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Separation and characterization of antioxidant rich fractions from *Cordyceps sinensis* by semi preparative HPLC

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Abstract
Cordyceps sinensis (*C. sinensis*), is a high altitude medicinal mushroom that is widely revered in traditional Oriental medicine for its energy enhancing and rejuvenating values. *C. sinensis* is known to possess several bioactive constituents like steroids, terpenoids, polyphenolics, glycosides, glycoproteins and peptides which impart therapeutic effects like antiproliferative, antihypercholesterolemic, immunomodulatory, prebiotic, etc. In the present paper, antioxidant fractions were prepared from aqueous extract of Indian variety of *C. sinensis* by semi preparative high performance liquid chromatography (SP-HPLC). Fractionation was brought about on a Waters Sunfire® C18 column (10 mm x 250 mm), using 0.5% glacial acetic acid and acetonitrile (95:5, v/v) as the mobile phase, maintained at an isocratic flow rate of 5 mL/min. Eight fractions namely, F1 to F8 were collected in separate vials. Evaluation of total phenolic content (TPC) and total flavonoid content (TFC) confirmed significant antioxidant potential of these fractions. This observation was further substantiated by HPTLC fingerprinting that verified the presence of numerous antioxidant compounds in F1 to F8. Characterization by Fourier Transform Infrared (FTIR) spectroscopy, ^{1}H and ^{13}Cnuclear magnetic resonance (NMR) spectroscopy, and electrospray ionization mass spectroscopy (ESI-MS) manifested the presence of novel bioactive compounds in the fractions e.g.: n-heptanyl-beta-D-glucuronopyranosyl-(4'-1")-beta-D-glucopyranosyl-(4"-1"') beta-D-xylopyronoside, 1-hexadecanyl glycerol, 1-pentadecanyl glycerol, myristinylxylosidetetradecanoylxyloside, n-octadecanoic acid, arachidyl-beta-D-glucopyranosyl-(6'-1")-beta-D-glucopyranosyl-(6"-1"')-beta-D-glucopyranoside, nonacosan-13-beta-ol and beta-D-glucopyranosyl-(6-1)-o-beta-D-glucopyranoside.

Keywords
Cordyceps sinensis; semi preparative HPLC; HPTLC; FTIR; NMR; ESI-MS.

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Introduction

Medicinal herbs have been used since ages in traditional medicine for improvement of overall human health by boosting immunity and averting diseases of the vital organs. (Karjalainen *et al.*, 2010; Zhou *et al.*, 2008). Therapeutic herbal products derived from plants and mushroom sources can largely be categorized into two forms: (i) bioactive drugs isolated from medicinal herbs such as digoxin, digitoxin, reserpine, taxol, vinblastine, vincristine etc. (Raskin *et al.*, 2002) (ii) bioactive herbal isolates of characteristic novel structure derived directly and indirectly from natural products such as steroids, terpenoids, alkaloids, phenolic compounds, polysaccharides, protein and peptides etc. One such prominent source of traditional medicines is the high altitude medicinal mushroom, *Cordyceps sinensis*, commonly known as the 'caterpillar fungus', because of its parasitization on ghost moth. It is found predominantly in China, Tibet and India at higher elevations of around 4000-6000 m and has been extensively reported to wield beneficial effects on circulatory, hematogenic, cardiovascular, respiratory, digestive and immune systems, under various detrimental conditions (Nie *et al.*, 2013).

The medicinal properties of *C. sinensis* could be ascribed to the abundance of bioactive compounds i.e., amino acids, glycoproteins, nucleobases, polysaccharides, steroids, terpenes, vitamins, etc. (Pal *et al*., 2015; Rakhee *et al*., 2016). Different methods of separation are employed for the isolation and purification of these aforesaid compounds from varied herbal sources, in the present context, medicinal mushrooms. Owing to the complex composition of phytoconstituents, unique extraction procedures need to be

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employed for maximal separation of desired bioactive compound in its purest possible form (Doughari, 2012). In purview of this, the present work was designed to isolate novel bioactive constituents specifically, antioxidant rich fractions from *C. sinensis* using semipreparative high performance liquid chromatography (SP-HPLC). Further characterization of these fractions was accomplished by advanced analytical techniques such as Fourier Transform Infrared Spectroscopy (FTIR), Proton nuclear magnetic resonance spectroscopy (^{1}H-NMR), ^{13}C-NMR, and electron spray ionization mass spectroscopy (ESI-MS). This type of an elaborate structure elucidation of various components collected from *C. sinensis* is necessary to explore its therapeutic properties in detail. Besides, the antioxidant potential of the semi preparative fractions was investigated by various assays and high performance thin layer chromatography (HPTLC).

