5.1 Discussion

The present study was carried out to explore the diverse presence of *Cordyceps* spp. present in the select areas of Eastern Himalayan region of India and Bhutan. Survey for the samples were carried out in the region from different locations of Lachung and Lachen valleys in Sikkim, Mechuka valley in Arunachal Pradesh, and Gasa and Lunana block in the districts of Bhutan. The study emphasized on the taxonomy, biochemical, nutritional and pharmaceutical analysis of the *Cordyceps* species. The study resulted in detailed identification of the species which will reduce the counterfeit trades due to its high economic value. The market of *Cordyceps* mushroom is enormous with price ranging from USD $20,000 to 40,000 per kg (Sharma, 2004). The knowledge of the edibility of the mushrooms collected by local collectors of *Cordyceps* may not be resourceful with traditional technique of identification. Proper nutritional status and identification of *Cordyceps* spp. through this study shall help in mycophagy of the zombie fungi.

The identification of medicinal plants, fungi, and animals, has advanced significantly with the recent rapid development of molecular techniques which is responsible for attracting a wide range of attention and recognition (Chen et. al., 2010, Xiang et. al., 2013, Zhang et. al., 2014, Luo et. al., 2013, Chen et. al., 2014). Given that DNA-based identification is efficient, accurate, and accessible to those without formal training in taxonomy, it has gained significance in the identification of medicinal plants (Ali et al., 2014). Owing to rapid concerted evolution, the internal transcribed spacer (*ITS*) of nuclear rDNA has many variable sites and potential informative sites among related species, which has been proven to be an important molecular marker in phylogenetic and evolutionary studies. The Consortium for the Barcode of Life recently decided to designate the *ITS* sequence as the official marker for fugal genetic identification (Das and Deb, 2015). As an environmental DNA barcode, *ITS* sequence amplification was utilised to detect fungus from soils or water (Bellemain et al., 2010). While *COI*, *COII*, and *Cytb* are all useful for analysing the phylogenetic relationships, the Mantel test revealed that *COI* is a trustworthy marker to disclose the phylogenetic links and geographic distribution patterns of the host insects (Hebert et. al., 2003).

In our study, DNA was successfully extracted and amplification of the target gene was carried out from the fruiting body and host of wild samples collected from Sikkim (CBUS1, CBUS2, CBUS3 and CBUS4), Bhutan (CBUB1, CBUB2, CBUB3 and CBUB4) and Arunachal Pradesh (CBUAP1) and the standard sample i.e. *Cordyceps militaris* (CBUCM). Moreover, the DNA from the mycelia of wild sample collected from Sikkim (CBUS3- Mycelia) was also extracted. The concentrations of the isolated DNA of samples ranged from 178-258 ng/µl, among which DNA concentration of Bhutan (CBUB1) sample was highest and Arunachal Pradesh (CBUAP1) sample exhibited the lowest. Using these isolated genomic DNA, amplification of the *ITS* and *COI* sequences were carried out successfully from the samples collected from different regions of study areas with specific primers set. The PCR amplified *ITS* sequences of samples collected from Sikkim (CBUS1, CBUS2, CBUS3 and CBUS4) was 528 bp*,* 526 bp,559 bpand 583 bp respectively. Similarly, the amplicon of the samples collected from Bhutan (CBUB1, CBUB2, CBUB3 and CBUB4) was 579 bp, 571 bp, 582 bp and 583 bp respectively. CBUAP1 collected from Arunachal Pradesh resulted in sequence length of 612 bp. The finding is consisting with Wu et al., 2016, where they reported the sequence length of *ITS* and *COI* region of Bhutanese *Cordyceps* to be 562 bp and 658 bp. Conversely, Chen et al., 2001 reported that the total length of the *ITS1*, 5.8S and *ITS2* regions of *Cordyceps sinensis* and *ITS* assumed anamorphs or allies ranged from 486 to 537 bp (Chen et al., 2001). Based on the amplified *ITS* and *COI* sequences, Nucleotide Blast was carried out and the phylogenetic tree for samples collected from Sikkim (CBUS1, CBUS2, CBUS3 and CBUS4), Bhutan (CBUB1, CBUB2, CBUB3 and CBUB4) and Arunachal Pradesh (CBUAP1) were constructed using MEGA11. The analysis from the phylogenetic tree revealed the identification of the stroma of sample collected from Sikkim and Bhutan to be *Ophiocordyceps sinensis,* while the stroma of CBUAP1 was identified as *Ophiocordyceps liangshanensis.* Moreover, the larva of samples collected from Sikkim, Bhutan and Arunachal Pradesh was identified as *Thitarodes* spp. The finding of molecular identification is consistent with Wu et al., 2016, where they identified *Cordyceps* collected from of Bhutan as an alternative to natural Chinese *Cordyceps*.

