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Research Article

PHYTOCHEMICAL AND PROTEOMIC ANALYSIS OF A HIGH ALTITUDE MEDICINAL MUSHROOM *CORDYCEPS SINENSIS*

Rakhee[1], Niroj Kumar Sethy[1], Vijay Kumar Singh[1], Shruti Sharma[3], Raj Kishore Sharma[2], Renu Deswal[3], Kalpana Bhargava[1] and Kshipra Misra[1]*

[1]*Defence Institute of Physiology and Allied Sciences, Lucknow Road, Timarpur, Delhi 110054, India*
[2]*Department of Chemistry, University of Delhi, Delhi 110007, India*
[3]*Department of Botany, Plant Molecular Physiology and Biochemistry Laboratory, University of Delhi, Delhi 110007, India*

Abstract: *Cordyceps sinensis* (*C. sinensis*) is well established as a traditional Chinese medicine (TCM) that has been valued as a health food for centuries. It is an entomopathogenic fungus in Ascomycetes that naturally occurs at high altitude in Himalayan region and has received considerable attention due to the abundance of various biologically active compounds. Despite having reported health benefits and economic importance, qualitative phytochemical analysis, proximate composition and proteome study of Indian isolates of *C. sinensis* grown at high altitude remains untapped. In the present study, qualitative phytochemical analysis was carried on powdered whole body of *C. sinensis* (CS_{Wb}) and its aqueous extract (CS_{Aq}) prepared by accelerated solvent extraction technique which indicated the presence of several bioactive constituents such as alkaloids, amino acids and proteins, carbohydrates, flavonoids and phenols, gums, mucilages and saponins. We evaluated chemical composition of the Indian Himalayan medicinal mushroom *C. sinensis* in terms of its carbohydrate (55.68%) content, crude fiber (6.40%), fat (1.80%), moisture (7.18%), protein (21.46%) and total ash (7.48%). Furthermore, soluble protein identification of both CS_{Wb} and CS_{Aq} by SDS-PAGE followed by MALDI-TOF-TOF analysis revealed the presence of various types of most abundant proteins such as P-type II A ATPase, TE1b [Blumeriagraminis f. sp. hordei], Chitin synthase Chs [Penicilliummarneffei ATCC 18224], Serine/threonine-protein kinase CLA4, DEHA2C06820p [Debaryomyceshansenii CBS767], YALI0E29887p [Yarrowialipolytica] etc. In conclusion, the present study provides a comprehensive qualitative phytochemical analysis, proximate composition and proteome study on Indian isolate of *C. sinensis* which could endorse its use as a functional food.

Keywords: *Cordyceps sinensis*; phytochemical analysis; proximate composition; proteome study.

Note: Coloured Figures available on Journal Website in "Archives" Section

Introduction

C. sinensis popularly known as "Yartagunbu" or "Dong Chong Xia Cao" (winter worm summer grass) is a high value medicinal mushroom, naturally distributed in China, India, Nepal and Bhutan (Holliday and Cleaver, 2008). *C. sinensis* is a parasitic fungus found at altitude of more than 3,200 meters. It has a characteristic life cycle on the larva of a moth; belongs to *Clavipitaceae* family and the genus *Ascomycetes* (Jang *et al.*, 2015). The wild fungus along with the cultivated varieties as well as cultured mycelia, fruiting body and extracts reportedly possesses diverse medicinal properties (Valverde *et al.*, 2015). Owing to these properties, it has been employed to treat various rehabilitation disorders such as arrhythmias, asthenia after severe illness, bronchitis, cancer, hyperglycaemia, hyperlipidaemia, hyposexuality, liver disease, lungs disorders, night sweating, renal dysfunction and renal failure etc. (Donohue, 1996; Han, 1995; Manfreda *et al.*, 1989; Qiuo and Ma, 1993; Tuli *et*

Corresponding Author: **Kshipra Misra**
E-mail: kmisra99@yahoo.com

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al., 2014; Zheng and Deng, 1995; Zhou *et al.*, 2009; Zhu and Rippe, 2001). Considering, its wide medicinal properties, the fungus is regarded as "soft gold" in China and is extremely high in price; approximately USD $20,000 to 40,000 per kg (Jang *et al.*, 2015; Lo *et al.*, 2013).

