ferricyanide/Prussian blue assay

The extract solutions with different concentrations (0.5 ml) were mixed with sodium phosphate buffer (200 mmol/l, pH 6.6, 0.5 ml) and potassium ferricyanide (1% w/v, 0.5 ml). The mixture was incubated at 50 °C for 20 min, and trichloroacetic acid (10% w/v, 0.5 ml) was added. The mixture (0.8 ml) was poured in a48 wells plate, mixed with deionized water (0.8 ml) and ferric chloride (0.1% w/v, 0.16 ml), and the absorbance was measured at 690 nm in ELX800Microplate Reader (Bio-Tek Instruments, Inc; Winooski, USA).

2.6.3. DPPH radical-scavenging activity

This methodology was performed using the Microplate Reader mentioned above. The reaction mixture on 96 well plate consisted of different concentrations (30 µl) of the extract and methanolic solution (270 µl) of DPPH (6×10^{-5} mol/l). The mixture was left to stand for 30 min in the dark, and the absorption was measured at 515 nm. The radical scavenging activity (RSA) was calculated as a percentage of DPPH discoloration using the equation: $\%RSA=[(A_{DPPH}-A_S)/A_{DPPH}]\times100$, where A_S is the absorbance of the solution containing the sample, and A_{DPPH} is the absorbance of the DPPH solution.

2.6.4. Inhibition of β-carotene bleaching or β-carotene/linoleate assay

A solution of β-carotene was prepared by dissolving β-carotene (2 mg) in chloroform (10 ml). Two milliliters of this solution were pipetted into a round-bottom flask. The chloroform was removed at40 °C under vacuum and linoleic acid (40 mg), Tween 80 emulsifier (400 mg), and distilled water (100 ml) were added to the flask with vigorous shaking. Aliquots (4.8 ml) of this emulsion were transferred into test tubes containing different concentrations (0.2 ml) of the extract. The tubes were shaken and incubated at 50 °C in a water bath. As soon as the emulsion was added to each tube, the zero time absorbance was measured at 470 nm. β-Carotene bleaching inhibition was calculated using the following equation: (Absorbance after2 h of assay/ initial absorbance)×100.

2.6.5. Thiobarbituric acid reactive substances (TBARS) assay

Porcine (*Sus scrofa*) brains were obtained from freshly slaughtered animals, dissected, and homogenized with Polytron in an ice cold Tris-HCl buffer (20 mM, pH 7.4) to produce a 1:2 w/v brain tissue homogenate which was centrifuged at 3000 g for10 min. An aliquot (100 µl) of the supernatant was incubated with the different concentrations of the sample solutions (200 µl) in the presence of $FeSO_4$ (10 mM; 100 µl) and ascorbic acid (0.1mM; 100 µl) at 37 °C for 1 h. The reaction was stopped by the addition of trichloroacetic acid (28% w/v, 500 µl), followed by thiobarbituric acid (TBA, 2%, w/v, 380 µl), and the mixture was then heated at 80 °C for 20 min. After centrifugation at 3000 g for 10 min to remove the precipitated protein, the color intensity of the malondialdehyde (MDA)-TBA complex in the supernatant was measured by its absorbance at 532 nm. The inhibition ratio (%) was calculated using the following formula: Inhibition ratio (%)=[(A−B)/A]×100%, where A and B were the absorbance of the control and the sample solution, respectively.

2.7. Evaluation of the antimicrobial activity of C. militaris methanolic extract

Successive dilutions were made from the stock solution and submitted to antibacterial and antifungal assays.

2.7.1. Antibacterial activity

The following Gram-negative bacteria: *Escherichia coli* (ATCC 35210), *Pseudomonas aeruginosa* (ATCC 27853), *Salmonella typhimurium* (ATCC 13311), *Enterobacter cloacae* (ATCC 35030), and Gram-positive bacteria: *Staphylococcus aureus* (ATCC 6538), *Bacillus cereus* (clinical isolate), *Micrococcus flavus* (ATCC 10240), and *Listeria monocytogenes* (NCTC 7973) were used. The microorganisms were obtained from the Mycological laboratory, Department of Plant Physiology, Institute for biological research "Sinisa Stanković", University of Belgrade, Serbia.

