3 Materials and Method

3.1 Study Area

3.1.1 North Sikkim

The state of Sikkim, one of the unique states from India's northeast, is famous for the spectacular topography, abundant wildlife, and unique mushrooms, like *Ophiocordyceps sinensis*. Out of the nine districts of Sikkim, North Sikkim is the foundation district, a place offering peeks into the amazing bounties of the state. The region is crossed by multitudinous rivers and streams that enhance its distinctive beauty. Biodiversity in North Sikkim is exceptionally rich due to its varied environments. Abundant temperate forests, alpine meadows, and vistas laden with rhododendrons adorn the area. The area contains a diverse range of plant species, including rare orchids, medicinal herbs, and mosses. North Sikkim is home to a diverse variety of wildlife species, from the subtle snow leopard to the Himalayan black bear, musk deer, red panda and countless species of birds. North Sikkim's Khangchendzonga National Park's sanctuary (Kanchenjunga Biosphere Reserve) is home to a number of species, with the focus on protecting their natural environments. An outstanding representation of the biodiversity of North Sikkim is that of *Ophiocordyceps sinensis*, a highly valued medicinal fungus, which is renowned for its healing properties. *Ophiocordyceps sinensis*, commonly referred to as the "Himalayan Viagra" or "Yarsagumba," thrives in the elevated regions of the Himalayas, such as North Sikkim. It's highly desired for its health benefits and is used in traditional medicine for some diseases.

3.1.2 Mechuka Valley, Arunachal Pradesh

Mechuka Valley (in the Memba dialect of Arunachal Pradesh, the word 'Men' signifies medicine, 'Chu' signifies water in the form of streams and rivers, and 'Kha' signifies snow, meaning "the land blessed with medicinal snow-fed water-rivers and streams") is located in the Tshi-yomi district of the state of Arunachal Pradesh, India, which is known to be a pictorial region for its stunning landscapes, rich biodiversity, and unique cultural heritage. Mechuka Valley is nestled amidst the Eastern Himalayas, offering breathtaking views of snow-capped peaks and verdant valleys. The pristine Siyom River traverses the valley, contributing to its natural beauty. The flora of Mechuka Valley is diverse and lush, with dense forests of rhododendron, pine, and oak trees covering its slopes. The valley is also home to a variety of medicinal herbs, orchids, and other endemic plant species. The fauna of Mechuka Valley is equally rich, with the region

serving as a habitat for diverse wildlife, including snow leopards, Himalayan black bears, red pandas, and numerous bird species such as monal pheasants and hornbills. Mechuka Valley's high elevation influences its climate, resulting in cool summers and cold winters. The valley experiences heavy rainfall during the monsoon season, which contributes to its lush greenery and fertile soil. Situated at an elevation of approximately 2000 meters above sea level, Mechuka Valley offers a tranquil escape amidst the rugged Himalayan landscape, attracting nature enthusiasts and adventurers alike. ron, pine, and oak trees covering its slopes. The valley is also home to a variety of medicinal herbs, orchids, and other endemic plant species.

3.1.3 Lunana and Laya of Gasa District, Bhutan.

Cordyceps are mostly harvested in the two primary untouched alpine meadows of Laya, Lunana, and Bumthang in Bhutan. The government initially established sustainable harvest guidelines in 2004. Only registered local villagers are permitted to harvest *Cordyceps* in Bhutan, namely within the village's proximity, for a certain duration each year. In order to safeguard the environment, ensure the lasting viability of the *Cordyceps* and protect the collectors, the collecting process is supervised by local leaders and forestry services, who also monitor for any illegal hunting activities.

