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First report of *Ophiocordyceps liangshanensis* from Arunachal Pradesh, India- Its molecular characterization, phylogenetic studies, nutritional and metabolomic analysis

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ABSTRACT

The present study aims to identify *Ophiocordyceps* collected from Arunachal Pradesh through molecular tools and phylogenetic analysis and also evaluate its nutraceutical properties. The molecular approach involved the amplification of internal transcribed spacer (ITS) region from the stroma and cytochrome oxidase subunit-I (COI) from host larva. The sequences were processed with BioEdit version 7.2.5 and multiple sequence alignments were executed using fast fourier transform (MAFFT) database. Model testing for the phylogenetic analysis was performed using Mega X version 10.2.5, and the best model was chosen to construct the maximum likelihood tree. A Bayesian tree was also created using MrBayes 3.2.7 to verify the findings of the maximum likelihood tree. The study confirms the sample collected from Arunachal Pradesh to be *Ophiocordyceps liangshanensis* which is an entomopathogenic fungi belonging to the family Ophiocordycipitaceae and genus *Ophiocordyceps*. The total protein content in the sample CBUAP1 was found to be 12.4 % w/w and Total Dietary fibre was found to be 38.55 w/w. Similarly, HPLC analysis for quantification of bioactive compounds adenosine and cordycepin was performed, where the concentrations of Adenosine were found to be .02µg/µg of extract in CBUAP1 whereas cordycepin was not detected in the sample. This study is the first report on the identification of *Ophiocordyceps liangshanensis* from India.

1. Introduction

Ophiocordyceps liangshanensis previously known as *Metacordyceps liangshanensis* (Zang M, Liu DQ, Hu RY, 1982) is an entomopathogenic fungi belonging to the family *Ophiocordycipitaceae* and genus *Ophiocordyceps* (Wang et al., 2021). It has long been used as herbal medicine in China like *Ophiocordyceps sinensis* (Berk.) (G.H. Sung et al., 2007). The spore of *O. liangshanensis* parasitizes larvae of Hepialidae, colonizing the hyphae inside the larva from which a fruiting body (stalk) emerges from the head portion of the host (Biswa et al., 2021). The primary hosts of *Ophiocordyceps* pertain to Lepidoptera, Coleoptera, Hymenoptera, Hemiptera, Diptera, Orthoptera, and Odonata, most of which are largely larvae of Lepidoptera or Coleoptera inhabiting wood or soil (Sung et al., 2007). *Cordyceps* is one of the largest genus, having roughly 500 species (Sung et al., 2007). *Ophiocordyceps sinensis, Cordyceps sobolifera, Cordyceps cicadicola, Ophiocordyceps liangshanensis, Cordyceps ophioglossoides, and Cordyceps militaris* have all been cultivated for their therapeutic potential (Tuli et al., 2015). *O. liangshanensis* is reported from China and Nepal at an altitude of 3500 m above sea level. The species diversity of *Ophiocordyceps* appears to be the maximal in East and Southeast Asia (Barseghyan et al., 2011). Furthermore, despite the challenges in harvesting and transportation, it is still regarded as a highly prized mushroom due to its extensive natural bioactive component resources with a variety of robust biological activities and nutraceutical importance (Yu et al., 2013). The fruiting body of fungus along with the larva has been frequently used for the treatment of asthma, chronic cough, lumbago, impotence, hemoptysis, and seminal emissions, and other diseases in traditional Chinese medicine (Yang et al.,2011).