The minimum inhibitory (MIC) and minimum bactericidal (MBC) concentrations were determined by the microdilution method (Espinel-Ingroff, 2001). Briefly, fresh overnight culture of bacteria was adjusted by the spectrophotometer to a concentration of 1×10^5 CFU/ml. The requested CFU/ml corresponded to a bacterial suspension determined in a spectrophotometer at 625 nm (OD625). Dilutions of inocula were cultured on solid medium to verify the absence of contamination and check the validity of the inoculum. Different dilutions of the methanolic extract were added to the wells containing 100 µl of Tryptic Soy Broth (TSB) and afterwards, 10 µl of inoculum was added to all the wells. The microplates were incubated for 24h at 37 °C. The MIC of the samples was detected following the addition of 40 µl of iodonitrotetrazolium chloride (INT) (0.2 mg/ml) and incubation at 37°C for 30 min. The lowest concentration that produced a significant inhibition (around 50%) of the growth of the bacteria in comparison with the positive control was identified as the MIC. The minimum

inhibitory concentrations (MICs) obtained from the susceptibility testing of various bacteria to the extracts were determined also by a colorimetric microbial viability assay based on reduction of a INT color and compared with positive control for each bacterial strain (CSLI, 2006; Tsukatani et al., 2012). MBC was determined by serial sub-cultivation of 10 µl into microplates containing 100 µl of TSB. The lowest concentration that shows no growth after this sub-culturing was read as the MBC. Standard drugs, namely streptomycin and ampicillin were used as positive controls. 5% DMSO was used as negative control.

2.7.2. Antifungal activity

For the antifungal bioassays, the following microfungi were used: *Aspergillus fumigatus* (1022), *Aspergillus ochraceus* (ATCC 12066), *Aspergillus versicolor* (ATCC 11730), *Aspergillus niger* (ATCC 6275), *Penicillium funiculosum* (ATCC 36839), *Penicillium ochrochloron* (ATCC 9112), *Trichoderma viride* (IAM 5061), and *Penicillium verrucosum* var *cyclopium* (food isolate).The organisms were obtained from the Mycological Laboratory, Department of Plant Physiology, Institute for Biological Research "Siniša Stanković", Belgrade, Serbia. The micromycetes were maintained on malt agar (MA) and the cultures were stored at +4 °C and subcultured once a month (Booth, 1971).

The fungal spores were washed from the surface of agar plates with sterile 0.85% saline containing 0.1% Tween 80 (v/v). The spore suspension was adjusted with sterile saline to concentration of approximately 1.0×10^5 in a final volume of 100 µl/well. The inocula were stored at +4 °C for further use. Dilutions of the inocula were cultured on solid MA to verify the absence of contamination and to check the validity of the inoculum.

Minimum inhibitory concentrations (MICs) determination was performed by a serial dilution technique using 96-well microtitre plates. The extract was dissolved in 5% DMSO and added to broth malt medium with fungal inoculum. The microplates were incubated for 72 h at 28 °C. The lowest concentrations without visible growth (at the binocular microscope) were defined as MIC. The minimum fungicidal concentrations (MFCs) were determined by serial subcultivation of 2 µl in microtitre plates containing 100 µl of malt broth per well and further incubation for 72 h at 28 °C. The lowest concentration with no visible growth was defined as the MFC, indicating 99.5% killing of the original inoculum. 5% DMSO was used as a negative control, while the antibiotics bionazole and ketokonazole were used as positive controls.

2.8. Evaluation of the anti-proliferative activity of C. militaris methanolic extract

Successive dilutions were made from the stock solution and tested against five human tumor cell lines: MCF-7 (breast adenocarcinoma), NCI-H460 (non-small cell lung cancer), HCT-15 (colon carcinoma), HeLa (cervical carcinoma) and HepG2 (hepatocellular carcinoma).Cells were routinely maintained as adherent cell cultures inRPMI-1640 medium containing 10% heat-inactivated FBS (MCF-7,NCI-H460 and HCT-15) and 2 mM glutamine or in DMEM supplemented with 10% FBS, 2 mM glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin (HeLa and HepG2 cells), at 37ºC, in a humidified air incubator containing 5% CO_2. Each cell line was plated at an appropriate density (7.5×10^3 cells/well for MCF-7, NCI-H460 and HCT-15 or 1.0×10^4 cells/well for HeLa and HepG2) in 96-well plates and allowed to attach for 24 h. Cells were then treated for 48h with the different diluted sample solutions. Following this incubation period, the adherent cells were fixed by adding cold 10% trichloroacetic acid (TCA, 100 µl) and incubated for 60 min at 4ºC. Plates were then washed with deionized

water and dried; sulforhodamine B solution (0.1% in 1% acetic acid, 100 µl) was then added to each plate well and incubated for 30 min at room temperature. Unbound SRB was removed by washing with 1% acetic acid. Plates were air dried, the bound SRB was solubilised with10 mM Tris (200 µl, pH 7.4) and the absorbance was measured at 540 nm (Monks et al., 1991) in the microplate reader mentioned above. The results were expressed in GI_{50} values (sample concentration that inhibited 50% of the net cell growth). Ellipticine was used as positive control.

