Quantification of Flavonoids and Nucleoside by UPLC-MS in Indian *Cordyceps sinensis* and its *In-vitro* Cultures

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ABSTRACT

Background: *Cordyceps sinensis*, a comestible mushroom growing in Himalayan regions, is extensively familar in traditional system of medicine. The paper presents the results of quantification of some of the flavonoids and nucleosides (kaempferol, isorhamnetin and cordycepin) in the Indian variety of *Cordyceps sinensis.* Since, the plant is known for its high therapeutic potential and not easily available, hence it was also tissue cultured in the laboratory as an alternative source of medicine. **Methods:** Ultra Performance Liquid Chromatography (UPLC) along with Q-TOF–MS detector was used for the characterization and quantification of active components in the aqueous extracts of both the samples of *Cordyceps sinensis* (natural as well as *in vitro* cultured). **Results:** Results indicate that both the extracts have almost similar concentration of the kaempferol, isorhamnetin and cordycepin which supports use of *in vitro* biomass as an alternative source of medicine.

Key words: Cordycepin, *Cordyceps sinensis*, Isorhamnetin, Kaempferol, UPLC-MS/MS.

INTRODUCTION

Cordyceps sinensis is a medicinal herb that grows at an altitude more than 3800 m above sea level in cold, grassy, alpine meadows of Himalayan Mountains and is used for various ailments by the local people. *Cordyceps sinensis* is a parasitic mushroom, a type of gascomycetes fungus and has found its use in traditional Chinese medicine for many centuries. Various studies have shown that *C. sinensis* modulates immune response,[1] inhibits tumor cell proliferation,[2] improves liver function,[3] regulates insulin sensitivity,[4] decreases plasma cholesterol levels,[5] has hypotensive and vasodilatory activity,[6] and increases physical performance.[7] It has also shown protection against cerebral ischemia reperfusion injury, oxidative damage,[8] and radiation induces bone marrow and intestinal injury.[9] The various medicinal properties of *C. sinensis* have been reviewed in detail by Zhu *et al.*[10]

Historically, plants and medicinal fungus provide the basic building blocks for a number of highly effective drugs and they remain an attractive option for discovery of the new molecular entities, due to their largely tapped chemical diversity.[11] Flavanoids are polyphenolic compounds found in plants and are known to possess antioxidant, cyto protective and anti-inflammatory activities.[12-14] The nucleosides, which are some of the bioactive compounds present in *C. sinensis* are found responsible to increase mitochondrial activity and play major role in the production of ATP.[15] The flavonoids like kaempferol (Figure 1c) and isorhamnetin (Figure 1b) have been reported in *C. sinensis*.[16] Cordycepin (Figure

Submission Date : 11-12-2014
Revision Date : 02-04-2015
Accepted Date : 23-04-2015

DOI: 10.5530/ijper.49.4.12

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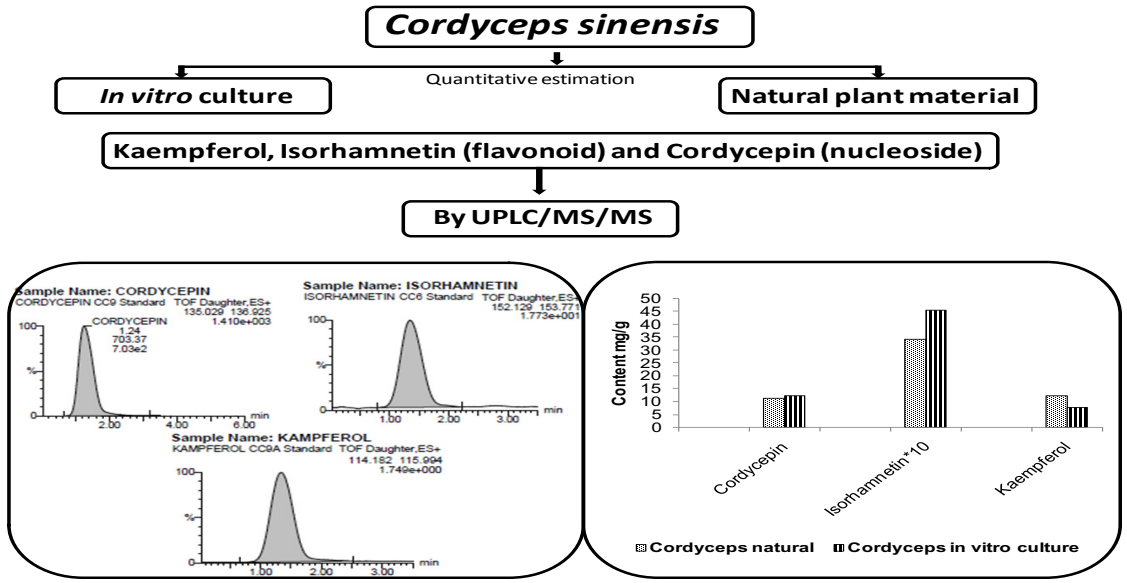