The Indian variety of *C. sinensis* is found at high altitude between 12,000 and 16,000 ft at Himalayan plateau of Kumaon and Garhwal hills, Arunachal Pradesh, Uttarakhand and Sikkim (Negi, 2007; Panda and Swain, 2011; Winkler, 2009). In India, it is known by the name of *Ghas Ka Kira* or *Kira Jhar* or *Kira ghas* or *Yartsa Gumba* in hilly regions of Darma and Johaar Valleys in Pithoragarh (Negi, 2007). The fungus contains several idiosyncratic secondary metabolites such as cordycepic acid, cordycepin, ergosterol, fatty acids, nucleobases, polyphenols, polysaccharides, steroids, terpenes and vitamins (Arora *et al.*, 2008; Mamta *et al.*, 2015; Singh *et al.*, 2014). The Indian isolates of *C. sinensis* possess medicinal properties like anti-bacterial activity, anti-oxidant activities, anti-stress effect, anti-inflammatory, anti-microbial, muscular endurance, immunomodulating effects, enhancement of neuromuscular activity, endurance enhancing activity (Kumar *et al.*, 2011; Mamta *et al.*, 2015; Meena *et al.*, 2013; Negi *et al.*, 2006; Rathor *et al.*, 2014; Singh *et al.*, 2014) and tolerance activity to hypoxia at high altitude (Singh *et al.*, 2013). As mentioned by Thakur *et al.* (2011), the qualitative and quantitative fingerprinting of Indian isolates of *C. sinensis* has not been reported so far although several studies have been reported on its pharmacological activities (Thakur *et al.*, 2011).

In the current study, we carried out a comprehensive qualitative phytochemical and proximate analysis along with SDS-PAGE followed by MALDI-TOF-TOF of the Indian isolate of *C. sinensis.* As, proteomic one dimensional approach (1-DGE) combined with mass spectrometry (MS) has been widely used for fungal protein identification, it was employed for identifying soluble proteins, isolated through accelerated solvent extraction technique (Yin *et al.*, 2012). Our results catalogued the hydrophilic proteins present in *C. sinensis* which may be beneficial for its future medicinal purposes.

Materials and methods

Mushroom material

C. sinensis used in this study was the same as used in earlier studies (Pal *et al.*, 2015). The material was collected from Pithoragarh at the steeped region of western Himalayas at an altitude above 3,200 meters. The ethno-botanical identification of species was done, ICS-I gene sequence was established and deposited in NCBI (National centre for Biotechnology Information) vide accession No. JF705859 by Defence Institute of Bio Energy Research (DIBER), Pithoragarh. The voucher specimen (DIP-CS/2011) is preserved at DIBER. *C. sinensis* was ground into fine powder using grinder and stored in an air tight polythene bag at room temperature until required for use.

Preparation of extract

C. sinensis aqueous extract was prepared in triplicate manner using Accelerated Solvent Extraction system (ASE 350) equipped with a solvent controller unit from Dionex Corporation (Sunnyvale, CA, USA) at room temperature. The extracted aqueous sample was lyophilized in Lyophilizer (Allied frost FD-5, India). The lyophilized extract was stored at 4^0C until use.

Phytochemical analysis

Three independent both the samples (CS_{Wb} and CS_{Aq}) were analyzed for phytochemicals in qualitative manner. The analysis included detection of alkaloids (Mayer's test), carbohydrates (Molish's test, Fehling's test and Benedict's test), saponins (foam test), proteins and amino acids (ninhydrin test) and phenolic compounds and flavonoids (lead acetate test) (Khandelwal, 2009; Raaman, 2006; Shah *et al.*, 2014).

Proximate analysis

Proximate analysis as a quantitative evaluation of phyto-constituents of the dried powder of *C. sinensis* was carried out using methods described by Raghuramulu *et al.*, 2003. The sample was analyzed for proximate parameters such as carbohydrates, crude fiber, crude protein, fat moisture contents and total ash using standard

procedures in triplicate . The moisture content was obtained by drying the samples in a halogen moisture analyzer (Wensar HMB100) at 100^{0}C until circulation was accomplished; ash, from burn up residue obtained at 550^{0}C after 8 h by using muffle furnace (Metrex); crude protein by the Kjeldahl method (kes-125/kelvac/distyl-em/kel freez) with a conversion factor of 4.38 (Hsu *et al.*, 2002) and soxhlet extraction method with petroleum ether was used for the gravimetric determination of fat content (Sanmee *et al.*, 2003). The total carbohydrate was calculated as: 100% - [%moisture+%ash+%crude protein+%fat+%fibre] (Güner *et al.*, 1998; Mattila *et al.*, 2002).