The term ‘dietary fibre’ refers to complex carbohydrates, entirety of plant-based edible components, and various other substances that can be fully or partially fermented in the large intestine of human as like lignins, oligosaccharides, polysaccharides, and

associated plant substances, but cannot be broken down and absorbed by the small intestine. Dietary fibre, an essential component of functional foods and a frequently discussed subject in food studies, is ranked as the seventh nutrient, behind protein, fat, carbohydrate, cellulose, minerals, and water (Elleuch et. al., 2011). It is said that dietary fibre acts as a 'gut scavenger'. Consequently, consuming more edible fungus that are high in dietary fibre can help prevent a number of diseases and fulfil the need to alter the standard diet structure that is recommended both domestically and internationally (Wu, 2003). Analysis of dietary fiber content revealed significant variability among samples, with *Ophiocordyceps sinensis* exhibiting the highest content. Among the wild samples, the total dietary fibre was significantly higher in CBUS3 (*Ophiocordyceps sinensis*) of 42.72%, while CBUS4, CBUS1, CBUS2, and CBUAP1 accounted for 41.33%, 40.01%, 39.56% and 38.55%, respectively. The finding is consistent with Manzi et al., 2001, where they reported the average total dietary fibre in mushrooms ranges from 5.5 to 42.6 dry weight. There was a little consensus between the finding of Kumar et al., 2023, where the reported the dietary fibre content of *Cordyceps militaris* to be 1.97 % of dry weight.

The present study also assessed the free radical scavenging properties of the samples by means of DPPH, ABTS, and FRAP assays. The mushroom's high polyphenolic content may have contributed significantly to the reported antioxidant activity, given that single electron transfer can scavenge free radical atoms (Joshi and Sagar, 2014, Palacios et. al., 2011). The DPPH assay rests on the principle that a hydrogen donor is an antioxidant. DPPH free radical, stable at room temperature, is reduced in the presence of an antioxidant molecule, showing a transition from violet to yellow colour (Mishra et. al., 2012). The results of the DPPH assay indicated that CBUS3 had the best activity, with a median inhibitory concentration (IC_{50}) of 62.2 mg, CBUS3-Mycelia came in second, with an IC_{50} of 61.52 mg, and CBUCM grown on brown rice had the lowest, with an IC_{50} of 44.55 mg. Dong and Yao, 2008 reported that DPPH radical scavenging activity of the extract of *C. sinensis* and found inhibition of 80% at 4–8 mg/ml. Similarly, Uhrinova and Polancikova 2018, used methanol extract of *C. sinensis* to evaluate DPPH scavenging activity and reported 39.6- 67.5 % inhibition and it is in line with our result.

Similar to this, the ABTS assay compares an antioxidant's relative potency to that of a standard antioxidant in order to scavenge the ABTS free radical (Shalaby and Shanab, 2013). The results specified that all the samples were proficient of scavenging ABTS free

radical. However, CBUS3 (IC_{50}= 389.95 mg) exhibited the best activity, followed by CBUS3-Mycelia (IC_{50}= 385.25 mg), while CBUCM grown on barni rice showed the lowest activity (IC_{50}= 345.21 mg). Le et al., 2022, reported the ABTS scavenging activity of *Ophiocordyceps soboliphera*, they found the inhibition of 39-59 % in the concentration range of 2-6 mg/mL.

High DPPH and ABTS activity in CBUS3 suggest that it has the capacity to donate protons. Therefore, CBUS3 may function as a primary antioxidant as well as an inhibitor or scavenger of free radicals (Patil et. al., 2009). The FRAP assay also validated CBUS3's antioxidant capacity and corroborated the findings of the DPPH and ABTS assays.