Graphical Abstract

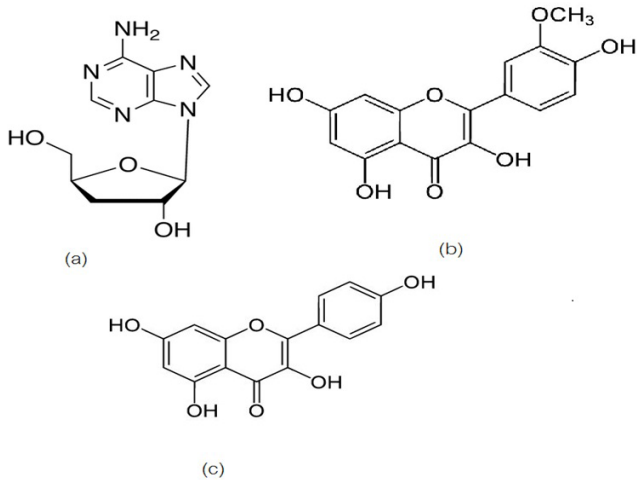

Figure 1: Chemical structures of (a) Cordycepin (b) Isorhamnetin (c) Kaempferol

1a) one of the major nucleoside and important active component of *C. sinensis* considered as a known marker to evaluate its quality.[17] Few analytical methods have been reported on *C. sinensis* for cordycepin, isorhamnetin and kaempferol using HPLC, LCMS etc. However, no one has reported till date its production using *in vitro* condition followed by quantification of its metabolites to check its completeness in having all the enzyme and co-enzyme system. This can be used as an alternative source of medicine, since the drug is only growing at Himalayan region.

UPLC uses small columns packed with 2 μm particles and has emerged as a powerful method in recent years. The UPLC separation is 5–10 times faster and has much better resolution than the normal analytical HPLC.[18] Combining UPLC with Q TOF-MS detector fulfills key requirements in terms of rapidity, sensitivity, selectivity, peak-assignment and quantification of the analytes at low concentrations in complex matrices.[19] Therefore, the present study was carried out to compare the potential *in vitro* produced culture of *C. sinensis* of Indian variety with natural drug in terms of the concentrations of kaempferol, isorhamnetin and cordycepin as determined by UPLC-QTOF-MS.

MATERIALS AND METHODS

Chemicals and Reagents

Cordycepin ($C_{10}H_{13}N_5O_3$; assigned purity> 99.5%), Isorhamnetin ($C_{16}H_{12}O_7$; assigned purity> 99.5%), Kaempferol ($C_{15}H_{10}O_6$; assigned purity> 99.5%), was purchased from Sigma-Aldrich, Germany. LC-MS grade acetonitrile (Assigned purity 99.9%; Lot No: 90525) was purchased from Sigma-Aldrich, Germany. MS grade ammonium acetate and ammonium formate were obtained from Fluka analytical, Sigma- Aldrich, Germany. Formic acid (assigned purity> 98%; Lot No: 1439632) was commercially obtained from Fluka analytical, Sigma-Aldrich, Germany. Water used in the entire analysis was of LC-MS grade. Other chemicals used were of analytical grade from commercial sources. The solvent for sample preparations were of analytical grade obtained from Merck (India). The *C. sinensis* (Natural) and *C. sinensis* (Cultured) were obtained from Defence Institute of Bio-Energy Research, Haldwani.

Plant Material

Cordyceps sinensis (natural) was collected during the months of May–April 2011, from hilly regions of Himalayas, India, where the plant grows widely under natural conditions. It was characterized by the Ethno-Botany Division of DIBER, Haldwani, where the voucher specimen of the plant is preserved in the herbarium. *C. sinensis* (cultured) was also obtained from of DIBER, Haldwani where it was tissue cultured in the laboratory and both the varieties were processed for the preparation of extracts.

The live specimens of *C. sinensis* were collected carefully from their natural habitat in the high altitude region (above 12000 fts) of Central Himalayan hills. The specimens were washed with the tap water to remove the adhering dust particles on it. Further, it was washed cleanly thrice with double distilled water followed by sterilization using mercuric chloride or sodium hypochlorite or propanol or ethanol. Finally the sterilized specimens were washed several times with sterile distilled water. In order to propagate the mycelium *in vitro*, tissues were excised from the different parts of the fungus body like spores, stalk tissue and tissue from stroma region. These tissues were excised from the fungus body with the help of a sterilized blade inside a laminar flow and cultured on to the various culture media. Eight different types of the culture media were used to grow the pure culture of the fungus.

The culture media utilized for the pure mycelium culture are potato dextrose agar (PDA), casein hydrolysate dextrose agar (CHDA), beef extract dextrose agar (BEDA), soybean seed extract dextrose agar (SEDA), rice extract dextrose agar (REDA, mushroom extract dextrose agar (MEDA), soyabari extract dextrose agar (SBEDA) and black soya seed extract dextrose agar (BSEDA). pH of these media varied from 5.5 to 6.5. The cultures were incubated at the various range of temperature (5 to 15) inside an incubator. Maximum growth of mycelium was observed on potato dextrose agar (PDA) which was used for growth of *In vitro* cultures. Twelve months old culture was used for analysis.

Preparation of Extracts

Both the mycelium were cleaned and washed thoroughly with water and dried under shade, in a clean dust free environment and crushed using a laboratory blender. Aqueous extracts were prepared using Accelerated Solvent Extraction system (ASE) equipped with a solvent controller unit (ASE 350, Dionex Corporation, Sunnyvale, CA, USA). Extractions were carried out using distilled water at 25°C for 15 min. in 33 mL stainless steel extraction cells containing 2 g of sample and equal volume of diatomaceous earth in three cycles. The extraction procedure followed was as per the method described previously by Misra *et al.*[16] The crude yield of the lyophilized extract was determined gravimetrically by weighing the dried residues obtained from the known volumes of extract. The percent-yields of both the extracts (*C. sinensis* natural and cultured) ranged between 25-30 % w/w.