Protein extraction

Protein extraction was carried out for whole body *C. sinensis* powder as well as lyophilized powder of *C. sinensis* aqueous extract. 500mg of each CS_{Wb} and CS_{Aq} was ground thoroughly to a fine powder in liquid nitrogen in a ceramic mortar and pestle. Protein was extracted using Tris-Glycerol buffer and protein quantification was done by Bradford assay. Both samples were ground with extraction buffer (Tris-pH 7.4, 100mM + glycerol 30%) in ratio of sample and buffer 1:10 followed by centrifugation for 20minutes at 12,000 rpm and finally discarded the residue/debris with the collection of the supernatant. Protein pellets were prepared using Pro-Q method. In this method, methanol was added to 150µl supernatant of both samples, further to this sequentially added $CHCl_3$ then Milli-Q and vortexed after each step. Protein discs were formed after centrifugation at 12,000rpm for 5minutes at 4^{0}C and were washed by methanol with centrifugation at same rpm for 5minutes. After that these pellets were dried on ice and used for protein separation (Jiang *et al.*, 2015).

Protein separation by one-dimensional gel electrophoresis (1-DGE)

One-dimensional well established SDS-PAGE gel electrophoresis technique (Weber & Osborn, 1969) was applied on 15% polyacrylamide gel for the separation of proteins. Bromophenol blue was added to the sample in a ratio 1:3 and then boiled for 5 minutes at 95^{0}C prior to gel electrophoresis. 30µl of both the samples were loaded then gel was stained with Coomassie brilliant blue R-250 and the separated proteins were visualized. Prestained SDS-PAGE standards of lower molecular weight range (14-97 kDa) were used as molecular marker.

Trypsin digestion

The CS_{Wb} gel was cut into five sections, zone first (W1), zone second (W2), zone third (W3), zone fourth (W4) and zone fifth (W5) and similarly CS_{Aq} gel was cut into five sections, zone first (A1), zone second (A2), zone third (A3), zone third (A3), zone fourth (A4) and zone fifth (A5) (Figure 1) and each sectioned gel fragment was washed thrice times with Milli-Q followed by destaining with reagent 30mM Potassium ferricyanide and acetonitrile (1:1). The destained gel pieces were washed three times with Milli-Q and then mixture of Milli-Q/ACN/10mM NH_4HCO_3 (1:0.5:0.5) followed by washing in ACN. The washed gel pieces were dried using Speed Vac Concentrator (SAVANT SC250EXP). In-gel tryptic digestion was performed overnight with incubation at 37^{0}C. The supernatant was collected and washed by 50% ACN followed by reaction termination by acidification with 0.1% TFA solution(Ahmad *et al.*, 2013) The micro centrifuge tubes were sonicated, dried over speed-vac and supernatants were collected for MALDI-TOF analysis.

Mass spectrometry

MALDI-TOF was performed on an ABSciex 5800 TOF/TOF™ system. For peptide mass fingerprinting, tryptic digested peptides of each zone were mixed with an acidic solid matrix of á-cyano-4-hydroxy cinnamic acid (CHCA) of 10mg/mL concentration. The matrix was prepared in 70% acetonitrile (ACN) and 0.01% trifluoroacetic acid (TFA). 0.5 µL of digested protein and 0.5 µL of prepared matrix were mixed together and manually spotted onto a plate (384 opti-TOF 123mm x 81mm stainless steel, Applied Biosystem, ABSciex, USA) and further dried at ambient temperature. The peptide mass spectra were recorded in reflectron positive ion mode using the above mentioned mass spectrometer equipped with a 384-sample scout source and the ion acceleration voltage was 29,000 V after pulsed extraction. The MS and MS/MS data were

recorded automatically on the MALDI-TOF/TOF system according to peptide mass fingerprinting (PMF) spectrum with the use of three most abundant peptide signals. The monoisotopic peak list was generated in Post Processing s/w and True peptide mass list was generated without using the smoothing function by Protein pilot software version 3.2 (ABSciex). Database search with the peptide masses was performed against Global proteome server (GPS) explorer workstation installed with the MASCOT search engine (Matrix science) to search the database of the National Center for Biotechnology Information (NCBInr) with following search parameter: Mass Tolerance : ±250 ppm species, *Homo sapiens*; maximum number of missed cleavages was set to 1 for all the samples.

Results

In the present study, C. *sinensis* powder was used for extract preparation and lyophilized to obtain 33% of dried aqueous extract powder. All the further studies including qualitative, proximate and proteomics study were performed on the whole body *C. sinensis* and lyophilized aqueous extract obtained using ASE technique.