2.9. Evaluation of hepatotoxicity of C. militaris methanolic extract

A cell culture was prepared from a freshly harvested porcine liver obtained from a local slaughter house. It was designed as PLP2. Briefly, the liver tissues were rinsed in Hank's balanced salt solution containing 100 U/ml penicillin and 100 µg/ml streptomycin and divided into 1×1 mm^3 explants. Some of these explants were placed in 25 cm^2 tissue flasks in DMEM supplemented with 10% fetal bovine serum, 2 mM nonessential amino acids and 100 U/ml penicillin, 100 mg/ml streptomycin and incubated at 37 ºC with a humidified atmosphere containing 5% CO_2. The medium was changed every 2 days. Cultivation of the cells was continued with direct monitoring every 2-3 days using a phase contrast microscope. Before confluence, cells were sub-cultured and plated in 96-well plates at a density of 1.0×10^4 cells/well, and cultivated in DMEM medium with 10% FBS, 100 U/ml penicillin and 100 µg /ml streptomycin (Abreu et al., 2011). Cells were treated for 48 h with the different diluted sample solutions and the same procedure described in the previous section for SRB assay was followed. The results were expressed in GI_{50} values (sample concentration that inhibited 50% of the net cell growth). Ellipticine was used as positive control.

2.10. Statistical treatment

Three samples were used and all the experiments were carried out in triplicate. The results are expressed as mean values and standard deviation (SD).

3. Results and discussion

The results obtained for the quantification of free sugars are presented in **Table 1**. The free sugars found in C. *militaris* were mannitol (2.01 g/100 g dw) and trehalose (24.71 g/100 g dw), this one in higher quantities. Mannitol is the most abundant polyol in the sporocarps of Basidiomycetes and Ascomycetes (former classification; Lewis and Smith, 1967). This sugar alcohol arises in the available literature as one of the compounds of *C. militaris* with pharmacological activity, holding diuretic, anti-tussive and free-radical activities (Paterson and Russell, 2008; Das et al., 2010). Trehalose is a common sugar component of most immature sporocarps and it may function as a reserve which is metabolized when the sporocarps are maturing (Koide et al., 2000). The levels found (of both polyol and sugar) are in agreement with other studies, where these molecules were found in higher amounts in *C. militaris* fruiting bodies from Taiwan, despite other sugars also have been quantified, specifically arabinose, fructose and ribose (Huang et al., 2006). The mentioned sugars were not identified in the sample herein studied (**Figure 1A**).

The organic acid found in higher amounts was citric acid (7.97 g/100 g dw; **Table 1**). It was also possible to quantify oxalic acid (0.33 g/100 g dw) and fumaric acid (0.13 g/100 g dw) (**Figure 1B**). Citric and fumaric acids play an important role in the Krebs cycle, being essential for human metabolism, but they have much more applications; citric acid is a crystal thickener in bones (Hu et al., 2010) and fumaric acid possesses interesting biological effects such as anti-inflammatory, neuroprotective and

chemopreventive activities. It also acts as an antimicrobial agent for fruit and vegetable preservation (Baati et al., 2011).Regarding phenolic acids, *p*-hydroxybenzoic acid was the only compound found in the studied species (0.02 mg/100g dw). However, cinnamic acid was also found (0.11 mg/100g dw; **Table 1**) (**Figure 1C**). Phenolic acids are composed of hydroxycinnamic and hydroxybenzoic acids. They have antioxidant activity as chelators and free radical scavengers with special impact on hydroxyl and peroxyl radicals, superoxide anions and peroxynitrites (Carocho and Ferreira, 2013), hence the importance of their detection in biological sources.

The main fatty acids found in this mushroom species were palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1n9) and linoleic acid (C18:2n6) (**Table 2**; **Figure 2A**). Other studies evaluate the fatty acids profile in different *Cordyceps* species (wild and cultivated), from different regions, and described similar profiles (Yang et al., 2009). Polyunsaturated fatty acids (68.87% of total FA) predominated over saturated fatty acids (23.40% of total FA) and monounsaturated fatty acids (7.73% of total FA) due to the higher levels of linoleic acid (68.00% of total FA; **Table 2**). Zhou et al. (2009) stated that unsaturated fatty acids content reaches 57.84% in *Cordyceps* species, the linoleic acid content being the highest one, followed by oleic acid. The saturated fatty acids content was 42.16%, palmitic and octadecanoic acids content being the highest. This way, the results obtained are similar to those reached by other research groups (Zhou et al., 2009). Unsaturated fatty acids are effective physiologically active components, which decrease blood lipids and protect against cardiovascular disease (Zhou et al., 2009). Therefore, *C. militaris* proved to be a source of the so called "good fat".