Preparation of Sample Solutions

Weighed, 50 mg of the sample extract was dissolved in 5 mL of water in a volumetric flask, sonicated for 30 minutes. Filtered the mixture using 0.2 µm syringe filter and collected the filtrate sample, which was used for UPLC analysis.

Preparation of calibration standards and quality control (QC) samples

The standard stock solution containing 1000 µg mL^{-1} of cordycepin, isorhamnetin and kaempferol were prepared by dissolving requisite amount in LC-MS grade Methanol:Water 50:50 v/v (Sonicated: 44 kHz, 250 W at 25ºC for 20 min). The stock solutions were appropriately diluted to prepare a series of standard working solutions and then stored at 4ºC. The solutions were brought to room temperature and filtered through 0.22 µm membrane filter before UPLC/Q-TOF-MS analysis. Calibration curve of standards consisting of a set of ten non-zero concentrations (A-J) were prepared by making different dilutions of above stock solution for cordycepin, isorhamnetin and kaempferol in methanol. However, QC samples were prepared at three levels; 800 ng mL^{-1} (HQC, high quality control), 400 ng mL^{-1} (MQC, middle quality control) and 10 ng mL^{-1} (LQC, low quality control). All the solutions were stored at 2 - 8ºC until use.

Ultra Performance Liquid Chromatography Conditions

UPLC was performed on a Water's ACQUITY UPLC™ system (Serial No# F09 UPB 920M; Model Code# UPB; Waters Corp., MA, USA) equipped with a binary solvent delivery system, an auto-sampler, column manager and a tunable MS detector (Serial No# JAA 272; Synapt; Waters, Manchester, UK). Chromatographic separation was performed on a Water's ACQUITY UPLC™ BEH C18 (100.0 mm x 2.1 mm; 1.7 µm) column at 40 ± 5ºC. The mobile phase used for UPLC analysis consisted of acetonitrile–20 mM ammonium acetate (90:10; v/v), which was degassed before analysis. For isocratic elution, the flow rate of the mobile phase was kept at 0.20 mL min^{-1} and 10 µL of sample solution was injected in each run. The total chromatographic run time was 3.50 min. The column and auto-sampler were maintained at 40 ± 5 and 25 ± 5ºC, respectively and the pressure of the system was set to 15000 psi.

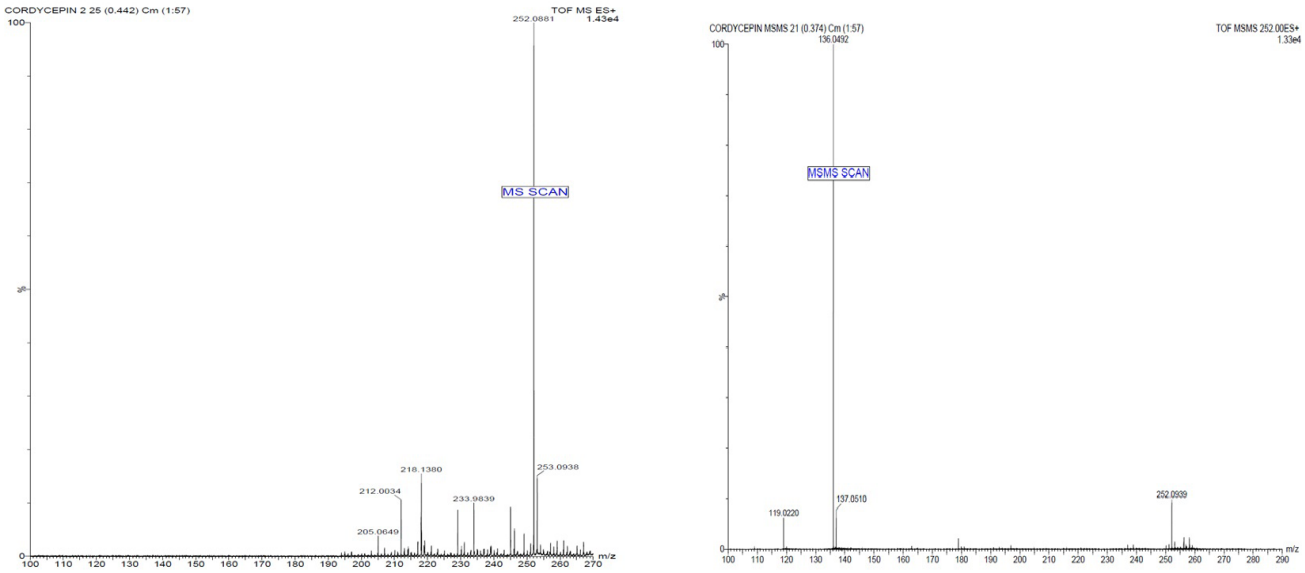

Figure 2: Mass spectra (m/z 252.08) and MS/MS product ion spectra (m/z 252.08 →136.04) of Cordycepin