Lunana

The Lunana Block is situated in the farthest northwest region of the Bhutan. It is positioned at an elevation of 3400 meters above sea level. The region undergoes a wide range of temperature conditions, varying from temperate to alpine. The winters are exceedingly cold, while the summers are brief but enjoyable. In winter, Lunana experiences significant snowfall, which results in the mountain routes becoming inaccessible to neighboring Blocks and district Dzongkhags. The typical annual rainfall in Lunana is 2000mm.The residents of Lunana predominantly communicate in Dzongkha, characterized by a unique accent and their native dialect. The Block also faces harsh weather conditions characterized by prolonged winters, which hinder the diversification of agricultural production. Nevertheless, dry land cropping involves the cultivation of crops such as wheat, buckwheat, and horticulture production, which includes potatoes, radishes, turnips, and other similar crops are conducted on a limited or reduced scale. The inhabitants of this Block rely exclusively on *Ophiocordyceps* spp. (yartsagunbup) and cattle farming, which they exchange through trade with nearby Blocks and Dzongkhags.

Laya

Laya Block is situated in the northwestern region of Gasa District. Among the four Blocks of Gasa District, it is the second largest in terms of size. The region shares an international boundary with Tibet to the north. The geographical extent of Laya Gewog is around 981.5 square kilometres (equivalent to 242,528.65 acres. The main attraction of the Laya block is the scenic Laya Village which is situated at an altitude of around 3,850 meters above sea level. The Layaps are the primary inhabitants of Laya block, they possess a diverse cultural legacy that is intricately intertwined with the local ecosystem. The ecology and wildlife of Laya is similarly stunning, characterized by undisturbed forests that are abundant with a diverse range of plant and animal species.

3.2 Survey and Sample Collection:

Permission to conduct survey and sample collection from the Office of PCCF, Arunachal Pradesh and Sikkim helped immensely for robust survey and selective collection. Four varieties (Table 3.1) of samples were collected from Bhutan. Similarly, samples were collected from North Sikkim (Lachung and Lachen valley) in four batches and listed in Table 3.1. 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 used from DMR (Directorate of Mushroom Research), Solan.

Table 3.1. Sample Details

Sl. No.	Sample Name (local name/ variety)	Type of sample	Source of Collection	Assigned Abbreviation for the study
1	Super A	Dry	Laya, Bhutan	CBUB1 (*Cordyceps* Bodoland University Bhutan 1)
2	A1	Dry	Lunana, Bhutan	CBUB2 (*Cordyceps* Bodoland University Bhutan 2)
3	B1	Dry	Lunana, Bhutan	CBUB3 (*Cordyceps* Bodoland University Bhutan 3)

4	C1	Dry	Laya, Bhutan	CBUB4 (*Cordyceps* Bodoland University Bhutan 4)
6	Yartsagumba	Dry	Mechuka Valley, Tsi-Yomi District, Arunachal Pradesh	CBUAP1 (*Cordyceps* Bodoland University Arunachal Pradesh 1)
7	Yarsagumba	Dry	Lachung Valley, North Sikkim	CBUS1 (*Cordyceps* Bodoland University Sikkim 1)
8	Yarsagumba	Dry	Lachung Valley, North Sikkim	CBUS2 (*Cordyceps* Bodoland University Sikkim 2)
9	Yarsagumba	Fresh	Yumesamdong Lachen Valley	CBUS3 (*Cordyceps* Bodoland University Sikkim 3)
10	Yarsagumba	Dry	Lachung Valley, North Sikkim	CBUS4 (*Cordyceps* Bodoland University Sikkim 4)
11	*Cordyceps militaris*	Pure Culture	DMR, Solan	CBUCM (*Cordyceps* Bodoland University *Cordyceps militaris*)

3.3 Morphological & Microscopic Studies:

3.3.1 Morphological Studies:

For morphological studies, length and diameter of the stroma, larva and colour of host were studied.

3.3.2 Microscopic studies:

The samples were collected, cleaned and dried for the microscopic study. During the initial screening of samples, it was found that apart from the sample CBUS3, other samples did not qualify for the study due to incomplete fruiting structure i.e the top part of the fruiting which is a fertile region important for microscopic studies.