Vitamin E is composed of eight isoforms, with four tocopherols (α-tocopherol, β-tocopherol, γ-tocopherol and δ-tocopherol) and four tocotrienols (α-tocotrienol, β-

tocotrienol, γ-tocotrienoland δ-tocotrienol). This vitamin halts lipid peroxidation and is the only major lipid-soluble chain breaking antioxidant found in plasma, red cells and tissues, allowing the protection of the integrity of lipid structures, mainly membranes (Burton and Traber, 1990). In the studied sample, δ-tocopherol was the only isoform identified (55.86 μg/100 g dw; **Table 2**; **Figure 2B**).

There are no studies available specifically with the vitamin E, organic and phenolic acids profiles of *C. militaris*; so the present work offers new information about the biomolecules profile of this species, and increases the knowledge about the compounds with pharmacological interest that could be extracted from *C. militaris*.

Analyzing the results presented for the antioxidant potential (**Table 3**), the methanolic extract of *C. militaris* revealed the lowest EC_{50} value for lipid peroxidation inhibition (1.05 mg/ml). The highest EC_{50} value was verified in DPPH radical-scavenging activity (12.17 mg/ml) and also in *Folin-Ciocalteu* assay (15.04 mg GAE/g). Pereira et al. (2012) reported the antioxidant activity of twenty different wild edible species revealing EC_{50} values for DPPH radical-scavenging activity ranging from 20.02 to 0.68 mg/ml, and 4.58 to 58.14 mg GAE/g for *Folin-Ciocalteu* assay; therefore the EC_{50} value presented herein could be considered within the range of the results presented by the mentioned authors. *C. militaris* revealed the presence of some antioxidant molecules such as δ-tocopherol or *p*-hydroxybenzoic acid, which may be related to its antioxidant activity (Heleno et al., 2010; Pereira et al., 2012; Reis et al., 2012b), as well as others which have been reported in literature (such as polysaccharides, Ferreira et al., 2010).

Concerning the antibacterial activity (**Table 4**), the methanolic extract of *C. militaris* revealed the highest antibacterial potential against *Bacillus cereus* (MIC- 0.015 mg/ml; MBC- 0.03 mg/ml) and *Pseudomonas aeruginosa* (MIC- 0.015 mg/ml; MBC- 0.03 mg/ml). The most resistant bacterium to the effect of the extract was *Salmonella*

typhimurium (MIC- 3.00 mg/ml; MBC- 6.25 mg/ml). In the cases of *Bacillus cereus* and *Pseudomonas aeruginosa*, the extract possessed higher activity than the commercial antibiotics streptomycine and ampicilline. Regarding the antifungal activity (**Table 5**), the highest inhibitory potential was verified for *Aspergillus* species (MIC- 0.04 mg/ml). The highest fungicidal activity was equally expressed on *Penicillium funiculosum*, *Penicillium ochrochloron* and *Trichoderma viride* (MFC- 0.17 mg/ml). The lowest inhibitory and fungicidal activity was revealed for *Penicillium verrucosum* var *cyclopium* (6.25 and 12.5 mg/ml). Comparing the antifungal activity of bifonazole and ketoconazole to the investigated extract, activity of the latter was more efficient for *Penicillium ochrochloron*, *Penicillium funiculosum* and *Trichoderma viride*.

Ahn et al. (2000) reported that culture filtrates and methanol extracts of *C. militaris* and its constituent, cordycepin, had strong growth-inhibiting activity towards *Clostridium* species, but had no adverse effects on the growth of eight lactic acid producing bacteria. Interestingly, the methanol extract of *C. militaris* was sequentially partitioned into hexane, chloroform, ethyl acetate, butanol and water portions, and only the ethyl acetate and butanol fractions had strong growth inhibitory activity. Little data is available on the antifungal activity of *Cordyceps* species, with only ophiocorin isolated from *C. ophioglossoides* known to have antifungal activity (Kneifel et al., 2004). Nevertheless, there are other studies that deal with antimicrobial properties of natural matrices, in which the individual components are correlated to their antimicrobial activity (López et al., 2005; López et al., 2007; Goñi et al., 2009). Therefore, *C. militaris* was shown to be a rich source of natural bioactive compounds that can be correlated to its antimicrobial activity.

Currently, only a limited number of antifungal agents are available for the treatment of fungal infections. The *C. militaris* extract that was active against major food-borne

pathogens, plant, animal, mushroom and human pathogenic species, may be used as an alternative to synthetic chemicals that are being applied in mushroom and plant cultivation, food production, and pharmacy, to prevent and cure the most important diseases. It is also a candidate for future studies of synergism, compatibility, and activity in food or food-processing systems.