Materials and Methods

Chemicals and Reagents

All chemicals and reagents used in the experimental procedures were purchased from Sigma Aldrich (USA) and belonged to HPLC grade. Water used throughout was of Millipore grade (Merck, USA).

Instruments

Cordyceps sinensis aqueous extract (CSAq) was prepared using an accelerated solvent extraction (ASE 350) system equipped with a solvent controller unit from Dionex Corporation (Sunnyvale, CA, USA). Extracts were freeze dried under vacuum in an Allied Frost 5 lyophilizer. Fractions collected were concentrated with the help of Savant™ SpeedVac™ High Capacity Concentrators, Thermo Scientific™). All absorbances were measured using BiotekPowerwave XS2 microplate reader. HPTLC analysis was performed using a system from CAMAG, Switzerland. Here, a Linomat 5 applicator was used for sample application, Reprostar 3 for documentation and Scanner 3 for densitometric scanning. Fractionation was accomplished using Waters® semi preparative HPLC set up, where stationary phase was Waters® SunFireTM 10 mm x 250 mm, C18 column. A 500 MHz Bruker Avance III nuclear magnetic resonance spectrometer, Bruker FTIR and Agilent 1100 ESI-MS was used for structural characterization of the fractions.

Preparation of Cordyceps sinensis aqueous extracts

Cordyceps sinensis (CS) whole bodies were commercially procured from M/s Aryan Enterprises, Delhi, India (batch number CO-1601). The CS whole bodies were powdered to an ultrafine texture using a grinder. CSAq was prepared by ASE according to a method described by Mamta *et al.*, 2015. Here, 10 g of dried of CS powder was mixed with diatomaceous earth (3:1, w/w) and filled neatly in extraction cells. Aqueous extract of CS was eluted taking water as the solvent, and pre optimized extraction parameters being pressure 1200 psi, temperature 25 °C, time 15 mins. The extract collected was concentrated by freeze drying in a lyophilizer to obtain pure CSAq and it was preserved at 4°C until further use.

Sample preparation for semipreparative HPLC separation from CSAq

Firstly, 500 mg of CSAq was weighed and dissolved in methanol (1:10, w/v) for extraction of active ingredients. This solution was placed in an ultrasonic bath for four hours at ambient temperature with pulse rate of 5 mins at frequency 40 kHz. Then the mixture was filtered through a 0.25 µm pore syringe membrane filter (Millipore, Durapore®) and the solution thus, obtained was stored at 4 °C for further separation of active ingredients.

Separation of bioactive fractions using semipreparative HPLC System

Separation of bioactive fractions from the sample prepared as aforesaid was achieved by semi-preparative HPLC using a mobile phase constituted of 0.5% glacial acetic acid (A) and acetonitrile (B), (A:B::95:5). An isocratic mode of elution was followed where flow rate was kept at 5 ml/min. Pressure range was maintained at around 3000 psi. A photodiode array detector was used for peak identification at a wavelength of 260 nm. Specific fractions were collected which were concentrated to a volume of 1-2 mL with speed vac evaporator. Eight such semi preparative fractions (henceforth, collectively referred to as "SPFs", and individually labelled as F1, F2 … F8) were collected. All these SPFs were lyophilized and stored at 4 °C for further characterization.

Estimation of antioxidant potential

Total phenolic content

Total phenolic content of the SPFs was determined by a method described by Mishra *et al*., 2018. In this assay, gallic acid was taken as a standard and values were expressed as gallic acid equivalent (GAE) per gram of fraction (gGAE/g fraction). A series of concentrations of gallic acid over a range from 1mg/ml to 1µg/ml were used to plot the standard curve. Then, 20 µl of standard or sample was mixed with 150 µl of water and 20 µl of Folin-Ciocalteu reagent. After incubation of 3 minutes, 25 µl of 1N Na_2CO_3 was added to this solution, followed by another incubation under dark for two hours at RT. Thereafter, absorbance was measured at 725nm using a microtiter plate reader. All readings were taken in triplicates.

Total flavonoid content

Determination of total flavonoid content was performed by a method described in previous literature (Mishra et al., 2018).Here, rutin was taken as the standard and values were denoted in terms of rutin equivalent (RT) per gram of fraction. Various concentrations of rutin from 1mg/ml to 1 µg/ml were used to plot the standard curve. 25µl of standard or sample was added to 125 µl of water and 7.5 µl of 5% $NaNO_2$, and allowed to react for 5 minutes. Following this, 25 µl of

$AlCl_3$ solution and 50 µl of 4% NaOH was added and volume was made up to 250 µl with the help of water. This solution was then incubated at dark for 15 minutes at RT. Absorbances were measured at 510 nm. Analysis was performed in triplicates.

