6.1. Summary

Mycophagy is a common practice from time immemorial and used for traditional healing process to treat numerous ailments. Entomopathogenic fungi offer several advantages over conventional mushroom. *Cordyceps* spp. is a genus of parasitic fungi known for its diverse biological activities and potential health benefits. These fungi, primarily found in mountainous regions, have been used in traditional Chinese medicine. They exhibit immunomodulatory, anti-inflammatory, antioxidant, and has potential anti-tumor properties. *Cordyceps* spp. is characterized by its resistance against low temperatures, low oxygen levels, and UV radiation. The fungus spreads by rain, air, and insects, contaminating its host during the larval stage and germinating during the summer. *Cordyceps* spp. is a fungus with a rich history in traditional Chinese medicine and has been recognized for its therapeutic properties. These fungi can boost the immune system, enhance energy levels, and act as adaptogens and antioxidant properties, leading to extensive scientific studies into its potential applications for various health conditions. The fungi's ability to improve respiratory function, support cardiovascular health, and exhibit anti-inflammatory along with anti-tumor activities has fueled its popularity in modern herbal supplements. *Cordyceps* spp. is a fungus rich in essential amino acids, proteins, peptides, and polyamines, with rare cyclic dipeptides and polyamines. Nucleotides Cordycepin (3′-deoxyadenosine) and adenosine exhibit immunomodulatory and antioxidant activities. Fungi contain sterols, including ergosterol, which plays a crucial role in vitamin D2 synthesis. Unsaturated fatty acids are more prevalent, with 28 unsaturated and saturated fatty acids and their derivatives, as well as polar substances like alcohols and aldehydes. *Cordyceps* spp. also contains proteins, peptides, polyamines, essential amino acids, and distinctive cyclic dipeptides. Cordycepin from *Cordyceps* spp., has shown potential in restraining inflammatory mediators in neurodegenerative diseases. It inhibits the overproduction of NO, prostaglandin E2, and pro-inflammatory cytokines suggesting its potential role in neurodegenerative diseases. Artificially cultivated *Cordyceps* spp. exhibit stronger antitumor activity against various cancer cell lines. With these consideration, the present study was carried out to collect and identify *Cordyceps* spp. through morphological, microscopic and phylogenetic analysis from select areas of Eastern Himalayan belt.

Keeping the above-mentioned issues in view, the present research has addressed the following objective:

1. To conduct thorough survey of select areas of Eastern Himalayan region for detailed study of candidate *Cordyceps* spp. and its micro-habitat

Under this objective, application for permission to conduct survey and sample collection was granted from the office of the PCCF, Arunachal Pradesh and Sikkim. Four varieties of samples were obtained from Bhutan. Similarly, samples were collected from North Sikkim (Lachung and Lachen valley) in four batches. One batch of samples was also collected from Mechuka valley, Tsi-yomi District of the state of Arunachal Pradesh, India. To compare the nutraceutical properties of the collected samples, primary culture of *Cordyceps militaris* was also procured from ICAR-Directorate of Mushroom Research (Solan), Himachal Pradesh.

2. To study the identification of the candidate *Cordyceps* spp*.,* nutritional status and genetic diversity by following standard protocols

Under this second objective, morphological studies (macro and micromorphological) was carried out. Further, the samples were cleaned and dried for the microscopic evaluation. Microscopic study revealed CBUS3 collected from Sikkim to be *Ophiocordyceps sinensis*. Sample collected was cultured in petri plates containing potato dextrose agar media. The cultivation of *Cordyceps militaris* was carried out on different rice varieties to study the importance of rice as a substrate and also to compare its nutritional properties with the sample collected from wild. For molecular identification, the DNA was isolated from the collected samples and subjected to electrophoresis for confirmation. PCR amplification was carried out using *ITS*, *COI* and *Cytb*. The primer *ITS* was used for amplification of target gene from the fruiting body of the fungi, whereas *COI* and *Cytb* was used for amplification of target gene from the host larva. Sequencing was carried out in an applied biosystem (Model No-AB13730XL) DNA Sequencer following sanger sequencing method. After obtaining the raw sequence data, it was processed in Bioedit version 7.2.5 and consensus sequence was made for further research. Nucleotide BLAST tool in NCBI database was used for every sample to know the homologous and similarity with existing database. With the similarity of our target gene, reference sequence data were downloaded from NCBI database and phylogenetic tree was