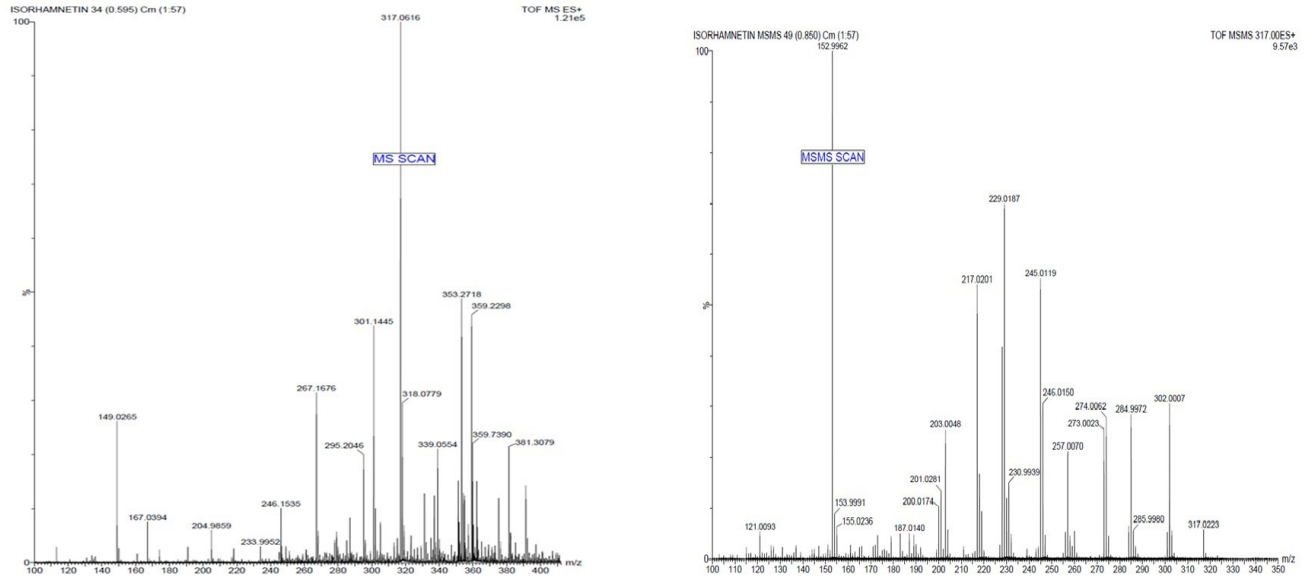

Figure 3: Mass spectra (m/z 317.06) and MS/MS product ion spectra (m/z 317.06→152.99) of Isorhamnetin

RESULTS AND DISCUSSION

Q-TOF-MS conditions for the calibration of cordycepin, isorhamnetin and kaempferol

The mass spectrometry was performed on a quadruple orthogonal acceleration time of flight tandem mass spectrometer (Waters Q-TOF PremierTM). The nebulizer gas was set to 500 L h^{-1}, the cone gas was set to 50 L h-1 and the source temperature was set to 100ºC. The capillary voltages were set to 3.0 KV and sample cone voltages were set to 40 V, respectively. Argon was employed as the collision gas at a pressure of 5.3 x 10-5 Torr. The optimum values for compound-dependent parameters like trap collision energy (Trap CE). The Q-TOF Premier TM was operated in V mode with resolution over 8500 mass with 1.0 min scan time and 0.02 s inter-scan delay. The accurate mass and composition for the precursor ions and for the fragment ions were calculated using the Mass Lynx V 4.1 software incorporated in the instrument.

Cordycepin quantitation was performed using selective reaction monitoring (SRM) of the transitions of m/z 252.08⟶136.04 for cordycepin with a scan time of 1.0 and 0.02 s inter scan per transition and transfer collision

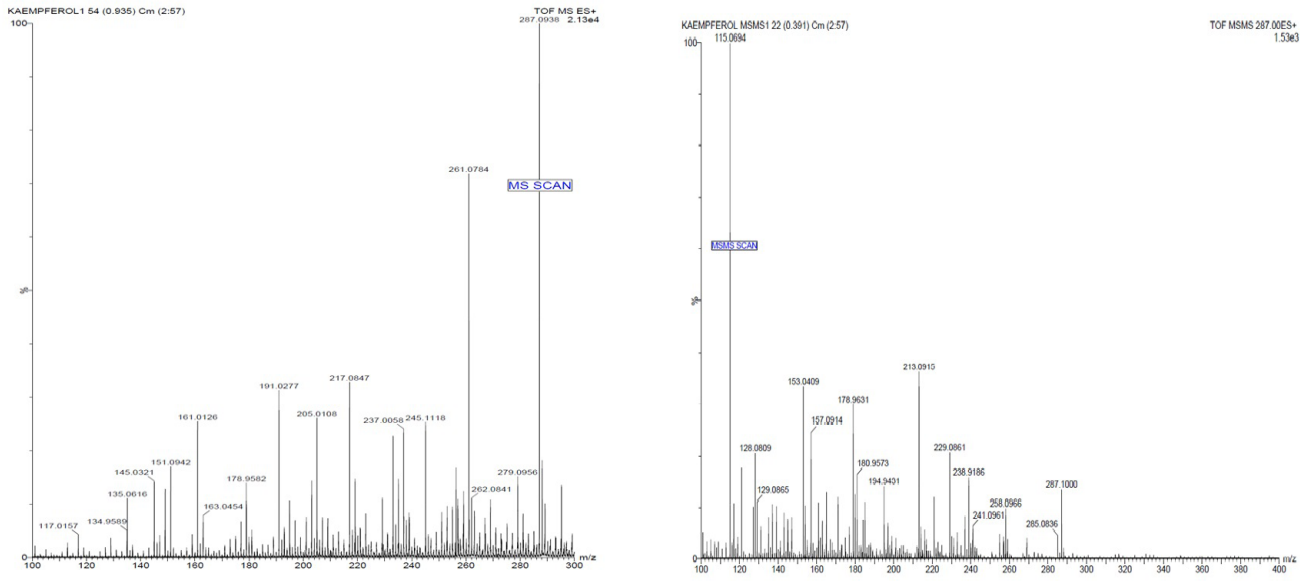

Figure 4: Mass spectra (m/z 287.09) and MS/MS product ion spectra (m/z 287.09 → 115.05) of Kaempferol