Phytochemical analysis

Phytochemical analysis was carried out as per the methods described in previous section of materials and methods which demonstrated the presence of alkaloids, carbohydrates, gums and mucilages, phenolic compounds and flavonoids, proteins and amino acids and saponins in both CS_{Wb} and CS_{Aq}. Results are presented in Table 1. However, interestingly phytosteroids could be identified in CS_{Wb} but were found absent in CS_{Aq} sample as confirmed by Liebermann-Burchard's test.

Proximate analysis

The proximate chemical composition results brought out that CS_{Wb} comprised 1.80±0.10% fat, 21.46±0.25% protein, 55.68±0.01% carbohydrate, 6.40±0.06% crude fibre, 7.18±0.03% moisture and 7.48±0.15% ash and the results are shown in Table 2.

Table 1
Phytochemical composition of both CS_{Wb} and CS_{Aq}

S.No.	*Phytochemical tests*	*Observation*	
		CS_{Wb}	CS_{Aq}
1.	**Alkaloids**		
	a. Mayer's test	**+ve**	**+ve**
2.	**Carbohydrates**		
	a. Molish's test	**+ve**	**+ve**
	b. Fehling's test	**+ve**	**+ve**
	c. Benedict's test	**+ve**	**+ve**
3.	**Saponins**		
	a. Foam test	**+ve**	**+ve**
4.	**Proteins and Amino acids**		
	a. Million's test	**-ve**	**-ve**
	b. Biuret test	**-ve**	**-ve**
	c. Ninhydrin test	**+ve**	**+ve**
5.	**Phytosteroids**		
	a. Liebermann-Burchard's test	**+ve**	**-ve**
6.	**Phenolic compounds and flavonoids**		
	a. Ferric chloride test	**-ve**	**-ve**
	b. Lead acetate test	**+ve**	**+ve**
	c. Magnesium and Hydrochloric acid reduction test.	**-ve**	**-ve**

+ve Present -ve Absent

Table 2
Chemical composition of CS_{Wb}

S.No.	Parameter	Contents percent (%)±SD
1.	Moisture	7.18±0.03
2.	Total ash	7.48±0.15
3.	Crude protein	21.46±0.25
4.	Fat	1.80±0.10
5.	Crude fiber	6.40±0.06
6.	Carbohydrate	55.68±0.01

Identification of soluble proteins

The protein profiling of both the samples was analyzed by 1D SDS-PAGE as shown in Figure 1. The characterization of protein zones by MALDI-TOF showed the presence of a number of proteins with above 40 mascot score in both samples CS_{Wb} & CS_{Aq} (Table 3). The comparative result using MALDI-TOF showed eighteen proteins in CS_{Wb} and seventeen proteins in CS_{Aq}.

In CS_{Wb}, five proteins (TE1b [*Blumeria graminis f. sp. hordei*]; TRAF-type zinc finger protein [*Arthroderma gypseum* CBS 118893]; RAD50 [*Saccharomyces pastorianus*]; DmbS [*Beauveria bassiana*]; Bromodomain containing protein [*Coccidioides posadasii* C735 delta SOWgp] were identified in W1, no protein in W2, eight protein (predicated protein [*Botryotinia fuckeliana* B05.10]; cytochrome P450 monooxygenase putative [*Penicillium marneffei* ATCC 1]; kinesin family protein [*Coccidioides posadasii str. Silveira*]; related to Progesterone 5-beta-reductase [*Sporisorium reilianum*], predicted protein [*Botryotinia fuckeliana* B05.10]; ribosomal protein 3/homing endonuclease-like fusion protein [*Leptographiumpityophilum*]; nmrA family transcriptional regulator [*Aspergillus niger* CBS 513.88]; KLTH0E11858p [*Lachancea thermotolerans*] in W3, five proteins (P-type II A ATPase [*Glomus diaphanum*]; predicted protein [*Botryotinia fuckeliana* B05.10]; predicted protein [*Laccaria bicolor* S238N-H82]; YALI0E29887p [*Yarrowia lipolytica*]; hydroxynaphthalene reductase [*Cochliobolus lunatus*] in W4 and no protein in W5.