O. liangshanensis was firstly recorded in “Sichuan

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Tongzhi, and it was treated as a new species and named *Cordyceps liangshanensis* (Zang et al., 1982). The medicinal properties of *O. liangshanensis* are also highly valued by herbalists as an alternative of natural *Ophiocordyceps sinensis* (Berk). Sung et al., (2007) proposed the genus *Metacordyceps* to place some species of Cordyceps which was characterized by solitary or grouped stromata which are simple or branched, with a fleshy or tough whitish stipe, a greenish yellow to greenish cylindrical to expanded fertile part, and perithecia moderately or completely immersed in stroma. However, based on multigene phylogenetic study conducted by Wang et al., (2021), *Metacordyceps liangshanensis* was placed to *Ophiocordyceps (Ophiocordycipitaceae)* genus. The bioactive components of natural *O. liangshanensis* are comparable to those of natural *O. sinensis*, which also contain alkaloids, organic acids, amino acids, mannitol, adenosine, ergosterol, and stearic acid. Adenosine and cordycepin (3′-deoxyadenosine) are the major active components. Adenosine is a nucleoside made up of adenine and d-ribose. Cunningham et al., (1951) were the first to extract cordycepin from *C. militaris*. It is a nucleoside analogue with a wide range of biological activities such as neuroprotection (Schmidt et al., 2003), lung and kidney protection (Nakamura et al., 2005), antitumor/anticancer/antileukemic activity (Dai et al., 2001), high antioxidant concentration (Li et al., 2006), antibacterial and antifungal (Lee et al., 2012) and anti-inflammatory properties (Yu et al., 2006), immunomodulatory effects (Yu et al., 2006) and prosexual activity (Lim et al., 2012). In this study, samples were collected from Mechuka valley, Shi Yomi district of Arunachal Pradesh state of India and molecular identification was carried out to reveal the species along with nutraceutical profiling.

2. Materials and Method

2.1 Sample Collection-

Samples were collected from Mechuka Valley, Arunachal Pradesh in the month of July, 2021. The samples were then examined at Bodoland University, Kokrajhar, Assam. The samples were cleaned with a fine art brush, photographs (Fig. 1) were taken and dried in a hot air oven at 55ºC for 24 hours. After drying, the samples were stored in an airtight container for further study.

2.2 DNA isolation, *ITS* and *COI gene* amplification-

The DNA isolation from the sample was carried out into two parts, from stroma (Fungal stalk) and the host (larva) separately. Fungal DNA isolation was carried out using DNeasy plant genomic kit (Qiagen, Germany) following manufacturer's protocol with some minor modifications. Wizard SV Genomic purification kit (Promega) was used for DNA isolation from host larva with some minor modifications. Isolated DNA samples were subjected to visualization in agarose gel (0.8 %) electrophoresis and quantified on a Qubit4 fluorometer (Thermo Fisher Scientific). For amplification of *ITS* region of fungal part of the sample, forward primer ITS-5 (GGAAGTAAAGTCGTAACAAGG-3') and reverse primer ITS-4 (5'-TCCTCCGCTTATTGATATGC-3') (Wu *et al.*, 2016) were used. For amplification of target *genes* from host larva, primer *cytochrome oxidase I* was used, forward primer COI-F (5'-GGTCAACAAATCATAAAGATATTG-3') and reverse primer COI-R (5'-TAAACTTCAGGGTGACCAAAAAAT3') (Quan and Zhou, 2011, Wu et al., 2016). The PCR (Polymerase Chain Reaction) for ITS region of fruiting body (stroma) was as follows: an initial denaturation step of 95 °C for 5 min,

Fig. 1: Fruiting body of CBUAPI (Arunachal Pradesh 1)

followed by 35 cycles of 95 °C for 30 s, 51 °C for 2 min, and 72°C for 1 min, and a final extension step of 72 °C for 10 min. The PCR amplification of *COI* gene of host larva consisted of an initial denaturation step of 95 °C for 5 min, followed by 35 cycles of 95 °C for 30 s, 50 °C for 2 min, and 72°C for 1 min, and a final extension step of 72 °C for 10 min (Wuet al., 2016). The amplification products were purified using the Qiaquick PCR purification kit (Qiagen, Germany) and confirmed on 1.5% agarose gel electrophoresis with a 100-bp ladder. The fragments were excised and purified using QIAEX gel extraction kit (Qiagen, Germany), and the concentration was measured on a Qubit 4 Fluorometer (Invitrogen, USA). Subsequently, the DNA was subjected to sanger sequencing with DNA sequencer (AB13730XL-15104-028, Applied Biosystem with sequence scanner 2.V2.0). The ITS and COI sequences were aligned by BLAST in the NCBI nucleotide blast portal (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch). Finally, the sequences were submitted, and the accession number was obtained from the NCBI database.