The results obtained for the anti-proliferative activity of the methanolic extract of *C. militaris* are presented in **Table 6**. The lowest GI_{50} value was obtained for non-small lung human carcinoma cell line (NCI-H460; 47.79 µg/ml). The tested extract also inhibited the proliferation of breast (MCF-7; 90.11 µg/ml), colon (HCT-15; 72.57 µg/ml) and cervical (HeLa; 66.32 µg/ml) human carcinoma cell lines. At the mentioned concentrations, the extract did not show toxicity against non-tumor liver primary cells (PLP2; GI_{50}=114.74 µg/ml). The studied mushroom proved not to inhibit the proliferation of human hepatocellular carcinoma cell line (HepG2), up to 400 µg/ml. Other studies stated that *C. militaris* was able to inhibit the growth of different tumor cell lines, such as the colon cancer cell line Colon 205 (Rao et al., 2010) or the human lung carcinoma A549 cells (Park et al., 2009b).

4. Conclusions

This study provides new data concerning the isolation and chemical characterization of bioactive compounds of the medicinal mushroom *Cordyceps militaris*. It was demonstrated that ascomycete mushrooms could be an excellent source of a wide range of interesting molecules with pharmacological activity or even with antitumor potential. The results obtained proved that *C. militaris* has antioxidant, antibacterial, antifungal and anti-proliferative properties. The anti-proliferative effects were exhibited in several tumor cell lines and did not show up in primary control cells. This is suggestive for an

antitumor effect of the *C. militaris* extract that should be confirmed (e.g. *in vivo* applications). This ancient medicinal fungus, *C. militaris*, which has been used as a crude drug for the welfare of mankind in old civilization, is now a matter of concern due to its unexplored potentials obtained by various modern techniques. In this regard, it needs evaluation on modern scientific lines such as accurate phytochemical analysis, biological screening sequencing, pharmacological investigation and clinical trials.

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Table 1. Composition of *Cordyceps militaris* in hydrophilic compounds (mean ± SD; n=3).

Hydrophilic compounds	Content
Mannitol (g/100g dw)	2.01 ± 0.18
Trehalose (g/100g dw)	24.71 ± 1.70
Total sugars (g/100g dw)	26.72 ± 1.88
Oxalic acid (g/100g dw)	0.33 ± 0.00
Citric acid (g/100g dw)	7.97 ± 0.23
Fumaric acid (g/100g dw)	0.13 ± 0.06
Total organic acids (g/100 g dw)	8.43 ± 0.17
p-Hydroxybenzoic acid (mg/100 g dw)	0.02 ± 0.00
Cinnamic acid (mg/100 g dw)	0.11 ± 0.00

dw- dry weight

Table 2. Composition of *Cordyceps militaris* in lipophilic compounds (mean ± SD; n=3).

Lipophilic compounds	Content
C6:0	0.36 ± 0.03
C8:0	0.06 ± 0.00
C10:0	0.04 ± 0.00
C12:0	0.09 ± 0.00
C14:0	0.35 ± 0.01
C14:1	0.01 ± 0.00
C15:0	0.55 ± 0.01
C16:0	12.76 ± 0.06
C16:1	0.34 ± 0.00
C17:0	1.79 ± 0.00
C18:0	5.71 ± 0.00
C18:1n9	7.29 ± 0.05
C18:2n6	68.00 ± 0.13
C18:3n3	0.41 ± 0.01
C20:0	0.28 ± 0.00
C20:1	0.06 ± 0.00
C20:2	0.07 ± 0.01
C20:3n3+C21:0	0.20 ± 0.01
C20:5n3	0.19 ± 0.00
C22:0	0.44 ± 0.01
C22:1n9	0.02 ± 0.01
C23:0	0.44 ± 0.01
C24:0	0.53 ± 0.04
C24:1	0.02 ± 0.00
Total SFA (% of total FA)	23.40 ± 0.05
Total MUFA (% of total FA)	7.73 ± 0.06
Total PUFA (% of total FA)	68.87 ± 0.11
δ-Tocopherol (μg/100 g dw)	55.86 ± 5.14

C6:0 (Caproic acid); C8:0 (Caprylic acid); C10:0 (Capric acid); C12:0 (Lauric acid); C14:0 (Myristic acid); C14:1 (Myristoleic acid); C15:0 (Pentadecanoic acid); C16:0 (Palmitic acid); C16:1 (Palmitoleic acid); C17:0 (Heptadecanoic acid); C18:0 (Stearic acid); C18:1n9c (Oleic acid); C18:2n6c (Linoleic acid); C18:3n3 (Linolenic acid); C20:0 (Arachidic acid); C20:1 (*cis*-11-Eicosenoic acid); C20:2 (*cis*-11,14-Eicosadienoic acid); C20:3n3+C21:0 (*cis*-11,14,17-Eicosatrienoic acid and Heneicosanoic acid); C20:5n3 (*cis*-5,8,11,14,17-Eicosapentaenoic acid); C22:0 (Behenic acid); C22:1n9 (Erucic acid); C23:0 (Tricosanoic acid); C24:0 (Lignoceric acid); C24:1 (Nervonic acid).SFA- Saturated fatty acids; MUFA- Monounsaturated fatty acids; PUFA- Polyunsaturated fatty acids. dw- dry weight.