On the other hand, in CS_{Aq} five proteins (serine/threonine-protein kinase CLA4, likely protein kinase [*Candida albicans* SC5314], CMGC/SRPK protein kinase [*Trichophyton tonsurans* CBS 112818], predicated protein [*Laccaria bicolor* S238N-H82], predicted protein [*Leptosphaeria maculans*]) were identified in A1, one protein i.e. predicted protein [*Chaetomium globosum* CBS 148.51] in A2, four proteins (methyltransferase (Ncl1) putative [*Metarhizium acridum* CQMa 102], predicted protein [*Aspergillus terreus* NIH2624], AGR078Cp [*Ashbya gossypii* ATCC 10895], expressed protein [*Schizophyllum commune* H4-8]) in A3, seven proteins (chitin synthase ChsE [*Penicillium marneffei* ATCC 18224]; predicted protein [*Paracoccidioides brasiliensis* Pb01]; solute carrier family 25 member 38 [*Ajellomyces capsulatus* G186AR]; DEHA2C06820p [*Debaryomyces hansenii* CBS767]; FAD linked oxidase, putative [*Aspergillus fumigatus* A1163]; YALI0E29887p [*Yarrowia lipolytica*]; 2OG-Fe(II) oxygenase family oxidoreductase, Putative [*Metarhizium acridum* CQMa 102]) in A4 and no protein in A5. All these proteins are having characteristic biological function(s) and have been given in Table 3 as obtained by InterPro, Uniprot KB-KW, UniprotKB-HAMAP and GO_Central on line protein and genomic data bases.

Comparative study of proteins between CS_{Wb} and CS_{Aq}

The results were also compiled in the form of Venn diagram which was drawn as per the method described elsewhere (http://bioinformatics.psb.ugent.be/webtools/Venn/) to compare the two samples with each other in terms of hydrophilic proteins. Venn diagram also depicts the presence of two common proteins namely *Laccaria bicolor* S238N-H82, YALI0E29887p [*Yarrowia lipolytica*] in both the samples. Sixteen proteins are present in CS_{Wb} and fifteen are in CS_{Aq} characteristically. The results are shown in Figure 2.

Discussion

Medicinal mushrooms have gained global scientific attention due to a wide range of pharmacological and nutraceutical properties. *C. sinensis* has been described as a fungal therapeutic biofactory due to the presence of secondary metabolites like cordycepin, cordycepic acid, cordyglucan, uridine, adenosine, guanosine, ergosterol etc. (Arora *et al.*, 2008; Arora, 2014;

Table 3
Protein identification and functional categorization in both CS_{Wb} and CS_{Aq} using MALDI-TOF/TOF

List of Proteins in CS_{Wb}

Zone	*Accession number*	*Mass (dalton)*	*Mascot score*	*Protein description*	*Peptide matched*	*Protein ID*	*References*	*MS; MSMS*	*Protein coverage %*
				Energy metabolism & cell membrane functions					
W1	gi|158534847	1.00E+05	54	TE1b [*Blumeriagraminis f. sp. hordei*]	20	ABW72065.1	InterPro	+;-	23.529
W1	gi|113913515	2.00E+05	52	RAD50 [*Saccharomyces pastorianus*]	53	ABI48901.1	InterPro	+;-	62.352
W3	gi|154314048	5742	54	predicted protein[*Botryotiniafuckeliana* B05.10]	4	XP_001556349.1	UniProtKB-HAMAP; EnsemblFungi	+;-	22.222
W3	gi|154294183	34311	40	predicted protein [*Botryotiniafuckeliana* B05.10]	8	XP_001547534.1	UniProtKB-HAMAP; EnsemblFungi	+;+	44.444
W3	gi|257097854	83282	40	ribosomal protein 3/homing endonuclease-like fusion protein [*Leptographiumpityophilum*]	12	ACV41147.1	InterPro; UniProtKB-KW	+;+	66.666
W3	gi|320040398	2.00E+05	41	kinesin family protein [*Coccidioidesposadasii str. Silveira*]	14	EFW22331.1	UniProtKB-KW; InterPro; UniRule annotation	+;+	77.777
W4	gi|89885365	42120	74	P-type II A ATPase [*Glomusdiaphanum*]	27	CAJ42038.1	InterPro	+;-	30.337
W4	gi|154316470	7781	45	predicted protein [*Botryotiniafuckeliana* B05.10]	5	XP_001557556.1	UniProtKB-HAMAP; EnsemblFungi	+;+	5.617
W4	gi|170091376	43522	44	predicted protein [*Laccariabicolor* S238N-H82]	17	XP_001876910.1	InterPro	+;+	19.101
W4	gi|50554323	27432	43	YALI0E29887p [*Yarrowialipolytica*]	18	XP_504570.1	GO_Central; UniProtKB-KW	+;+	20.224
W4	gi|23451229	29096	43	hydroxynaphthalene reductase [*Cochlioboluslunatus*]	20	AAN32707.1	InterPro	+;+	22.471
				Cell cycle control and genome maintenance					
W1	gi|303311489	89088	50	Bromodomain containing protein [*Coccidioidesposadasii* C735 delta SOWgp]	20	XP_003065756.1	InterPro	+;-	23.529
W3	gi|255715421	54858	40	KLTH0E11858p [*Lachanceathermotolerans*]	10	XP_0012553992.1	EnsemblFungi	+;+	55.555
				Miscellaneous					
W1	gi|315046904	51879	53	TRAF-type zinc finger protein [*Arthrodermagypseum* CBS 118893]	23	XP_003172827.1	InterPro	+;-	27.058
W1	gi|307340765	5.00E+05	50	DmbS [*Beauveriabassiana*]	48	ADN43685.1	InterPro; UniProtKB-KW	+;-	56.47
W3	gi|212536382	61995	41	cytochrome P450 monooxygenase,putative [*Penicilliummarneffei* ATCC 1]	8	XP_002148347.1	InterPro; UniProtKB-KW	+;+	44.444
W3	gi|323508194	43519	40	related to Progesterone 5-beta-reductase [*Sporisoriumreilianum*]	4	CBQ68065.1	InterPro	+;+	22.222
W3	gi|317036000	34110	40	nmrA family transcriptional regulator [*Aspergillusniger* CBS 513.88]	10	XP_001397387.1	Not found	+;+	55.555