2.3 Phylogenetic Analysis

Raw sequences were edited using BioEdit version 7.2.5. Phylogenetic analysis was performed using Mega X version 10.2.5 and reference sequences were downloaded from NCBI. The evolutionary history was inferred by using the Maximum Likelihood method and Tamura 3-parameter model (Kimura et al., 1980). The bootstrap consensus tree constructed from 1000 replicates (Felsenstein, 1985) was taken to represent the evolutionary history of the taxa analyzed (Felsenstein, 1985). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates were collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches (Felsenstein, 1985). Initial tree(s) for the heuristic search were obtained by applying the Neighbor-Joining method to a matrix of pairwise distances estimated using the Tamura 3 parameter model. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+*G*, parameter = 0.4124)). This analysis involved 67 nucleotide sequences. There were total of 2049 positions in the final dataset. Evolutionary analysis was conducted in MEGA X (Kumar et al., 2018).

2.4 Nutritional analysis

2.4.1 Protein Estimation

Total protein content of the sample was estimated using Kjeldahl method, AOAC Official Method SM 978.02. 0.5g sample was taken with catalyst mixture and 10 ml Conc. H_2SO_4 for digestion at 250-450ºC for 2 to 3.5 hrs. Kjeldahl instrument (Pelican Kelplus) was used for digesting the sample. 30 ml D.H_20 was added to the digested sample mixture, 4% boric acid was used for trapping NH_4/ [NH_4], 2-3 drops of methyl red and bromocresol green was added. For Titration 0.1 N H_2SO_4 was used.

Total Protein=

$$\frac{(b-a) \times 0.1 \times 14}{SW} \times 100 \times \frac{6.25}{1000}$$

Where b= Titrate value of blank, a= Titrate value of sample, SW= Sample weight, 1= Normality H_2SO_4 = 0.1 N, 14= atomic weight of nitrogen, 6.25= conversion factor.

2.4.2 Total Dietary Fiber-

Total dietary fiber extraction was carried out using total dietary fiber extraction assay kit (Sigma Aldrich, USA) following manufacturers protocol. 1 g sample was taken and ground to fine powder, 100 ml phosphate buffer was added to the sample in a conical flask, pH was adjusted to pH 6 and 0.10 ml α-amylase was pipetted in the sample mixture, the mixture was incubated at 95°C for 15 minutes. The pH of the solutions was adjusted to 7.5 ± 0.2 by adding 10 ml of 0.275 N NaOH to each beaker (sample and blank), 5 mg protease was added into each beaker, covering each beaker with aluminium foil and placed in 60°C under water bath. With continuous agitation the mixture was incubated for 30 minutes after the internal temperature of the beakers reach 60°C. The solution was allowed to cool under room temperature and pH was adjusted to 4.0, 0.1 ml of amyloglucosidase was added, the solution was incubated for 30 minutes after the internal temperature of the beakers reach 60°C. Four volumes of 95% ethanol were added to each beaker and kept overnight at room temperature to allow complete precipitation. Filtration was performed in a Pelican fibre extractor (Model- Pelican fibre plus), the residue was washed with three 20 ml portions of 78% ethanol, two 10 ml portions of 95% ethanol and two 10 ml portions of acetone. The crucibles containing residues was dried overnight in a 105° C air oven.

Calculation-

Residue weight= W_2-W_1, Ash weight=W_3-W_1, Blank=R_{BLANK}-P_{Blank}-A_{BLANK}

$TDF = [R_{SAMPLE} - P_{SAMPLE} - A_{SAMPLE} - B)\ SW] \times 100$

Where: TDF= Total Dietary Fiber
R = Average residue weight (mg)
P = Average protein weight (mg)
A = Average Ash weight (mg)
SW = Average sample weight (mg)

2.5 HPLC analysis for Cordycepin and Adenosine

2.5.1 Extract Preparation

One gram dried *Ophiocordyceps* sample was taken.

Sample was ground to fine powder and transferred to a 30 mL Borosil glass bottle with screw cap. 30 mL of Milli Q Type I water was taken and transferred into a conical flask. Extraction was carried at ultrasonicator with heating for 30 minutes. After ultrasonication, the sample was subjected to 60 minutes of thermal extraction at water bath, (the temperature was maintained at 85°C) Filtered through Whatman 41 filter paper. For second extraction, the left over sample after filtration was taken. To that 20 ml of solvent was added and subjected to 30 minutes of ultrasonication. Filtered with Whatman filter paper 41. The extract was then freeze dried for 48 hours. Total extract obtained after lyophilization was 190 mg.