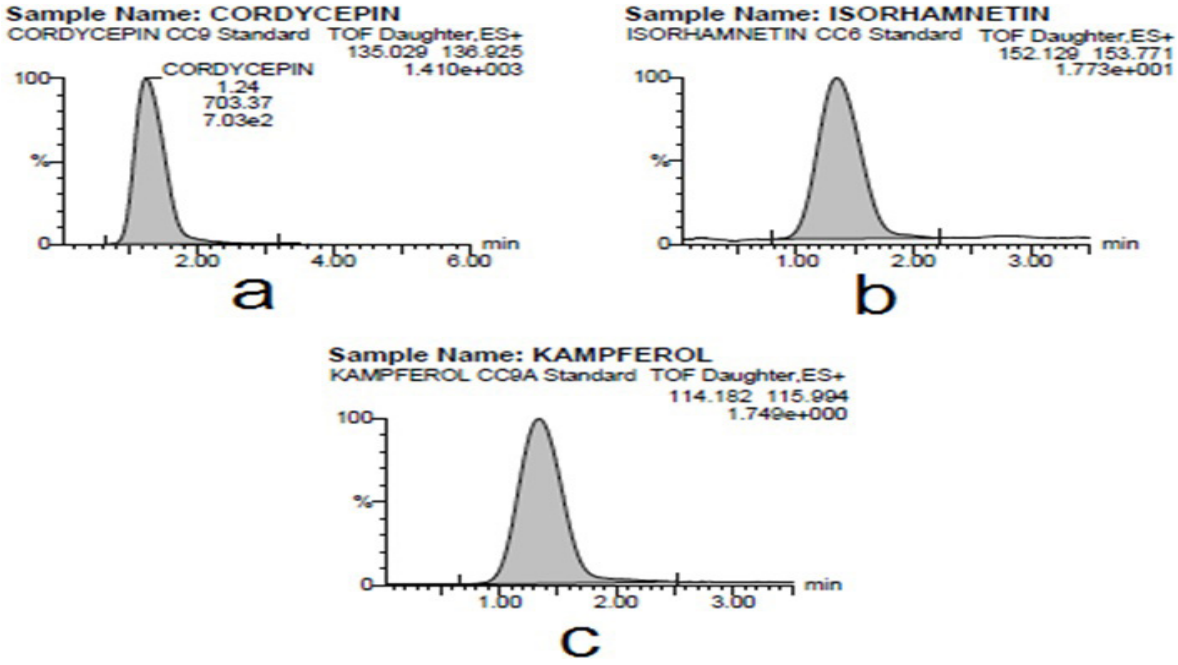

Figure 5: SRM Chromatograms of standard (a) Cordycepin, (b) Isorhamnetin and (c) Kaempferol

energy (Tran CE) were set to 16.0 and 1.0 V, respectively for fragmentation information.

Isorhamnetin quantitation was performed using selective reaction monitoring (SRM) of the transitions of m/z 317.06→152.99 for isorhamnetin with a scan time of 1.0 and 0.02 s inter-scan per transition. The optimum values for compound-dependent parameters like trap collision energy (Trap CE) and transfer collision energy (Tran CE) were set to 34.0 and 2.0 V, respectively for fragmentation information.

Kaempferol quantitation was performed using selective reaction monitoring (SRM) of the transitions of m/z 285.24→115.05 for kaempferol with a scan time of 1.0 and 0.02 s inter-scan per transition. The optimum values for compound-dependent parameters like trap collision energy (Trap CE) and transfer collision energy (Tran CE) were set to 37.0 and 4.0 V, respectively for fragmentation information. The MS scan and MS-MS spectra of cordycepin, isorhamentin and kaempferol are presented in the (Figure 2–6).

Calibration and Linearity

Calibration standards of nine concentrations of Cordycepin, Isorhamnetin and Kaempferol ranged 1–1000 ng mL^{-1}, were analyzed. The calibration graphs were plotted