List of Proteins in CS_{Aq}

S.No.	Accession number	Mass (dalton)	Mascot score	Protein description	Peptide matched	Protien ID	References	MS; MSMS	Protein coverage %
				Energy metabolism & cell membrane functions					
A1	gi|3334151	107109	58	Serine/threonine-protein kinase CLA4	36	O14427.1	UniProtKB-KW	+;-	40.9
A1	gi|68465372	107643	54	likely protein kinase[*Candida albicans* SC5314]	35	XP_723385.1	UniProtKB-KW; CGD	+;-	37.5
A1	gi|326470336	47884	53	CMGC/SRPK protein kinase [*Trichophytontonsurans* CBS 112818]	23	EGD94345.1	UniProtKB-KW	+;-	26.14
A1	gi|170091058	62663	50	predicted protein [*Laccariabicolor* S238N-H82]	23	XP_001876751.1	UniprotKB-HAMAP	+;-	26.14
A4	gi|212530178	206883	50	chitin synthase ChsE [*Penicilliummarneffei* ATCC 18224]	19	XP_002145246.1	InterPro	+;-	23.17
A4	gi|295668783	29825	47	predicted protein [*Paracoccidioidesbrasiliensis* Pb01]	6	XP_002794940.1	UniprotKB-HAMAP	+;+	7.79
A4	gi|159128538	63716	44	FAD linked oxidase, Putative [*Aspergillusfumigatus* A1163]	14	EDP53653.1	InterPro	+;+	15.55
A4	gi|50554323	27432	43	YALI0E29887p [*Yarrowialipolytica*]	14	XP_504570.1	UniprotKB-KW; GO_Central	+;+	21.87
A4	gi|322698135	37717	41	2OG-Fe(II) oxygenase family oxidoreductase, Putative [*Metarhiziumacridum*CQMa 102]	12	EFY89908.1	InterPro	+;+	15.55
				Cell cycle control and genome maintenance					
A1	gi|312214355	92552	50	predicted protein [*Leptosphaeriamaculans*]	28	CBX94347.1	InterPro	+;-	31.81
A2	gi|116194103	6615	41	predicted protein [*Chaetomiumglobosum* CBS 148.51]	2	XP_001222864.1	InterPro	+;+	50
A3	gi|322700995	93850	54	methyltransferase (Ncl1), Putative [*Metarhiziumacridum*CQMa 102]	18	EFY92747.1	InterPro	+;-	20
				Miscellaneous					
A3	gi|115390080	91171	52	predicted protein [*Aspergillusterreus* NIH2624]	29	XP_001212545.1	InterPro	+;-	32.22
A3	gi|45201173	82572	50	AGR078Cp [*Ashbyagossypii* ATCC 10895]	22	NP_986743.1	Not found	+;-	31.88
A3	gi|302673148	33697	50	expressed protein[*Schizophyllum commune* H4-8]	19	XP_003026261.1	InterPro	+;-	21.11
A4	gi|225558912	37574	45	solute carrier family 25 member 38 [*Ajellomycescapsulatus* G186AR]	15	EEH07195.1	UniprotKB-HAMAP	+;+	16.66
A4	gi|294655786	53022	45	DEHA2C06820p [*Debaryomyceshansenii* CBS767]	24	XP_457980.2	InterPro	+;+	26.66