2.5.2 HPLC Analysis

The quantification of cordycepin and adenosine was carried out following the method described by Huang et al., (2009). The samples were diluted in buffer (92:8 Water: Methanol) and then filtered using syringe filters and 10µL of the filtered sample was loaded into HPLC for the analysis. The analysis was performed in AGILENT (1200 series) HPLC. Isocratic gradient with buffer 92:8 Water: Methanol for 30 min. Standard adenosine and cordycepin were diluted with buffer and made a concentration of 1mg/ml. 10 µL from each of the above mentioned standard was taken and loaded into HPLC and then 5 µL of each standard were mixed and loaded into the instrument with standard mix as label. Flow rate was maintained 1ml/min and 254 nm wavelength was used. Column temperature was maintained at 30° C. 10 µL of injection volume was used

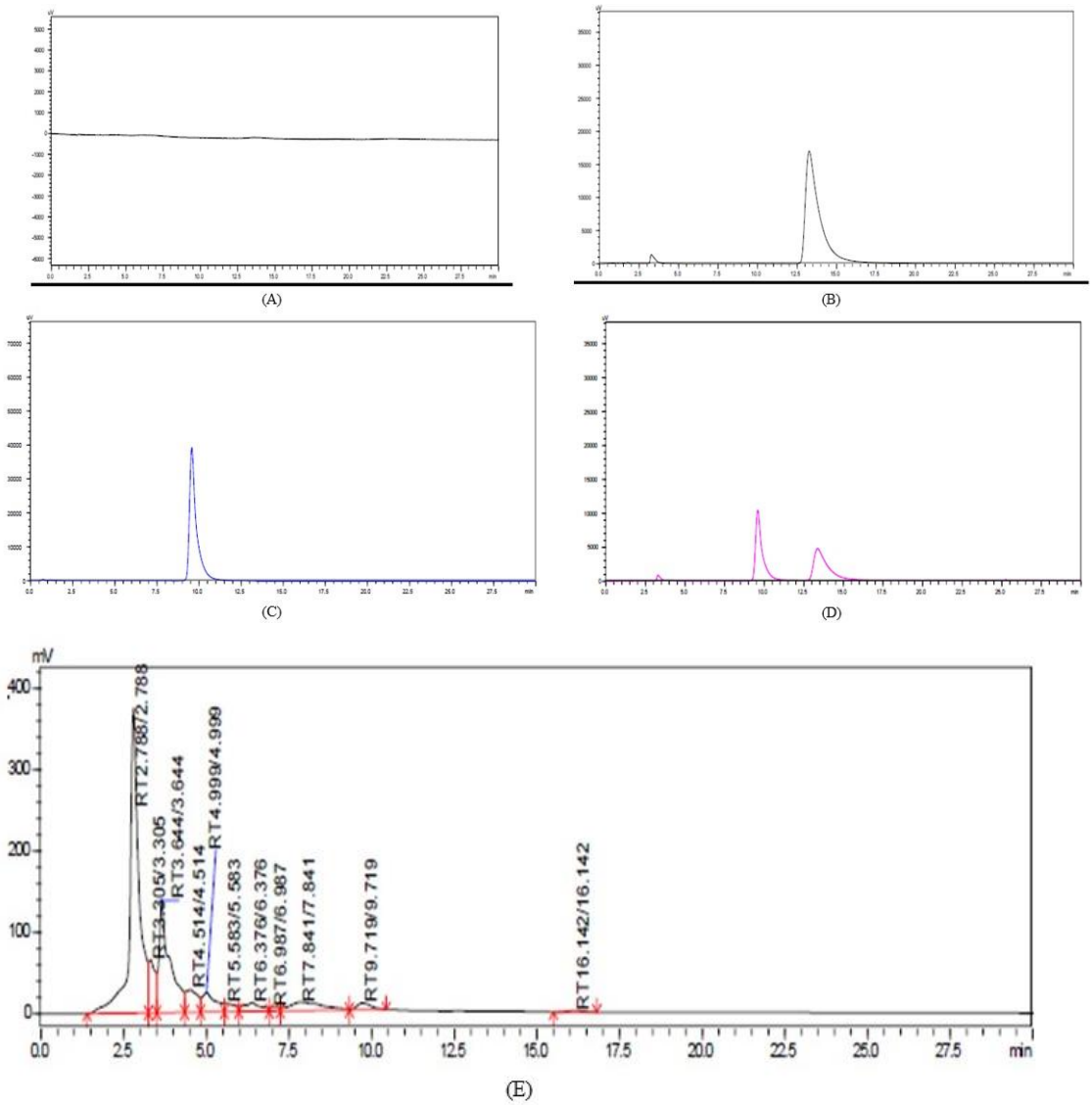

Fig. 2: HPLC Chromatogram; (A): Blank; (B): Standard Cordycepin; (C): Standard Adenosine; (D): Standard Mix; (E): CBUAP1