Characterization of SPFs by HPTLC

All the SPFs were dissolved in methanol and filtered by syringe filter before proceeding for HPTLC analysis. Identification of flavonoids under study namely, rutin, hesperidin, gallic acid, myricetin, and quercetin in the SPFs was facilitated using a mobile phase reported earlier (Bhardwaj *et al*., 2015). The mobile phase was composed of ethyl acetate: dichloromethane: formic acid: glacial acetic acid: Methanol (10: 10: 1: 1: 2, by volume) and glass backed silica gel 60 F_{254} HPTLC plates were used as the stationary phase. Standards and SPFs were prepared in methanol, having concentrations of 0.5 mg/ml and 5 mg/ml, respectively. The samples were applied on HPTLC plates as 7 mm bands. After sample application, the plate was allowed to develop till a solvent front of 90 mm at room temperature. After development, the plates were air dried for 15 min, and then documented by illuminating at 254 nm. Densitometry scanning and quantification were performed in absorbance mode at 254 nm where deuterium acted as the light source. Peak areas calculated by winCats software (version 1.4.4.6337) aided for determining the quantities of various flavonoids detected in the SPFs.

Structural characterization of SPFs

FTIR spectroscopy

The SPFs were mixed well with potassium bromide (KBr), to prepare homogenous mixtures. These mixtures were finely pelleted under high pressure and were subjected to FTIR spectroscopy for confirming the stretching and bending vibrations corresponding to amide bonds (Mishra et al., 2018).

NMR spectroscopy

Here, samples were prepared in d^6-DMSO. The ^1H NMR and 13C NMR spectra were recorded in the range of 0-220 ppm using tetramethylsilane (TMS) as reference internal compound. Chemical shifts observed aided in the identification of integral functional groups (Mishra *et al*., 2017).

ESI-MS

All the SPFs were characterized using ESI-MS fragmentation pattern by employing a mobile phase comprising 0.1% formic acid (A), acetonitrile (B) and methanol (C). The respective m/z values of the SPFs were recorded for structure elucidation (Pongratz *et al.*, 2004).

Results

The SPFs collected were thoroughly characterized for determination of structure and estimation of antioxidant potential. The results obtained are elaborated in succeeding paragraphs.

Collection of SPFs by semi preparative HPLC

Eight peaks, each representing a SPF, were obtained after subjecting CSAq to semi preparative HPLC according to a method described in previous section. The profile acquired is shown in Figure 1.1, along with the corresponding retention times (R_t) of every resultant peak. Over a course of several runs, each of the eight fractions was distinctly collected, lyophilized and stored at 4°C for further analyses. The percentage yields of the SPFs are depicted in Figure 1.2.

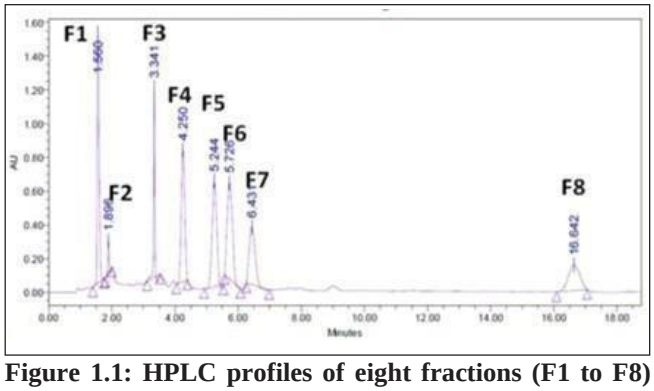

Figure 1.1: HPLC profiles of eight fractions (F1 to F8) obtained from aqueous extract of *C. sinensis.*

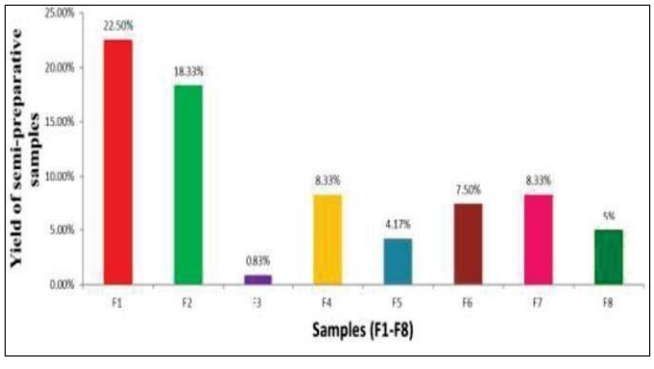

Figure 1.2: Percentage yields of F1 to F8.

Antioxidant potential of SPFs

Free radicals exert adverse oxidative effects in physiological system by rendering instability in various cellular and molecular processes. Antioxidants are able to inhibit this type of pro-oxidation by neutralizing free radicals (Yen *et al*., 2002). The antioxidant potential of any compound can be adjudged by various parameters for instance, total phenolic content (TPC) and total flavonoid content (TFC).