The investigation of the samples for potential antibacterial activity was prompted by observations from their antioxidant properties. Surveying antibacterial potential from natural sources has become increasingly crucial because to the rise in bacterial resistance to synthetic antibiotics and related medications (Alves et al., 2012). It was discovered that the different samples utilised in the investigation demonstrated varying degrees of antibacterial activity against the microorganism under test. In our study, we have analysed the antimicrobial activity of the wild and standard samples against *Escherichia coli, Mycobacterium smegmatis, Staphylococcus aureus, Bacillus cereus, Salmonella typhi,* and *Pseudomonas aeruginosa.* Antimicrobial activity against *E. Coli, P. aerugenosa,* and *B. subtilis* was demonstrated by all of the extracts. 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 heterogeneity in these strains' genotypes could be the cause of the variation in these extracts' sensitivity against different pathogens (Chattopadhyay et. al., 2004). The presence of phenolic compounds may potentially be responsible for the observed antibacterial activity in the current samples, as phenolic elements of plant extracts have demonstrated strong antimicrobial capabilities in previous investigations (Pereira et. al., 2007). Even though CBUS3 extract has the highest level of antioxidant activity, it has demonstrated very little inhibition against these strains. This implies that the mushroom extract's antioxidant activity and antibacterial activity might not be directly correlated, a theory reinforced by the earlier research (Borchardt et. al., 2008). The relationship between secondary metabolites and their antibacterial action in a specific extract, however, requires more research.

Biologically active compounds such as exo-polysaccharides, cordycepic acid, cordycepin, and adenosine, are found in *Cordyceps*, especially in extract. Due to its broad range of biological activity and possible medical benefits, cordycepin, also known as 3'-deoxyadenosine (9-(3-deoxy-D-ribofuranosyl) adenine), is the main active element that has drawn the greatest interest (Das et. al., 2021). Similar in structure to the cellular nucleotide adenosine, cordycepin operates as a nucleoside analogue. The most prevalent nucleosides among the different *Cordyceps* species is adenosine. The Pharmacopoeia of PR China 2000 uses adenosine concentration as a quality measure of *Cordyceps* and stipulates that herb medicine must contain at least 0.01% (by weight) of this compound (Wu et. al., 2007). The analysis of bioactive compounds revealed the presence of adenosine, a major nucleoside in *Cordyceps* species, and other bioactive components with potential pharmacological effects. All of the wild collected samples (CBUS3, CBUAP1, CBUB1, and CBUB2) contained adenosine, which is a major nucleoside in *Ophiocordyceps* species and its allies and plays an important role in biochemical processes in organisms. CBUS3 had the highest adenosine content of 119 µg/ mg of extract, while CBUAP1 had the lowest concentration of adenosine, recorded at 30 µg/ mg. With the exception of the finding that cordycepin is comparatively higher in CBUAP1 (*Ophiocordyceps liangshensis*) (10 µg/mg), it was found in small amounts or was not detected at all in the other wild samples. Huang et al., 2009, evaluated the concentration of *C. militaris* and *C. sinensis*, their results presented the mean contents of cordycepin and adenosine in the fruiting bodies of *C. militaris* were 2.654 ± 0.02 and 2.45

± 0.03 mg/g, those in *C. sinensis* were 0.9801 ± 0.01 and 1.643 ± 0.03 mg/g, while those in the mycelium of *C. militaris* were 0.9040 ± 0.02 and 1.592 ± 0.03 mg/g, respectively (Huang et al., 2009). Kaushik et al., 2020 reported the yield of higher amount of cordycepin with 466.48 ± 3.88, 380.23 ± 1.78, 434.97 ± 2.32, 269.78 ± 2.92, 227.61 ± 2.34, 226.02 ± 1.69 and 185.26 ± 2.35 mg/L respectively as compared to control with 13.66 ± 0.64 mg/L (Kaushik et al., 2020).

The most prevalent bioactive component in the CBUAP1 and CBUB1 samples in the current investigation was clofexamide (A.M. Tareq et al.,2023), which is well documented to have strong antidepressant effects. Cyclobarbital (Breyer-Pfaff, Jerg & Petruch, 1979), a key bioactive component in CBUS3, is utilised as an antidepressant and to treat insomnia. *Cordyceps* spp. has been widely used in the treatment of respiratory illness, the drug clenbuterol which is used to treat asthma, muscle atrophy (Jiang et al., 2011) is detected in our GCMS study. Another compound Flecainide which is detected in our sample CBUS3 is used for used for treatment of irregular heart beat (Arunachalam & Alzahrani, 2019.) All the bioactive compounds detected in our GCMS analysis indicated potent medicinal attributes and further extraction with different solvents may lead to the finding of novel bioactive compounds.

The exploration of various bioactivities of these wild samples was motivated by the well-known therapeutic properties of the bioactive compounds that constitutes these wild samples.