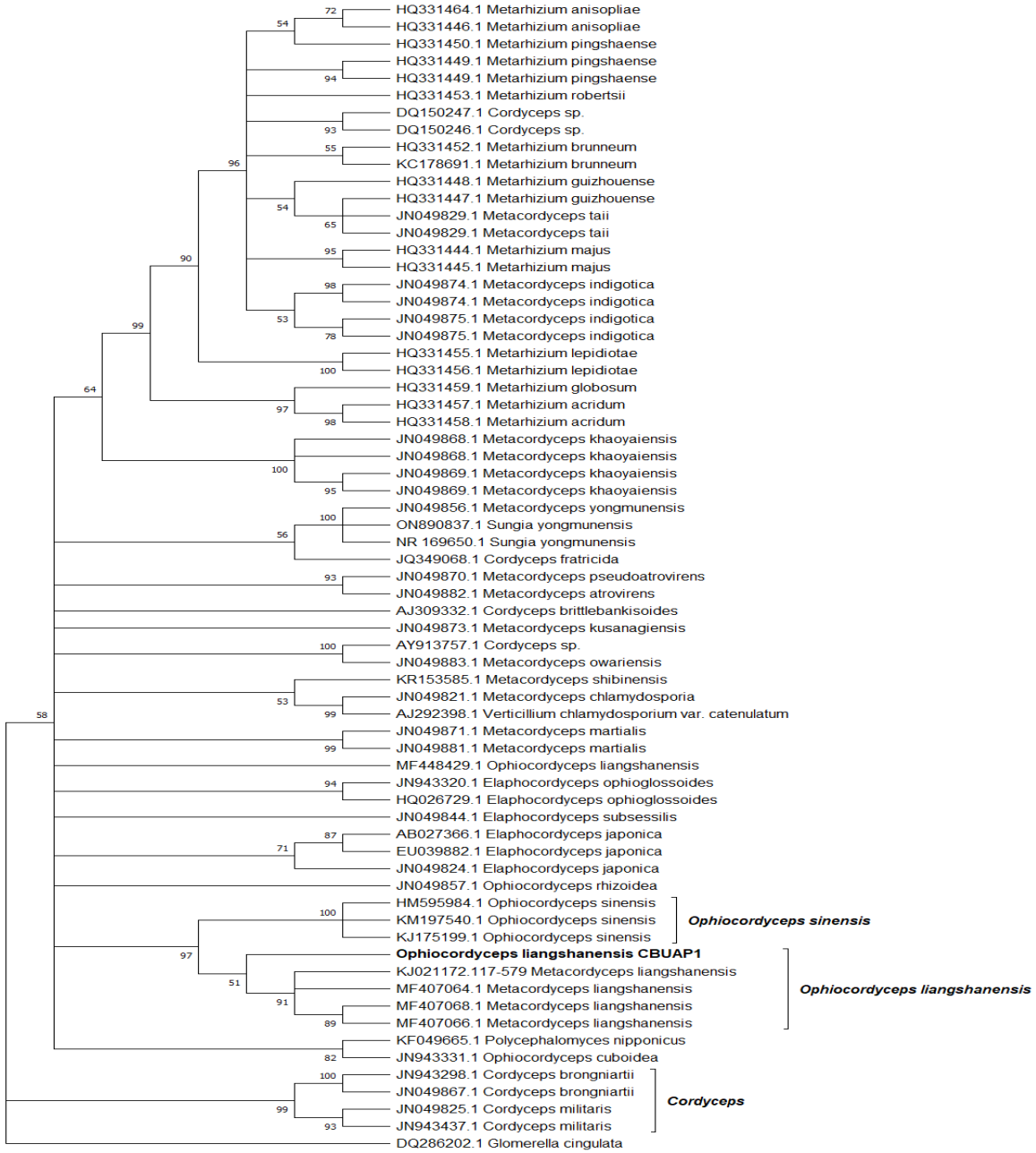

Fig. 3: Maximum likelihood tree of *CBUAP1 (ITS)*

3. Results and Discussion

The collected samples were cleaned and measurements were taken using a slide caliper, the average length and thickness of stalk of the fruiting body was found to be 48.66 mm and 2.56 mm respectively. Similarly, average length and thickness of the larva (host) was found to be 28.96 mm and 2.56 mm and photograph was taken (Fig. 1). DNA was extracted (DNeasy Plant Genomic kit, Qiagen). The DNA from stroma and host larva was isolated independently and amplified on a Thermal Cycler (2720, Applied Biosystems). The PCR product of ITS and COI was 612 bp and 628 bp respectively. The amplicon was then sequenced on an applied biosystem (Model No-AB13730XL) DNA Sequencer following sanger sequencing method. The sequenced raw data was processed in Bioedit version 7.2.5 and consensus sequence was made. The processed sequence of AP1- ITS and COI was blasted in NCBI database. The ITS amplicon of sample *Cordyceps* Bodoland University

Arunachal Pradesh-1 (CBUAP1 fruiting body) covered a query of 94%, which is identical with *O. liangshanensis*. The sequencing data of COI gene had query cover of 94% identity to *Hepialidae*. A total of 67 nucleotide sequences were taken up for the phylogenetic analysis including an outgroup from NCBI database. The sequences were subject to multiple sequence alignment using fast fourier transform (MAFFT) online tool. The alignment was optimized visually, and ambiguous regions were excluded from subsequent phylogenetic analysis. The best model was calculated by the model testing in Mega X (version 10.2.5), and the T92+G model was chosen (Kimura et al., 1980). Pairwise distance matrices were generated using Kimura models of nucleotide substitutions (Kimura et al., 1980, Kumar et al., 2018; Swofford et al., 1998), and the phylogenetic analysis was performed in the Mega version 10.2.5. An NJ tree with bootstrapping was constructed with distance measured by the Jukes Cantor distance model and Kimura's two parameter distance model (Jukes and Cantor, 1969). To assess the confidence of phylogenetic relationships, the bootstrap