constructed separately for *ITS* and *COI*/*Cytb* amplicon with an outgroup in Mega (version 11.0.13). Phylogenetic study of the *ITS* gene confirmed that CBUS1-CBUS4 collected from Sikkim to be *Ophiocordyceps sinensis*. Similary, phylogenetic study *COI* gene of CBUS1-CBUS4 confirmed the larva to be of *Thitarodes* spp. Furthermore, *ITS* amplicon of CBUB1-CBUB4 was confirmed be *Ophiocordyceps sinensis* and *COI* amplicon of host larva of CBUB1-CBUB4 was identified as *Thitarodes* spp. CBUAP1 collected from Mechuka valley was identified as *Ophiocordyceps liangshanensis* by studying the *ITS* amplicon. *COI* amplicon of host larva confirmed the larva belonging to *Thitarodes* spp.

3. To study the antioxidant and anti-microbial activity of *cordyceps* spp.

The protein content of all the samples were investigated. CBUS4 (*Ophiocordyceps sinensis*) exhibited the highest protein content of 14.1% (±0.01), followed by CBUCM (*Cordyceps militaris*) cultivated on Joha Rice of 13.96% (±0.02), CBUCM (*Cordyceps militaris*) grown on Brown Rice exhibited the lowest protein content of 5.25% (±0.02).

Total Dietary fiber of the samples was evaluated, CBUS3 (*Ophiocordyceps sinensis*) demonstrated the highest total dietary fiber content of 42.72% (±0.05), followed by CBUS4 (*Ophiocordyceps sinensis*) of 41.33% (±0.04) and CBUS1 (*Ophiocordyceps sinensis*) of 40.01% (±0.01). CBUS3- *Mycelia* (*Ophiocordyceps sinensis*) exhibited the lowest total dietary fiber content of 21.33 (±0.08). Among the *Cordyceps militaris* samples cultivated on different rice varieties, *Cordyceps militaris* grown on brown rice exhibited the highest fiber content of 34.25% (±0.03). Conversely, *Cordyceps militaris* grown on Black Rice and Joha Rice exhibited lower fiber percentages of 23.24% (±0.08) and 23.26% (±0.09) respectively.

In vitro free radical scavenging activity was studied of all the samples were performed by DPPH assay, FRAP assay and $ABTS^{+}$ cation scavenging assay. From the result of antioxidant activity, the sample CBUS3 was found to possess the highest overall antioxidative potential of all the samples.

The methanolic aqueous extract (70:30) of the samples was evaluated for its antimicrobial properties against six bacterial strains, the samples exhibited with variable intensity. CBUB2 and *Cordyceps militaris* grown on Joha rice exhibited the highest inhibition against *Staphylococcus aureus* with 27 mm zone of inhibition while other

samples also exhibited potential inhibition against the bacteria in the concentration of 200 µg/ mL extract concentration. Similarly, CBUS3 exhibited the highest inhibition against *Salmonella typhi* and *Mycobacterium smegmatis* with 20 mm and 19 mm zone of inhibition respectively, while *Cordyceps militaris* grown on Basmati rice exhibited lowest inhibition of 2 mm against *Salmonella typhi. Cordyceps militaris* grown on Basmati rice recorded the highest inhibition of *Pseudomonas aeruginosa* with 18 mm zone of inhibition, while CBUS3 and *Cordyceps militaris* grown on Black rice exhibited lowest inhibition zone of 9 mm. CBUS3 exhibited highest inhibition of 14 mm against *Escherrichia coli*, while *Cordyceps militaris* grown on Joha rice exhibited lowest inhibition zone of 7 mm. Similarly, CBUS3 exhibited highest inhibition of 14 mm against *Bacillus cereus*, while CBUB2 exhibited lowest inhibition zone of 8 mm. The detailed zone of inhibition of all tested the samples, standard antibiotic (Ampicillin 10 µg) and DMSO (Positive control).