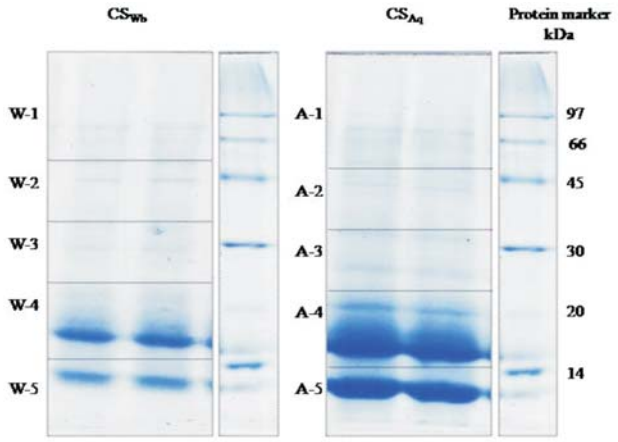

Figure 1: **SDS-PAGE band profile of proteins (MW ranging from 14kDa- 97kDa) in both CS_{Wb} and $CS_{Aq.}$ The grids indicate how the gel bands (W_1-5, A_1-5) were cut for mass spectrometry. The right of the figure indicates the molecular weight of the markers (kDa).**

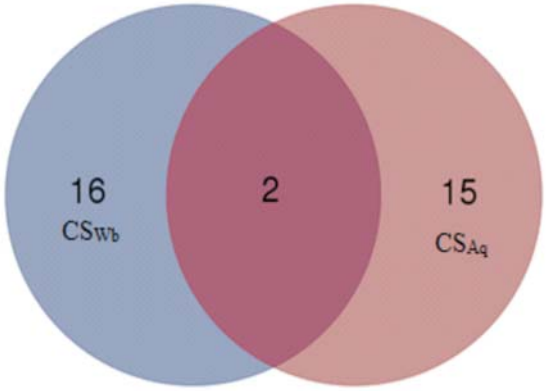

Figure 2: **Venn diagram demonstrating the distribution of proteins among both samples.**

Paterson, 2008; Seth *et al.*, 2014).The medicinal and pharmacological activities of *C. sinensis* are dependent on abiotic factors like altitude, temperature, humidity and biotic factors like host species. These factors account for differential levels of alkaloids, coumarins, flavonoids and steroids which could be responsible for reported medicinal properties (Sharma, 2012). Hence, this is the first time attempt to evaluate a comprehensive characterization of Indian variety of CS_{Wb} using proximate composition analysis and comparative study between CS_{Wb} and CS_{Aq} by phytochemical constituents and proteome analysis.

Screening of Phytochemical constituents in both the samples CS_{Wb} and CS_{Aq} as shown in the result section reveals the presence of phytochemicals with characteristic biological activities such as anti-inflammatory, anti-microbial activities, anti-oxidative and anti-stress. Amino acids present in both samples is also reported to have the property of quenching the deleterious radicals and reflects the proteins richness & its role in human diet. These phytochemical constituents also indicate the richness of medicinal value in *C. sinensis* (Chandran *et al.*, 2013). However, the presence of phytosteroids in CS_{Wb} shows its tendency to reduce cholesterol levels and have potential to inhibit lung, stomach, ovarian and breast cancers which is congregation of previous studies (Arora and Singh, 2009; Chang and Buswell, 1996).

Furthermore, proximate analysis is most established method as reported by Raghuramulu *et al.*, (2003) (Raghuramulu *et al.*, 2003). The results of proximate analysis of CS_{Wb} clearly manifested the presence of high protein (21.46±0.25%), carbohydrate (55.68±0.01%) and low fat content (1.80±0.10%). The less moisture content (7.18±0.03%) in CS_{Wb} indicated the high shelf life of the sample as previously suggested by Azanha & Faria (2005) (Azanha and Faria, 2005). Ash content (7.48±0.15%) is mostly considered as mineral content of the original food. CS_{Wb} has shown 6.40±0.06% crude fibre, which enhances the digestibility and promote health benefits such as reduction of blood cholesterol etc.

Naknaen *et al.*(Naknaen *et al.*, 2015) have clearly mentioned that all dried mushrooms are good sources of proteins with high quality therefore these are used as supplementary diet for many purposes such as nutraceuticals, pharmaceuticals and sports etc. In this study, the presence of proteins was determined by phytochemical (qualitative) and proximate (quantitative) analyses. These analyses established the enrichment of the samples with substantial amount of proteins. These observations prompted us to carry out the proteome analysis for the identification of different kind of proteins present in CS_{Wb} and CS_{Aq}.