Evaluation of TPC and TFC of SPFs, as indicated in Figure 2.1 and Figure 2.2 demonstrated that SPFs possess promising antioxidant potential. It was observed that the highest antioxidant potency in terms of TPC was seen in F1 fraction (22.25 ± 1.04 µg GAE/g of fraction) and least value was found in F7 fraction (7 ± 0.50 µg GAE/g of fraction). Similarly, the antioxidant potential in terms of TFC indicated the maximum

value in F1 fraction (70.67 ± 3.21 µg RE/g of fraction) and minimum value in F3 fraction (8.33 ± 2.89 µg RE/g of fraction).

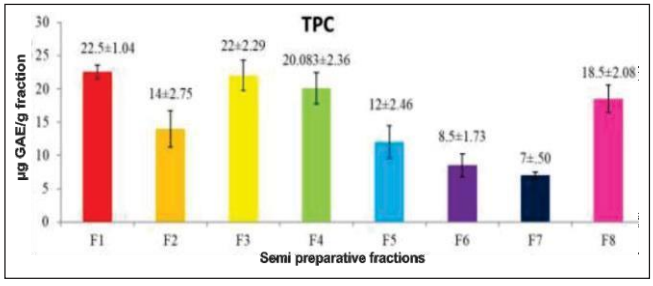

Figure 2.1. Antioxidant potential of semi preparative fractions, F1 to F8 in terms of total phenolic content (TPC).

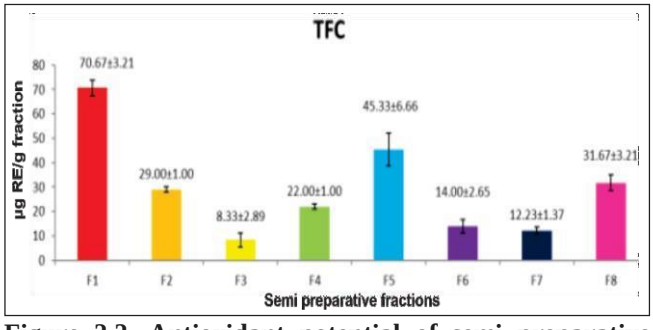

Figure 2.2. Antioxidant potential of semi preparative fractions, F1 to F8 in terms of total flavonoid content (TFC).

HPTLC analysis of the SPFs

HPTLC fingerprinting of all the SPFs confirmed their richness in terms of flavonoids and phenolic compounds. HPTLC profiles affirmed each SPF to be a mixture of many bioactive constituents as indicated by the presence of multiple bands in each fraction, except in F8 which displayed only a single band. Derivatization by different visualization reagents further asserted the presence of bioactive compounds in SPFs.

Figure 3.1 shows the profiling at 254 nm. It can be clearly seen that F1 and F4 contained only hesperidin. F2, F3 and F6 had rutin and hesperidin in them. F6 and F8 were seen to comprise only rutin. F5 and F7 did not contain any of the standards under consideration but they certainly had other antioxidant compounds in them, which were manifested as distinct bands.

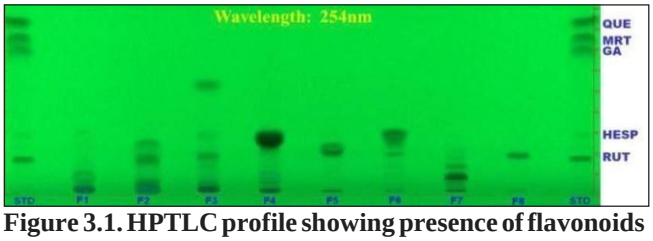

Figure 3.1. HPTLC profile showing presence of flavonoids in fractions F1 to F8.

HPTLC profiles after derivatization may be seen in Figures 3.2 and 3.4. It was observed that after derivatization with DPPH, some of the bands in F1 to F7 turned yellow (Figure 3.2) which is a clear indication of the antioxidant potential of the compounds corresponding to these yellowish bands (Bhardwaj *et al.*, 2015).

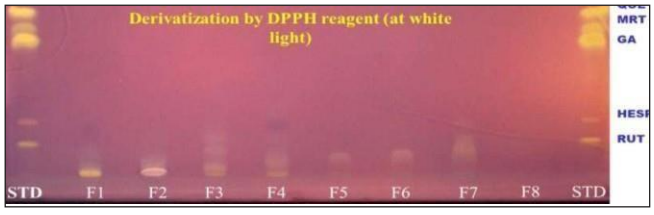

Figure 3.2. Derivatization with DPPH showing presence of antioxidant compounds in F1 through F7.