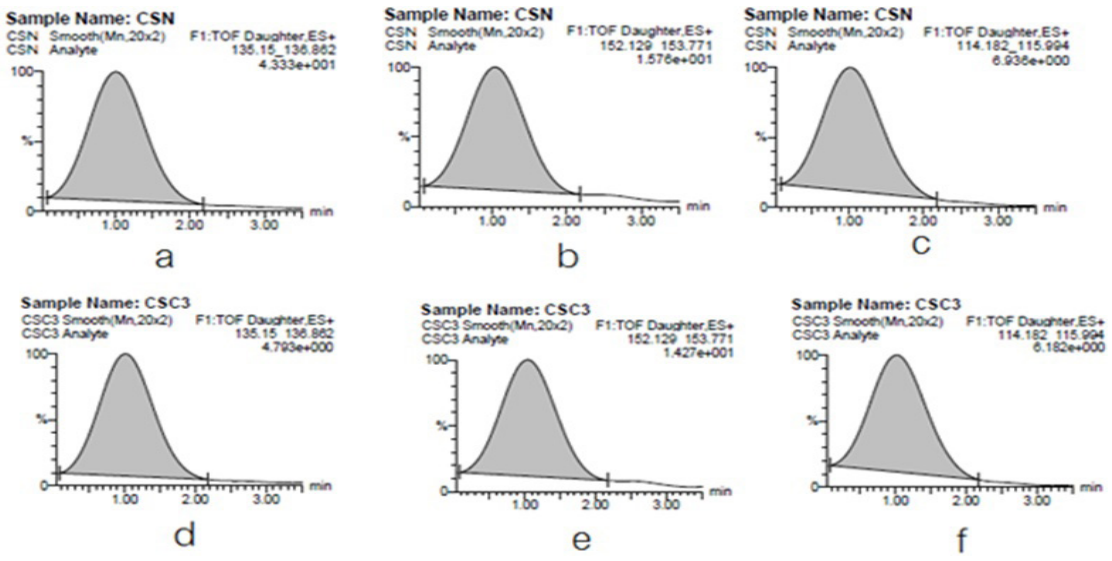

Figure 6: SRM Chromatograms of of Cordycepin, Isorhamnetin and Kaempferol in *Cordyceps sinensis* (Natural) and (Cultured) samples

Table 1: Linearity parameters for quantification of cordycepin, isorhamnetin and kaempferol by UPLC MSMS

Parameters	Range		
	Cordycepin	Isorhamnetin	Kaempferol
Linearity	1-1000ng mL^{-1}	1-1000ng mL^{-1}	1-1000ng mL^{-1}
Intercept	1.529	0.023	2.319
Slope	0.702	0.007	0.107
Regression equation	Y= 0.702x + 1.529	Y=0.007x+ 0.023	Y=0.107x+ 2.319
Correlation Coefficient (rr)	0.998	0.997	0.996
LOD	310 pg	305 pg	317 pg
LOQ	929 pg	913 pg	950 pg

*pg = pico gram

using peak areas versus drug concentrations. For assessing the linearity, the least square regression equation and correlation of coefficient were determined. The proposed method was validated as per the ICH guidelines for linearity, accuracy, precision, LOD & LOQ similar to other method reported by laboratory for salannin, [20] silybin.[21] glabridin[22] gallic, ellagic and ascorbic acid.[23]

Linearity

Linearity was assessed with the aid of serially diluted calibration solutions as mentioned above. Calibration graphs were plotted on the basis of triplicate analysis of each calibration solutions by using peak area against concentration. The proposed method was found to be linear over a wide range of concentration 1-1000 ng mL^{-1} for cordycepin, isorhamnetin and kaempferol with good regression coefficient of 0.998, 0.997 and 0.996 respectively. The slope and intercept was found 0.702 ± 0.001, 0.007 ± 0.0003, 0.107 ± 0.005 and 1.529 ± 0.04, 0.023 ± 0.0005, 2.319 ± 0.02 respectively, for cordycepin, isorhamnetin and kaempferol. The results of the experiment are incorporated in (Table 1).

Accuracy

The accuracy of the method was evaluated as recovery by standard addition method. The pre analyzed samples were spiked with standard at three different concentration levels i.e. 50,

100 and 150% and the mixtures were re-analyzed by the proposed method. The results of the experiment are incorporated in (Table 2,3).

Precision

The precision of the methods were carried out by doing intermediate precision. In intermediate precision, intra-day and inter-day precisions were carried out.

Intra-day and inter-day precisions were done by preparing and applying three different concentrations of

Table 2: Accuracy of the method (n=3) natural sample				
% of standard spiked to the sample	**Theoretical content (ng mL^{-1})**	**Amount of drug recovered (ng mL-1±SD[a])**	**% of drug recovered**	**% RSD[b])**
Cordycepin				
0	114	110.8 ± 2.14	97.22	1.92
50	171	178.2 ± 2.39	104.23	1.33
100	228	229.06 ± 0.67	100.46	0.29
150	285	286.26 ± 1.74	100.44	0.60
Isorhamnetin				
0	343.3	350 ± 2.57	101.95	0.73
50	514.9	517.73 ± 5.25	100.54	1.01
100	686.6	690.66 ± 1.15	100.59	0.16
150	858.2	863.16 ± 2.13	100.57	2.13
Kaempferol				
0	124	105.1 ± 1.3	102.03	1.23
50	186	155.16 ± 1.47	100.43	0.95
100	248	206.33 ± 2.95	100.16	1.42
150	310	260.66 ± 1.15	101.22	0.44

a) Standard deviation; b) Relative standard deviation.