In the current study, a number of peptides of molecular weight 14-97 kDa (Table 3) were separated by one-dimensional gel electrophoresis method. 1D SDS-PAGE is an important and useful technique used to separate different class of

proteins present in any traditional or folk medicine (Panda & Swain, 2011; Petrovska *et al.*, 2004). This method is preferred as it is easy and many samples can be analyzed in a more directive manner at the same time. The protein bands separated on polyacrylamide gel could be further cut and used for protein characterization through MALDI-TOF analysis. In the present study, we analyzed the protein profiles of CS_{Wb} and CS_{Aq} to explore the similarities and differences in both the studied samples. Figure 1 shows more number of protein bands in CS_{Aq} as compared to CS_{Wb}. This may be attributed to the sample preparation protocol. It has been reported that use of ASE method for sample preparation provides higher yield and concentrates the constituents (Richter *et al.*, 1996). Higher number of proteins in CS_{Aq} also suggests that CS is a rich source of edible protein. Very limited studies are available for protein isolation and identification from *C. sinensis*.

Several proteins were identified on proteome analysis and some of these have been discussed as follows. P-type II A ATPases are a large group of integral membrane transporter protein with vital importance in life (Bublitz *et al.*, 2011). These are ion pumps that carry out many fundamental processes in medicinal biology ranging from generation of membrane potential to muscle contraction and the removal of toxic ion by use of energy stored in ATP (Kühlbrandt, 2004). TE1b [Blumeriagraminis f. sp. hordei] protein has nucleic acid binding and RNA-DNA hybrid ribonuclease activity. Serine/threonine-protein kinase CLA4 has protein kinase activity with the catalysis of the phosphorylation of an amino acid residue in a protein and ATP–binding activity. Chitin synthase, Chs [Penicilliummarneffei ATCC 18224] is an integral membrane bond protein that participates in the biosynthesis of chitin and plays an important role in cell wall synthesis, hyphal growth and differentiation (Lenardon *et al.*, 2010), DEHA2C06820p [Debaryomyceshansenii CBS767] has metal ion-binding & nucleic acid binding activity and YALI0E29887p [Yarrowialipolytica] has GTP binding, intracellular protein transportation and Rab protein signal transduction activities. Future studies will focus on multidimensional protein identification technological (MudPIT) and metabolomic analysis to identify the active protein ingredients in the *C. sinensis*.

Conclusion

The phytochemical analysis suggested that both the samples (CS_{Wb} and CS_{Aq}) are rich in flavonoids, phenolic compounds, steroids, saponins and alkaloids. Proximate analysis of CS_{Wb} also highlighted the importance of edible mushrooms, CS in diet as determined by the quantitative estimation of ash, carbohydrate, crude fibre, crude protein, fat and moisture content. These phytochemicals observed in both the S samples are supposed to be biological response modifiers which are known to boost the immune system and generate many cellular defensive functions. The 1-D SDS PAGE gel electrophoresis of CS_{Wb} and CS_{Aq} was used to isolate proteins of low molecular weight ranging from 14kDa to 97kDa.The isolation of these lower molecular weight proteins from CS_{wb} and CS_{Aq} is very characteristic of this class that makes this particular fungus to be used as a functional food because these proteins play an essential role in many biological processes such as ribosome formation, stress adaptation for e.g. temperature reduction and cell cycle control.

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Abbreviations

C. sinensis, Cordyceps sinensis; CS, *C. sinensis*; CS_{Wb}, whole body CS, CS_{Aq}, CS aqueous extract; HA, High altitude; ASE, Accelerated Solvent Extraction; rpm, revolution per minute, 1-DGE, one-dimensional gel electrophoresis; W1, zone first of CS_{Wb}; W2, zone second of CS_{Wb}; W3, zone third of CS_{Wb}; W4, zone fourth of $CS_{Wb}$and W5, zone fifth of CS_{Wb};A1, zone first of CS_{Aq}; A2, zone second of CS_{Aq}; A3, zone third of CS_{Aq}; A4, zone fourth of CS_{Aq} and A5, zone fifth of CS_{Aq}; ACN, acetonitrile; NH_4HCO_3, ammonium bicarbonate; $CHCl_3$, chloroform; TFA, trifluoro acetic acid.

Conflict of interest

The authors declare that there is no conflict of interest with the contents of the article.

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