test (Felsenstein, 1985) was conducted with 1000 resampling for NJ analysis. The phylogenetic relationships of *O. liangshanensis* were analyzed using the Bayesian method (Ronquist et al., (2012). *Glomerella cingulata* was used as an outgroup for phylogenetic analysis of the ITS region. The comparative phylogenetics results using ITS sequence indicated that the presence of the sample collected from Arunachal Pradesh clubbed with the sequences of *Metacordyceps liangshanensis* sequences (China) retrieved from NCBI. The presence in the same clade diverging from the other related species confirmed that the sample belongs to *O. liangshanensis* (*Metacordyceps lianshanensis*), which is an entomoparasitic fungus infecting larva of various genera (*Thitarodes*, *Endoclita*, *Napialus*). The processed sequence of CBUAP1 was submitted to gene bank and accession number MZ318360.1 was obtained. The total protein content in the sample CBUAP1 was found to be 12.4 % w/w, similarly total dietary fibre was found to be 38.55 w/w which indicate potent source of nutraceuticals in the sample. Sample comparison for concentration of adenosine and cordycepin was done with standard and noted in terms of retention time. The concentrations of adenosine were found to be 0.02μg/μg of extract in CBUAP1 whereas cordycepin was not detected in the sample (Fig.-2E).

4. Conclusion

The present study was carried out to document the diversity of *Ophiocordyceps* from Arunachal Pradesh using molecular tools and also evaluate its nutraceutical properties. The ITS region of the fungal part and cytochrome oxidase subunit-I (COI) from host was studied. The phylogenetic analysis confirms the sample collected from Arunachal Pradesh is *Ophiocordyceps liangshanensis* belonging to the family Ophiocordycipitaceae. The nutritional content indicates that it is a potent source for nutraceuticals, however cordycepin was not detected. This is the first report of the presence of *Ophiocordyceps liangshanensis* from India.

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6. Conflict of Interest

The authors declare no conflict of interest.

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