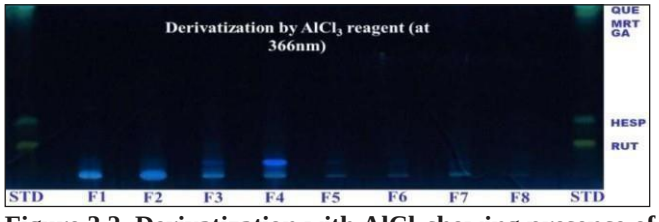

Figure 3.2. Derivatization with $AlCl_3$ showing presence of antioxidant compounds in F1 through F7.

Spectroscopic structure elucidation of SPFs

Structure elucidation of major compounds in each isolated SPF was accomplished by spectroscopic techniques namely FT-IR, ^{1}H-NMR, ^{13}C-NMR and ESI-MS. FT-IR helps in identifying characteristic stretching and bending frequencies of various functional groups which are present in any compound. FTIR spectra of *C. sinensis* SPFs revealed stretching and bending vibrations indicative of ethyl, methyl, ester, acetyl, etc functional groups. Similarly, ^{1}H-NMR and ^{13}C-NMR, which are based on magnetic resonance of nuclei, demonstrated the presence of hydroxyl, acetal, cyclic ether, ester, methyl, etc chemical moieties in the SPFs. ESI-MS uses characteristic fragmentation pattern, in order to identify several types of functionalities with specific m/z values in a compound, here SPFs.

Thus, this type of an overall spectroscopic approach abetted substantial structure elucidation of all the SPF fractions. The wave numbers of various bonds (in cm^{-1}) as acquired by FTIR spectroscopy, chemical shifts (in ppm) by ^{1}H-NMR and ^{13}C-NMR and m/z values by ESI-MS are compiled in Table 1.

Table 1. Combined spectroscopy data depicting structural characterization of SPFs.