Table 3. Antioxidant activity of the methanolic extract of *Cordyceps militaris* (mean ± SD; n=3).

	Assay	*Cordyceps militaris*	Trolox (standard)
Reducing power	*Folin-ciocalteu* (mg GAE/g extract)	15.04 ± 0.34	-
	Ferricyanide/Prussian blue (EC_{50}; mg/ml)	5.55 ± 0.03	0.03 ± 0.00
Radical scavenging activity	DPPH scavenging activity (EC_{50}; mg/ml)	12.17 ± 0.72	0.04 ± 0.00
Lipid peroxidation inhibition	β-carotene/linoleate (EC_{50}; mg/ml)	1.05 ± 0.07	0.003 ± 0.00
	TBARS (EC_{50}; mg/ml)	0.77 ± 0.03	0.004 ± 0.00

Concerning the *Folin-Ciocalteu* assay, higher values mean higher reducing power; for the other assays, the results are presented in EC_{50} values, what means that higher values correspond to lower reducing power or antioxidant potential. EC_{50}: Extract concentration corresponding to 50% of antioxidant activity or 0.5 of absorbance for the Ferricyanide/Prussian blue assay.

Table 4. Antibacterialactivity of the methanolic extract of *Cordyceps militaris,* mg/ml (mean ± SD; n=3).

Bacteria		*Cordyceps militaris*	Streptomycin	Ampicillin
Staphylococcus aureus	MIC	0.75±0.03	0.04±0.00	0.25±0.05
	MBC	1.75±0.00	0.09±0.00	0.37±0.02
Bacillus cereus	MIC	0.015±0.003	0.09±0.00	0.25±0.00
	MBC	0.03±0.003	0.17±0.01	0.37±0.02
Micrococcus flavus	MIC	1.5±0.14	0.17±0.02	0.25±0.03
	MBC	3.0±0.30	0.34±0.02	0.37±0.00
Listeriamonocytogenes	MIC	0.35±0.03	0.17±0.01	0.37±0.01
	MBC	3.0±0.60	0.34±0.00	0.49±0.03
Pseudomonas aeruginosa	MIC	0.015±0.00	0.17±0.04	0.74±0.02
	MBC	0.03±0.003	0.34±0.03	1.24±0.00
Salmonella typhimurium	MIC	3.0±0.14	0.17±0.00	0.37±0.01
	MBC	6.25±0.14	0.34±0.01	0.49±0.03
Escherichia coli	MIC	2.25±0.00	0.17±0.03	0.25±0.05
	MBC	3.0±0.14	0.34±0.02	0.49±0.05
Enterobacter cloacae	MIC	1.5±0.30	0.26±0.01	0.37±0.05
	MBC	3.0±0.00	0.52±0.02	0.74±0.07

MIC- minimum inhibitory concentration; MBC- minimum bactericidal concentration

Table 5. Antifungal activity of the methanolic extract of *Cordyceps militaris,* mg/ml (mean ± SD; n=3).

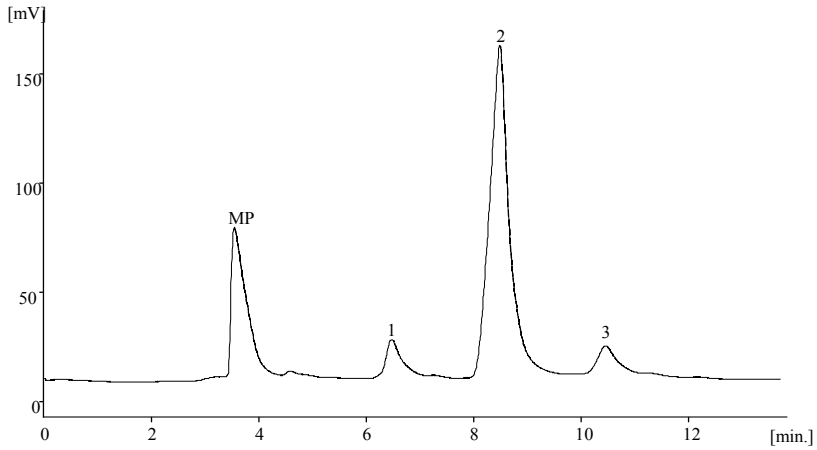
Fungi		*Cordyceps militaris*	Bifonazole	Ketoconazole
Aspergillus fumigatus	MIC	0.04±0.06	0.15±0.03	0.20±0.00
	MFC	6.25±0.14	0.20±0.03	0.50±0.10
Aspergillus versicolor	MIC	0.04±0.06	0.10±0.06	0.20±0.06
	MFC	1.5±0.14	0.20±0.06	0.50±0.06
Aspergillus ochraceus	MIC	0.04±0.00	0.15±0.03	1.50±0.00
	MFC	6.25±0.14	0.20±0.00	2.0±0.30
Aspergillus niger	MIC	0.75±0.10	0.15±0.03	0.20±0.03
	MFC	6.25±0.40	0.20±0.06	0.50±0.03
Trichoderma viride	MIC	0.09±0.00	0.15±0.03	1.0±0.00
	MFC	0.17±0.01	0.20±0.00	1.0±0.30
Penicillium funiculosum	MIC	0.09±0.03	0.20±0.06	0.20±0.03
	MFC	0.17±0.01	0.25±0.03	0.50±0.00
Penicillium ochrochloron	MIC	0.09±0.01	0.20±0.06	2.5±0.30
	MFC	0.17±0.01	0.25±0.03	3.5±0.10
Penicillium verrucosum var. *cyclopium*	MIC	6.25±0.00	0.10±0.00	0.20±0.00
	MFC	12.5±0.30	0.20±0.00	0.30±0.00