4. To study the *in-vitro* response of bioactive components of candidate *Cordyceps* spp. in different cell lines.

In vitro cytotoxicity of extracts prepared from the fruiting bodies and mycelia of CBUS3 (*Ophiocordyceps sinensis*) and CBUAP1 (*Ophiocordyceps liangshanensis*) collected from Sikkim and Arunachal Pradesh was studied in the MCF-7, HeLa, and SKOV3 cell lines. CBUAP1 exerted moderate cytotoxicity against MCF-7 with an IC_{50} of 292.8; CBUS3 (fruiting) did not exert active cytotoxicity (IC_{50} > 100 µg/mL); however, extract CBUS3 (mycelia) exhibited potent activity with an IC_{50} 77.48. Extracts CBUAP1 and CBUS3 (fruiting) were not active (IC_{50} > 100 µg/mL). However, CBUS3 (Mycelia) exerted moderate activity against MCF-7, with IC50 of 294.3 against the HeLa cell line. The extracts CBUAP1 and CBUS3 (fruiting) exerted low cytotoxic activity with no active activity (IC_{50} > 100 µg/ml); however, extract CBUS3 (mycelia) exhibited an IC_{50} of 185.6 against the SKOV3 cell line.

Similarly, LDH assays of sample CBUAP1, CBUS3 (fruiting), and CBUS3 (mycelia) showed increased levels of LDH at higher concentrations, i.e., 1891.54 U/L, 1554.91 U/L, and 1944.97 U/L at 320 µg/ml, when compared to control (untreated), which was 390.06 U/L in MCF 7 cells. At 100µM treatment, standard doxorubicin released 2324.35 U/L of LDH in MCF 7 cells. Comparing sample CBUAP1, CBUS3 (fruiting), and CBUS3 (mycelia) to Control (untreated), which had 368.69 U/L in HeLa cells, the three samples

had greater amounts of LDH (1656.43 U/L, 1496.13 U/L, and 1854.14 U/L at 320µg/ml). At 100 µM treatment, standard doxorubicin released 2527.40 U/L of LDH in HeLa cells. The LDH levels in CBUAP1, CBUS3 (fruiting), and CBUS3 (mycelia) were greater at higher doses (1683.15 U/L, 1474.76 U/L, and 1907.57 U/L at 320 µg/ml) than in the control (untreated) group, which had 384.72 U/L in SKOV3 cells. At 100µM treatment, standard doxorubicin released 2703.73 U/L of LDH in SKOV3 cells.

High Performance Liquid Chromatography was used for the quantification of adenosine and cordycepin because of its boasted medicinal attributes. The results demonstrated that the wild collected samples (CBUS3, CBUAP1, CBUB1 and CBUB2), CBUS3 had the highest adenosine content of 119 µg/ mg of extract and lowest concentration of adenosine was recorded from CBUAP1 of 30 µg/ mg. The bioactive compound Cordycepin was found only from CBUAP1 at a concentration of 10 µg/ mg among the wild collected samples. Similarly, among the standard *Cordyceps militaris* samples, *Cordyceps militaris* grown on Barni rice had the highest concentration of adenosine content of 260 µg/mg extract, followed by *Cordyceps militaris* grown on brown rice 250 µg/mg extract and the lowest concentration of adenosine was found to be on *Cordyceps militaris* grown on Basmati rice 145 µg/mg. The concentration of cordycepin was found to be highest *Cordyceps militaris* grown on brown rice on 300µg/ mg, followed by *Cordyceps militaris* grown on Basmati rice 295 µg/mg and the lowest concentration of cordycepin was recorded from *Cordyceps militaris* grown on Black rice 100 µg/mg of extract.

The samples were analysed using GCMS, which allowed for the identification of several bioactive chemicals based on their peaks observed on a chromatogram. The peaks were subsequently compared to the peaks of recognised chemicals recorded in NIST libraries. The fragmentation of molecules was compared based on their mass-to-charge ratio (m/z), and the compounds were identified. The bioactive chemicals found exhibited diverse actions and characteristics, including antibacterial, antioxidant, and antiviral effects.