Semi preparative fractions	^{1}H NMR chemical shift (δ value in ppm)	^{13}C NMR chemical shift (δ value in ppm)	FT-IR Wavelength (cm-1)	ESI-MS relative intensity (m/Z)
Fraction 1	**-OH** (4.02, 3.81, 3.58, 3.56, 3.52, 3.45, 3.42, 3.40, m) **-O-CH-O- or cyclic acetal** (5.10, 5.01, 4.82 1H, s) **-OCH2 group** (4.80, 2H, s) **-CH2 of OH group** (4.65, 2H, s) **cyclic CH of Hydroxyl groups** (3.38, 2H, m), (3.13, 2H, s) **Methylene groups (2.48,** 2H, m), (1.73, 6H, brs), (1.25, 2H, m) **Methyl group** (3H, t, J=6.5Hz, Me-1)	**-OH containing Carbon** (72.97,1C, s) (70.59, 1C, s) (72.07, 1C, s) (71.76, 1C, s) (61.21, 1C, s) (72.97, 1C, s) (71.09, 1C, s) (71.59, 1C, s) (75.43, 1C, s) **C of COOH** (175.01, 1C, s) **-O-CH-O- or cyclic carbon acetal** (93.67, 3C, s) (72.33, 1C, s) (71.59, 2C, s) **-OCH2 carbon group** (64.27, 1C, s) **Methylene carbon groups** (23.97,1C, s) (49.03, 1C, s) (49.03, 1C, s) (51.78, 1C, s) (53.67, 1C, s) (55.57, 1C, s) **Methyl carbon group** (17.41, 1C, s)	**Alcohol O-H** (3510-3200) **Alkyl C-H** (2950-2800) **O-H of COOH** (3400) **C=O of COOH at sugar moiety** (1643) **Alkyl CH2 bending** (1410-1557) **Alkyl CH3 bending** (1340) **CH2 bending** (410-654) cyclic C-O-C streching (1081)	388 $[M]^+$ ($C_{15}H_{32}O_{11}$) (100) 338 $[M]^+$ ($C_{12}H_{18}O_{11}$) (85.5) 295 $[M]^+$ ($C_{11}H_{19}O_9$) (38.9)
Fraction 2	**-OH** (4.06, 1H, m) **-CH2 of OH group** (3.11, 2H, m) **-CH of OH group** (3.51, 2H, s) **-OCH2 group** (3.07, 2H, t, J=6.8Hz) **Methylene groups** (2.07, 26H, brs, 13xCH2) 1.88 (2H,m) **Methyl group** (0.86, 3H, t, J=6.5Hz)	**-OH containing CH2 group** (72.92,1C, s) (61.22, 1C, s) **-OCH2 of carbon group** (72.07, 1C, s) (61.20, 1C, s) **Methylene carbon groups** (41.06, 1C, s) (31.16, 1C, s) (22.15, 12C, s) **Methyl carbon group** (19.01, 1C, s)	**Alcohol O-H** (3510-3225) **Alkyl C-H streching** (3061) **Ch2 group bending vibration** (416-542)	316 $[M]^+$ ($C_{19}H_{40}O_3$) (100) 317 $[M+H]^+$ ($C_{19}H_{41}O_3$) (23.55) 214 $[M]^+$ ($C_{12}H_{22}O_3$) (55.5)
Fraction 3	-OH (4.03, 1H, m) -CH2 of OH group (3.85, 2H,m) **-OCH2 group** (3.51, 2H, m), (3.15, 2H, m) **Methylene groups** (2.07, 2H, m), (1.22, 24H, brs, 12xCH2) Methyl group (0.83, 3H, t, J=6.6Hz)	**OH containing CH2 carbon** (-) **CH2 of carbon of OH group (-)** **-OCH2 group of carbon** (49.07, 1C, s) **Methylene group of carbon** (49.07, 1C, s) (40.60, 1C, s) (40.51, 1C, s) (40.44, 1C, s) (40.34, 1C, s) (40.27, 1C, s) (40.18, 1C, s) (40.01, 1C, s) (39.84, 1C, s) (39.68, 1C, s) (39.51, 1C, s) **Methyl group of carbon** (31.16, 1C, s)	**Alcohol O-H** (3364) **Alkyl C-H streching** (2950-2800) C-O-C stretching (1081) **Alkyl CH2 bending** (1402-1588) **CH2 bending (in plane and out plane bending)** (409-619)	303 $[M]^+$ ($C_{18}H_{39}O_3$) (55.8) 214 $[M]^+$ ($C_{12}H_{22}O_3$) (36.55)
Fraction 4	**-OH** (3.94, 1H, m), (3.58, 1H, m), (3.40, 1H, m) **-O-CH-O- or Cyclic acetal** (4.01, 1H, d, J=7.5Hz), 3.94, 1H, m), 3.40 (1H, m), 3.01 (2H, s) **-CH2 of OH group** (3.01, 2H, s), (2.34, 2H, m) **Methylene group** (2.64, brs, 10xCH2), (1.21, 2H, m) **Methyl group** (0.86, 3H, t, J=6.6Hz)	**Carbonyl C of ester** (169.11, 1C, s) **-OH group of carbon** (73.48, 1C, s) (71.37, 1C, s) (61.82, 1C, s) **-O-CH-O- or cyclic acetal of carbon** (102.67, 1C, s) **CH2 of carbon adjacent to ester** (49.06, 1C, s) **Methylene group of carbon** (21.26, 12C, s) **Methyl group of carbon** (14.16, 1C, s)	**Alcohol O-H** (3510-3225) **C=O of ester present at acetal carbon** (1687) **Alkyl C-H streching** (2950-2800) **C-O-C streching** (1107) **Alkyl CH2 bending** (1403-1560) **CH2 bending (in plane and out plane bending)** (410-550)	309 $[M-H]^+$ ($C_{19}H_{33}O_4$) (100) 360 $[M]^+$ ($C_{19}H_{36}O_6$) (20.0)