Table 3: Accuracy of the method (cultured samples)				
% of standard spiked to the sample	**Theoretical content (ng mL^{-1})**	**Amount of drug recovered (ng mL-1 ± SD[a])**	**% of drug recovered**	**% RSD[b])**
Cordycepin				
0	124	125.56 ± 2.21	101.26	1.76
50	186	187.5 ± 2.23	100.80	1.19
100	248	246.4 ± 2.60	99.35	1.05
150	310	309.2 ± 6.16	99.74	99.74
Isorhamnetin				
0	455.5	454.5 ± 7.60	99.78	1.67
50	683.25	671.1 ± 14.30	98.22	2.13
100	911	916.2 ± 10.96	100.57	1.19
150	1138.75	1133.36 ± 2.23	99.52	0.19
Kaempferol				
0	79	76.36 ± 1.15	96.66	1.50
50	118.5	116.03 ± 1.95	97.91	1.68
100	158	153.76 ± 3.52	97.32	2.29
150	197.5	196.3 ± 3.25	99.39	1.65

a) Standard deviation; b) Relative standard deviation.

standard in triplicate six times a day and similarly on six different days, respectively Assay for each analysis was calculated and % RSD was determined (Table 4).

Lower Limit of Detection (LLD) and Quantification (LLQ)

The lower limit of detection for each compound was stated to be the smallest concentration detectable by mass spectrometer with signal-to-noise ratio of 5:1. The limit of quantification was defined as the lowest concentration on the calibration curve which could be measured with an intra-assay precision and accuracy< 20%.[18]

Sample analysis

The analysis of aquesous extract of natural and cultured varieties of C. *sinensis* using proposed UPLC/MSMS method showed presence of cordycepin, isorhamnetin and kaempferol0 (Table 5). The content of these metabolites in *in vitro* cultures were showing no signifi-

Table 4: Precision of the method (n=6)				
Conc (ngmL^{-1})	Intra-day precision		Inter-day precision	
	Mean peak area ± S.D[a])	%RSD[b])	Mean peak area ± S.D	%RSD
Cordycepin				
100	74.58±1.34	1.8	79.80 ± 1.64	2.0
400	272.81±7.66	2.8	282.96 ± 3.77	1.3
800	577.67±8.56	1.4	584.52 ± 3.87	0.66
Isorhamnetin				
100	0.70 ± 0.3	1.6	0.74 ± 0.01	1.62
400	2.94 ± 0,01	0.39	2.91 ± 0.06	2.24
800	5.34 ± 0.01	0.56	5.48 ± 0.09	1.65
Kaempferol				
100	15.86 ± 0.26	1.68	15.36 ± 0.35	2.28
400	48.76 ± 1.0	2.05	48.68 ± 1.15	2.36
800	88.16 ± 1.7	1.94	88.60 ± 0.95	1.07

a) Standard deviation; b) Relative standard deviation.

Table 5: Results of analysis			
Sample	Cordycepin % ww^{-1}	Isorhamnetin % ww^{-1}	Kaempferol % ww^{-1}
***Cordyceps sinensis* (Natural)**	0.000114	0.003433	0.000124
***Cordyceps sinensis* (Cultured)**	0.000124	0.004556	0.000079

cant difference in total including all three metabolites as found in case of natural samples. Hence, it can be stated that all the enzyme and co-enzyme systems of natural C. *sinensis* are present in *in vitro* cultures, which may be considered as chemically equivalent with the natural drug. Thus produced biomass can be used as alternative source of medicine in place of *C. sinensis*, which is very rare and present only on Himalayan region.

In addition, the very sensitive, accurate and precise UPLC MS method developed and validated for nucleoside (cordycepin) and flavonoids (isorhamnetin and kaempferol) can used for routine analysis of these markers in any plant/formulation containing them in very minute quantity (even in pico gram level).

CONCLUSION

Based on the results of the study, it can be concluded that the tissue culture variety of Indian *Cordyceps sinensis* is at par with the natural variety in terms of the quantity of active principles as determined by UPLC-MS. The highly sensitive newly developed and validated UPLC MS method can be used for quality control of *C. sinensis*, its formulations available in market as well as other plant materials/formulations containing them as an ingredient.

ACKNOWLEDGMENT

Authors are thankful to Director, DIPAS, Delhi, India, for constant support.

Highlights of Paper

- *Cordyceps sinensis* is an edible parasitic mushroom.
- It is an important source of bioactive flavonoids and nucleosides.
- Kaempferol,isorhamnetin and cordycepin were quantified using UPLC/ MS method.
- In vitro cultures of *Cordyceps* were produced as an alternative source of medicine.
- Validated analytical method can be used for quality control.

Author Profile

- **Dr Ahmadhas** been associated inteaching and research since 2005 after completion of his Doctorate in Pharmacognosy and Phytochemistry. He has been honoured with the University Gold Medal, DST fast track young scientist award (2007), CST-UP young scientist award (2008-09), DST BOYSCAST, AICTE Career award (2009-10), PD Sethi award 2013 and Al Ameen College of Pharmacy award (2014). He did his postdoc from Albert Einstien College of Medicine New York (2011) and has to his credit >150 international publicationsandauthor of text book "Introduction to Pharmacognosy". He has been associated with more than sixteen Govt. of India sponsored research projects and supervised 20 PhDs till date.