Cardiovascular illnesses are the leading cause of death and morbidity, followed by cancer. As cancer is becoming a more serious global health issue, safer anti-cancer medications must be developed. The development process and therapeutic management of malignant tumours have seen significant advancements (Fan et. al., 2019, Macgregor and Squire, 2020). Several commercial anticancer medications, including 5-flurouracil, doxorubicin, paclitaxel, and cisplatin, have demonstrated exceptional pharmacological qualities in clinical settings (Paradisi et. al., 2013). Numerous natural extracts have been studied for their potential to have anticancer effects on tumour cells (Melo-Silveira et. al., 2014). Diverse pharmacological properties of hundreds of species of mushrooms have been verified via significant investigation. *C. sinensis* has been used more and more in Chinese medicine as an adjuvant of chemotherapy and radiation for the treatment of tumours due to its encouraging effectiveness, little side effects, and overwhelming

resistance to medications (Chen et. al., 2013; Santos et. al., 2018; Xie et. al., 2018). Numerous studies have reported that *C. militaris* extracts inhibit the growth of various cancer cell, such as HeLa, MCF-7, PC3, NL20, SKOV3, etc., (Sang et. al., 2020; Kong et. al., 2021). Evaluation of anticancer activity demonstrated promising results, with certain samples exhibiting significant growth inhibition against cancer cell lines. In this study, we examined the anticancer activity of our samples, where the wild samples CBUAP1, CBUS3(Fruiting), and CBUS3(Mycelia) underwent MTT assay on different cell lines, i.e., MCF-7 (breast-adenocarcinoma), HeLa (cervical), and SKOV3 (ovarian). According to the findings, CBUAP1 exhibited anticancer bioactivities only against MCF-7 cell line with an IC_{50} value of 292.8 µM while CBUS3(Mycelia) exhibited anticancer activities with an IC_{50} value of 77.48, 294.3, and 185.6 µM against MCF-7, HeLa, and SKOV3 cell lines respectively. At a dose of 160 µg/ml, CBUAP1 caused 38.34% growth inhibition of MCF-7 cell line after incubation. Whereas, CBUS3(Mycelia) was able to inhibit 83.77%, 61.95%, and 54.29% of cell growth for MCF-7, SKOV3, and HeLa respectively at a dose of 320 µg/ml. Song et al., 2016 reported dose- and time-dependent reductions in cell viability in the MCF-7 and HepG2 cells following incubation with *Cordyceps militaris*, They found the 24-h half maximal inhibitory concentrations of *Cordyceps militaris* in the MCF-7 and HepG2 cells to be ~1.096 and 0.791 mg/ml, respectively (Song et al., 2016)

Quan et al., 2022, studied anticancer activity of *C. militaris* against HL-60 and Meg-01 cell lines, they reported that the different extraction fraction of extract on Meg-01 was maximal at a concentration of 50 µg/mL; Of their total sample, Cod1 (37.89%) and Cod23 (30.60%) performed significant cytotoxicity on the Meg-01 cell line, Similarly, On the other hand, both Cod1 and Cod23 exhibit potent cytotoxicity on the HL-60 cell line, with IC_{50} values of 12.50 and 14.99 µg/mL, respectively (Quan et al., 2022).

An additional measure of cytotoxicity, LDH release, was used to look into the protective properties of the wild samples. When the cell membrane is damaged, the stable cytoplasmic enzyme LDH, which is found inside cells, is released into the cell culture supernatant. The cytotoxicity assessment determined by the release of LDH for sample CBUAP1, CBUS3 (Fruiting), and CBUS3(Mycelia) against MCF-7 cell was significantly increased at a concentration of 1891.54 U/L, 1554.91 U/L, and 1944.97 U/L, respectively at a dose of 320 µg/ml. Similarly, the LDH release against HeLa cell line was increased

at a concentration of 1656.43 U/L, 1496.13 U/L, and 1854.14 U/L; and against SKOV3 cell line at a concentration of 1683.15 U/L, 1474.76 U/L, and 1907.57 U/L at a dose of 320 µg/ml. The finding is consistent with Song et al., 2016, where they reported exposure to at doses between 0.2 and 2.0 mg/ml for 24 h, they observed8.45–16.80% increase in LDH release in the MCF-7 cells ($P<0.05$), Similarly, treatment with *C. militaris* for 24 h (0.2–2.0 mg/ml) enhanced LDH release in the HepG2 cells by 4.6–7.0% ($P<0.05$) (Song et al., 2016).