Semi preparative fractions	^{1}H NMR chemical shift (δ value in ppm)	^{13}C NMR chemical shift (δ value in ppm)	FT-IR Wavelength (cm-1)	ESI-MS relative intensity (m/Z)
Fraction 5	**Methylene group adjacent to COOH group** (2.72, 2H, t, J=15Hz) **Methylene groups** (2.62, 2H, m) (2.48, 2H, brs) (2.34, 36H, brs, 18xCH2) (2.06, 4H, s, 2xCH2) (1.89, 2H, m) (1.22, 2H, m) (1.13, 2H, m) **Methyl groups** (0.84, 3H, J=6.5Hz)	**Carbonyl C of COOH** (175.23, 1C, s) **Methylene carbon group adjacent to COOH group** (32.13, 1C, s) (24.73, 1C, s) **Methylene groups of carbon** (29.63, 10C, s) (24.73, 1C, s) (29.07, 1C, s) **Methyl group of carbon** (14.23, 1C, s)	**C=O of COOH** (1701) **O-H of COOH** (3400) **Alkyl CH2 bending** (1406-1081) **CH2 bending (in plane and out plane bending)** (488-405)	424 $[M]^+$ ($C_{28}H_{56}O_2$) (55.4)
Fraction 6	**-OH** (4.23, 4.13, 3.84, 3.72, 3.62, 3.55, 3.45, 3.42, 3.40, 3.37, m) **-O-CH-O-** [Cyclic acetal][4.86(1H, d,J=3.5Hz), 4.85 (1H, d, J=3.5Hz), 4.40 (1H, d, J=3.5Hz)] **Cyclic CH of hydroxyl group**(3.32 (1H,m), 3.28 (1H,m) 28 **CH2 groups of side chain** (1.22, 28H, brs) **Methyl group** (0.87, 3H, t, J=7Hz) **-CH2 groups** (2.48 ,2H,m) (2.06, 2H, m,) (1.84, 2H,m) (1.79,2H,m) **-OCH2 groups** (3.21, 2H, d, J=6.5Hz) (3.14, 2H, d, J=7.0) (3.07, 2H, d, J=7.0)	**Carbonyl C of ester** (172.35, 1C ,s) **OH group of C** (72.96, 2C, s) (71.80, 1C, s) (64.33, 1C, s) (72.87, 1C, s) (71.08, 1C, s) (63.56, 1C, s) (72.07, 1C, s) (70.01, 1C, s) (63.31, 1C, s) (60.02, 1C, s) **-O-CH-O- of cyclic acetal of C** (93.57, 1C, s) (93.05, 1C, s) (98.26, 1C, s) **Cyclic ether of C** (77.22, 1C, s) (73.33, 1C, s) (72.96, 1C, s) (61.71, 1C, s) (61.22, 1C, s) (60.02, 1C, s) **CH2 group adjacent to ester** (51.17, 1C, s) (41.06, 1C, s) (31.16, 1C, s) (38.12, 1C, s) **Methylene group C (29.53,13C, s) (19.32, 1C, s) Methyl C group (18.12, 1C, s)**	**Alcohol O-H (3510-3225) Alkyl C-H (2950-2800) C=O of ester (1713) Alkyl CH2 bending (1404-1600) Alkyl CH3 bending (1233-1390) cyclic C-O-C stretching (1079) CH2 bending (in plane and out plane bending) (409-544)**	774 $[M]^+$ ($C_{38}H_{78}O_{17}$) (1.1), 295[$(CH_3(CH_2)_{10}CO)^+$ (100)
Fraction 7	**-OH** (4.8, brs) **-CH of hydroxyl group** (3.12, 1H, m) **Methylene groups or -CH2 groups** (2.32, 4H, s, CH2-12, CH2-14) (1.25, 36H, s, 18 X CH2) (2.24, 2H, m, CH2) (1.56, 2H, m, CH2) (1.30, 2H, m) (1.12, 2H, m, Ch2) **Meth**yl group (1.01, 3H, t, J=6.5Hz), (0.68, 3H, t, J=6.9Hz)	**-OH containing C** (71.27, 1C, s) **-CH of C of hydroxyl group Methylene groups C** (48.30, 1C, s) (38.72, 1C, s) (30.39, 1C, s) (24.39, 5C, s) (20.79, 18C, s) **Methyl C group** (15.21, 1C, s) (11.64, 1C, s)	Alcohol O-H (3510-3154) Alkyl CH2 bending (1686-1401) Alkyl CH3 bending (1390-1231) Ch2 bending (in plane and out plane bending) (5152-413)	424 $[M]^+$ ($C_{28}H_{56}O$) (28.7), 199[$(CH_3(CH_2)_{10}CHOH]^+$ (21.2)
Fraction 8	**-OH** (1H, m) **-O-CH-O** (1H, m) **-CH2 of OH group** (2H, m) **Cyclic CH of hydroxyl group** (1H, s)	**-OH containing C** (74.07, 1C, s) (71.29, 1C, s) (64.94, 1C, s) (48.10, 1C, s) (47.93, 1C, s) **-Cyclic ether of C** (62.11, 2C, s) (47.59, 1C, s) **-O-CH-O- of cyclic acetal of C** (89.89, 2C, s) (86.82, 2C, s) **-OCH2 group of C** (42.17, 1C, s)		342 $[M]^+$ $C_{12}H_{22}O_{11}$ (100), 179($C_6H_{11}O_6$) (99.8)

The structures of novel most abundant bioactive compounds as characterized in all the fractions (F1-F8) using previously described spectroscopic technique sare given in Figure 4 below.

Discussion

Despite a wide range of metabolites and associated therapeutic activities reported for *C. sinensis* extracts, there is only limited literature available regarding the mechanism of action of the medicinal compounds isolated from this mushroom. Several researchers have established *C. sinensis* to possess varied beneficial health effects such as anti-aging, anti- carcinogenic, hyperlipidaemiatic, pharmacokinetic, energy enhancing, revitalizing, etc. (Shin *et al.*, 2003; Wang *et al.*, 2005). Advanced analytical techniques like FTIR and NMR spectroscopy have shed light on the presence of several characteristic compounds (Chen and Chu, 1996). There are a few reports that describe the clear and unique mechanisms of action of different *C. sinensis* extracts underlying its bioactivities (Paterson, 2008; Holliday *et al.*, 2004) whereas much of the research about other types of extracts prepared is yet unexplored.