MIC- minimum inhibitory concentration; MFC- minimum fungicidal concentration

Table 6. Anti-proliferative activity of the methanolic extract of *Cordyceps militaris* (mean ± SD; n=3).

	Cordyceps militaris	Ellipticine
Toxicity for human tumor cell lines		
MCF-7 (breast carcinoma) (GI_{50}, µg/ml)	90.11 ± 2.90	0.91 ± 0.04
NCI-H460 (non-small cell lung cancer) (GI_{50}, µg/ml)	47.79 ± 3.84	1.42 ± 0.00
HCT-15 (colon carcinoma) (GI_{50}, µg/ml)	72.57 ± 6.51	1.91 ± 0.06
HeLa (cervical carcinoma) (GI_{50}, µg/ml)	66.32 ± 5.25	1.14 ± 0.21
HepG2 (hepatocellular carcinoma) (GI_{50}, µg/ml)	>400	3.22 ± 0.67
Hepatotoxicity		
PLP2 (GI_{50}, µg/ml)	114.74 ± 4.74	2.06 ± 0.03

GI_{50} values correspond to the extract concentration achieving 50% of growth inhibition in human tumor cell lines or in liver primary culture PLP2.



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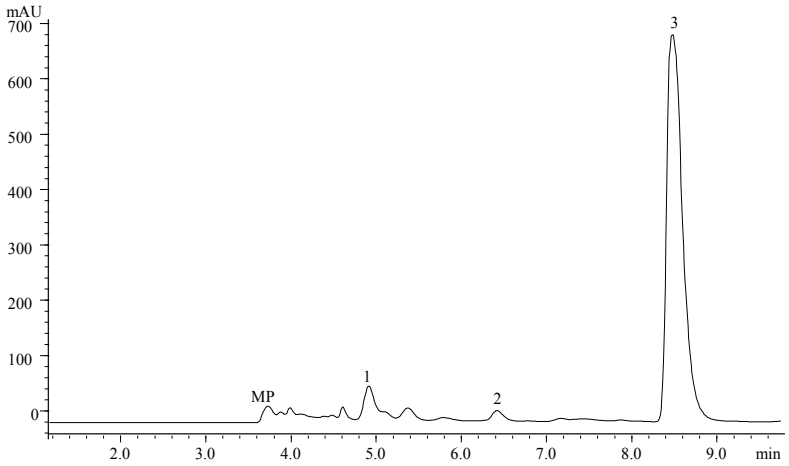
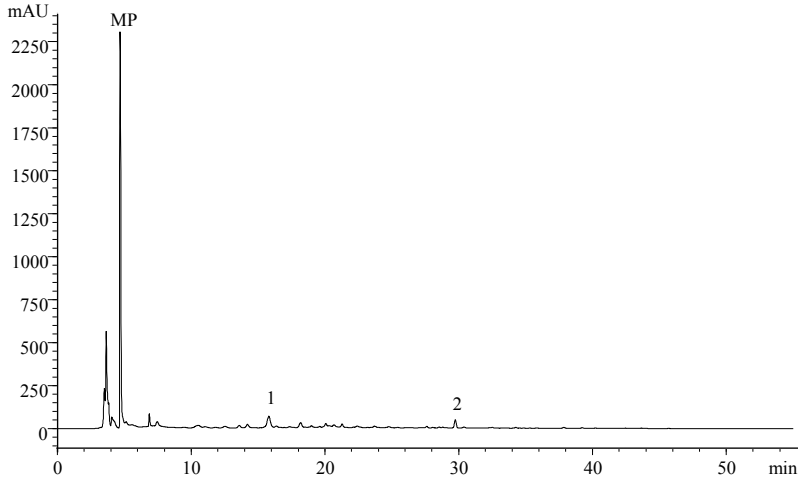
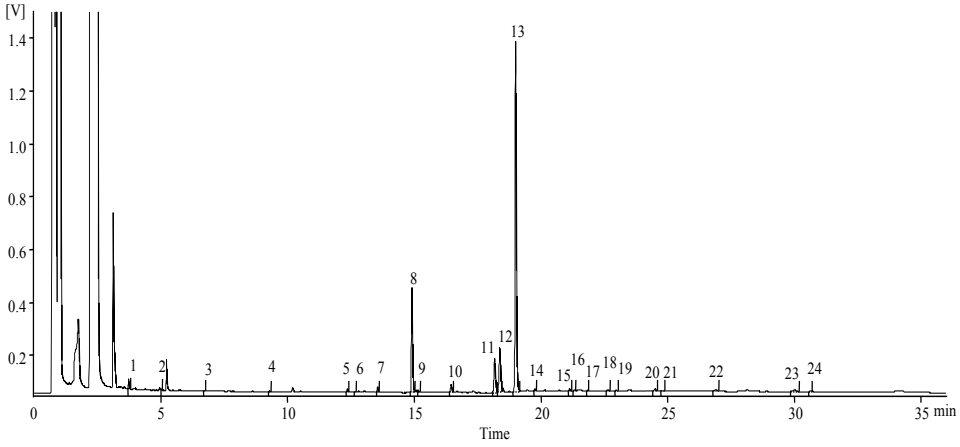