REFERENCES

1. Kuo YC, Tsai WJ, Shiao MS, Chen CF, Lin CY. *Cordyceps sinensis* as an immunomodulatory agent. Am J Chin Med. 1996; 24(02): 111-25.
2. Kuo YC, Lin CY, Tsai WJ, Wu CL, Chen CF, Shiao MS. Growth inhibitors against tumor cells in *Cordyceps sinensis* other than cordycepin and polysaccharides. Cancer Invest 1994; 12(6): 611-5.
3. Manabe N, Azuma Y, Sugimoto M, Uchio K, Miyamoto M, Taketomo N, *et al*. Effects of the mycelial extract of cultured *Cordyceps sinensis* on *in vivo* hepatic energy metabolism and blood flow in dietary hypoferric anaemic mice. Br J Nutr. 2000; 83(02): 197-204.
4. Balon TW, Jasman AP, Zhu JS. A fermentation product of *Cordyceps sinensis* increases whole-body insulin sensitivity in rats. J Altern Complement Med. 2002; 8(3): 315-23.
5. Koh JH, Kim JM, Chang UJ, Suh HJ. Hypocholesterolemic effect of hot water extract from mycelia of *Cordyceps sinensis*. Biol Pharm Bull. 2003; 26(1): 84-7.
6. Chiou WF, Chang PC, Chou CJ, Chen CF. Protein constituent contributes to the hypotensive and vasorelaxant activities of *Cordyceps sinensis*. Life Sci. 2000; 66(14): 1369-76.
7. Steve C, Zhaoping Li, Robert K, Marlon A, Woosong K, Christopher BC. Effect of *Cordyceps sinensis* on Exercise Performance in Healthy Older Subjects: A Double-Blind, Placebo-Controlled Trial. J Altern Complement Med. 2010; 16(5): 585–90.
8. Zhenquan L, Pengtao Li, Dan Z, Huiling T, Jianyou G. Protective effect of extract of *Cordyceps sinensis* in middle cerebral artery occlusion-induced focal cerebral ischemia in rats. Behav Brain Funct. 2010; 6(6): 61.
9. Liu WC, Wang SC, Tsai ML, Chen MC, Wang YC, Hong JH, *et al*. Protection against radiation-induced bone marrow and intestinal injuries by *Cordyceps sinensis*, a Chinese herbal medicine. Radiat Res. 2006; 166(6): 900-7.
10. Zhu JS, Halpern GM, Jones K. The scientific rediscovery of a precious ancient Chinese herbal regimen: *Cordyceps sinensis*: part II. J Altern Complement Med. 1998; 4(4): 429–57.
11. Butler MS. The role of natural product chemistry in drug discovery. J Nat Prod. 2004; 67(12): 2141-53.
12. Terao J, Kawai Y, Murota K. Vegetable flavonoids and cardiovascular disease. Asia Pac J Clin Nutr. 2008; 17(Suppl 1): 291-3.
13. Mandel S, Weinreb O, Amit T, Youdim MB. Cell signaling pathways in the neuroprotective actions of the green tea polyphenol(-)-epigallocatechin-3-gallate: implications for neurodegenerative diseases. J Neurochem. 2004; 88(6): 1555-69.
14. Mari H, Riina N, Pia V, Marina H, Moilanen E. Anti-Inflammatory Effects of Flavonoids: Genistein, Kaempferol, Quercetin and Daidzein Inhibit STAT-1 and NF-B Activations, Whereas Flavone, Isorhamnetin, Naringenin, and Pelargonidin Inhibit only NF-B Activation along with Their Inhibitory Effect on iNOS Expression and NO Production in Activated Macrophages. Mediators Inflamm. 2007; 45: 673.
15. Zhu JS, Halpern GM, Jones K. The scientific rediscovery of an ancient Chinese herbal medicine: *Cordyceps sinensis*: part I. J Altern. Complement. Med. 1998; 4(3): 289–303.
16. Misra K, Rajkumar T, Radhey S, Kumar DM, Gertrud M. Hyphenated High-Perfromance Thin-Layer Chromatography for profiling of some Indian natural efficiency enhancers. J of Liq Chrom & Rel Tech. 2012; 35(10): 1364-87.
17. Zheng HZ, Dong ZH, She J. Modern study of traditional Chinese medicine. Volume Two, Beijing: Xueyuan Press; 1997.
18. Dan M, Su M, Gao X, Zhao T, Zhao A, Xie G, *et al*. Metabolite profiling of Panax notoginseng using UPLC-ESI-MS. Phytochemistry 2008; 69(11): 2237-44.
19. Qi LW, Wang CZ, Yuan CS. Isolation and analysis of ginseng: advances and challenges. Nat Prod Rep. 2011; 28(3): 467-95.
20. Athar MT, Musthaba SM, Baboota S, Ahmad S. Validated UPLC/QTOF-MS method for quantitative determination of salannin in wistar rat plasma: *In vitro* assay, *ex vivo* stability effects and pharmacokinetic. Adv Sci Lett. 2012; 16(1): 173-8.
21. Parveen R, Batoota S, Ahmad S, Ali J, Ahuja A. Stability-indicating HPTLC method for quantitative estimation of silybin in bulk drug and pharmaceutical dosage form. Biomed Chrom. 2010; 24(6): 639-47.
22. Kamal YT, Singh M, Tamboli ET, Parveen R, Zaidi SMA, Ahmad S. Rapid RP-HPLC Method for the Determination of Glabridin in Crude Drug and Polyherbal Formulations. J Chromatogr Sci. 2012; 50(1): 779-84.
23. Singh M, Kamal YT, Parveen R, Ahmad S. Determination of Gallic acid, Ellagic acid and Ascorbic acid in *Emblica officinalis* Linn. and in Unani poly herbal formulations by validated HPLC method. J Liq Chromatogr Rel Tech. 2012; 35(17): 2493-502.