Keeping this in perspective, the present study aimed at separating eight SPFs from aqueous extract of *C. sinensis* by semi preparative HPLC technique and their antioxidant potential in terms of TPC and TFC was established. This antioxidant action could very well be correlated with the presence of flavonoids and phenolics in all the eight SPFs, i.e., F1 to F8 as confirmed by HPTLC. Additionally, flavonoid content in these fractions was also validated by the help of derivatizing reagents namely, DPPH and $AlCl_3$.

Structure elucidation of F1 to F8 fractions by spectroscopic techniques such as ^{1}H NMR, ^{13}C-NMR, FT-IR and ESI-MS, suggested the presence of distinct compounds in them, for instance glyosidic carbohydrates (disaccharides and trisaccharides), fatty acids, long chain alcohols, derivatized 5 membered sugars, etc. The list of these unique compounds detected in all the semi preparative fractions have been compiled in Figure 4. According to previous studies (Holliday and Cleaver, 2008; Holliday *et al*., 2004), several polysaccharides, nucleosides, fatty acids, alcoholic compounds have been isolated by HPLC from natural sources and their putative biological activities like antifungal, antioxidant, antimicrobial activity, insecticidal activity, anticancer agents etc. are also well reported. These above isolated distinctive compounds in F1-F8 fractions may have specific to nonspecific biological activity such as antioxidant, antitumor activity, improving ATP metabolism, etc. For instance, compounds similar to the one in F1 showed anticancer activity by inhibiting growth of transformed cells (Mani *et al*., 1998), hexadecanyl glycerol that was detected in F2 was previously reported for its antihypertensive action (Blank *et al*., 1984), and compounds similar to nonacosan-13-beta ol that occurs in F7 has significant nematicidal activity (Naz and Khan, 2013). However, further analyses need to be conducted to determine the exact mechanism of action of these SPFs.

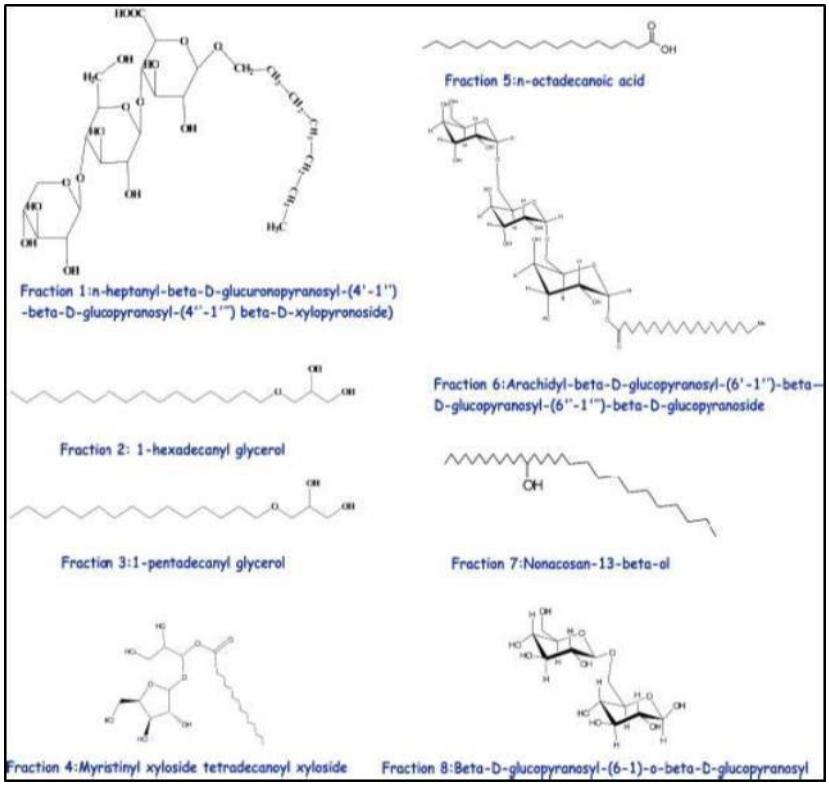

Figure 4: Structures of bioactive compounds detected in F1 to F8.

CONCLUSION

The eight different semi preparative fractions separated from *Cordyceps sinensis* were found to possess substantial antioxidant potential as manifested by their total phenolic and total flavonoid contents. Spectroscopic characterization aided in proving the presence of some novel bioactive compounds in these fractions. However, further scope remains in exploring these aforesaid putative bioactivities in various in vitro and in vivo models for establishing the fractions as promising therapeutic leads.

CONFLICT OF INTEREST

All the contributing authors declare that they have no conflict of interest.

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