B

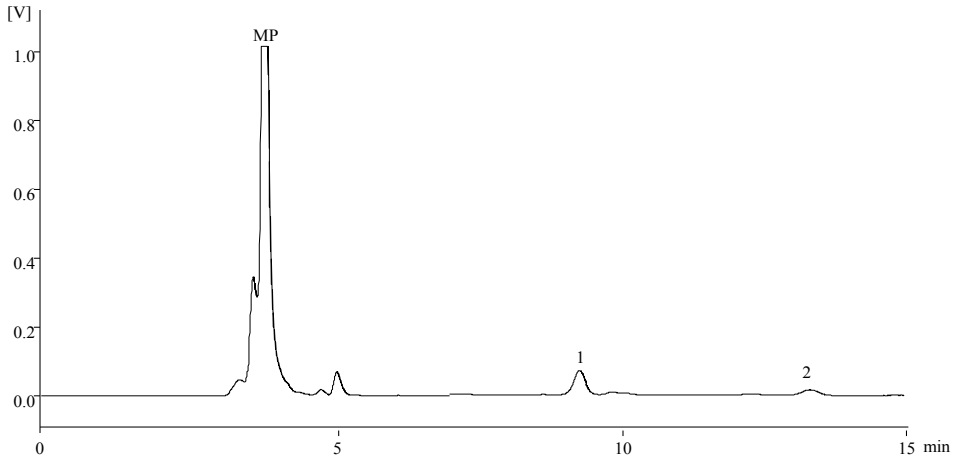


C

Figure 1. Individual chromatograms of *Cordyceps militaris*. A- Sugars profile: MP-mobile phase, 1-manitol, 2- trehalose, 3- raffinose (IS); B- Organic acids profile: MP-mobile phase, 1- oxalic acid, 2- citric acid, 3- fumaric acid; C- Phenolic acids profile: MP-mobile phase, 1-*p*-hydroxybenzoic acid, 2- cinnamic acid.



A



B

Figure 2. Individual chromatograms of *Cordyceps militaris*. A- Fatty acids: 1- Caproic acid (C6:0); 2- Caprylic acid (C8:0); 3- Capric acid (C10:0); 4- Lauric acid (C12:0); 5- Myristic acid (C14:0);6- Myristoleic acid (C14:1); 7- Pentadecanoic acid (C15:0); 8- Palmitic acid (C16:0); 9- Palmitoleic acid (C16:1); 10- Heptadecanoic acid (C17:0); 11- Stearic acid (C18:0); 12- Oleic acid (C18:1n9c); 13- Linoleic acid (C18:2n6c);14- α-Linolenic acid (C18:3n3); 15-Arachidic acid (C20:0); 16-Eicosenoic acid (C20:1c);17-*cis*-11,14-Eicosadienoic acid (C20:2c); 18- *cis*-11, 14, 17-Eicosatrienoic acid and Heneicosanoic acid (C20:3n3+C21:0); 19- *cis*-5,8,11,14,17-Eicosapentaenoic acid (C20:5n3); 20- Behenic acid (C22:0); 21- Erucic acid (C22:1n9); 22- Tricosanoic acid (C23:0); 23-Lignoceric acid (C24:0); 24- Nervonic acid (C24:1); B- Tocopherols profile: MP-mobile phase, 1-δ-tocopherol, 2- tocol (IS).