CBUS3:

The samples were wiped (cleaned) for any external dust and other debris with a brush and precise photographs were taken with placing a scale. For microscopic studies the dried sample were cut into thin sections with free hand using thermocol pith and sharp razor blade. The sections were then treated with 5 % KOH, Phloxin and Congo red for

five to ten minutes and visualized under biological microscope Olympus CX 43 at magnification up to 1000X. For microscopic studies transverse section of stroma, larva body and surface of larva body were studied. The captured images were visualized and measured with the software package Olympus viewer and LC micro.

Fig 3.1. Cleaning and processing of sample (CBUS3).

SEM (Scanning Electron Microscope) analysis:

For scanning electron microscopy (SEM), 1mm wide fruiting body of the samples were cut, fixed with 4% glutaraldehyde. The samples were placed in SEM stubs, coated with gold. Transverse section of fruiting body and its surface were examined with scanning electron microscope (Zeiss Evo-10) and photographed.

3.4. Isolation and Culture:

3.4.1 Isolation and culture of CBUS3 (Biswa et al., 2021)

a. The samples were cleaned with a sterile art brush and detached from the larva, followed by washing in sterile distilled water.
b. 0.1% mercuric chloride was used for surface sterilization. Subsequently, the samples were cut vertically using a sterilized scalpel.
c. The tissue from the center of the fruiting body was inoculated on potato dextrose agar (PDA) with 0.05% $MgCl_2$
d. The culture plates were incubated at 18 °C in a Bio-oxygen Demand (BOD) incubator.
e. Mycelial growth was observed after 4-5 days.

f. In order to obtain pure culture, small chunks of mycelia were sub-cultured on PDA plates containing $MgCl_2$ and 500 mg/L ampicillin.

g. The mycelia were then transferred to liquid media for DNA extraction to confirm the culture.

3.4.2 Culture of *Cordyceps militaris*:

The primary culture was sub-cultured in a Potato Dextrose Agar (PDA) media supplemented with 0.05% Magnesium Sulphate. Appropriate quantity of PDA and Magnesium Sulphate was measured and poured in 950mL of distilled water in a beaker. It was stirred well to avoid any lumps and the volume was adjusted to 1000 mL by adding distilled water. The mixture was heated in a microwave oven to completely melt the components. The media was poured in test tubes and culture bottles and sealed by non-absorbent cotton plugs. Sterilization was carried out in an autoclave at 121ºC, 15 psi for 30 mins. The media was cooled down and let to rest for 24-48 hours. The plates/ test tubes were inoculated with the standard culture of *Cordyceps militaris* obtained from DMR, solan. After inoculation, the plates were incubated in a BOD incubator at 18° C for 10 days in dark. The sub-cultured mycelia were used to prepare liquid spawn for cultivation of *Cordyceps militaris*.

Fig 3.2. Tissue Culture on PDA

3.4.3 Cultivation of *Cordyceps militaris*:

The cultivation of fruiting bodies of *Cordyceps militaris* was done following the method described by Lim et al., 2012 with some minor modifications. The liquid culture was prepared in a 500 mL conical flask containing Potato dextrose (PD) broth with 0.05 % Magnesium Sulphate. After sterilization at 121°C for 15 minutes, the flask was inoculated with pure culture of *Cordyceps militaris* after cooling to room temperature.

The liquid culture flask was placed in a BOD incubator with shaker at 80 rpm for 7 days. The inocula was cultivated in different varieties of rice (brown rice, black rice, basmati rozana rice, barni rice and joha rice) in a 750 mL polypropylene container with addition of 80 mL liquid media (30 g/L glucose, 5 g/L peptone, yeast extract 3 g/L, 1 g/L tri-ammonium citrate, 1 g/L potassium dihydrogen phosphate, 0.5 g/L magnesium sulphate, 50 mg/L thiamine hydrochloride, 10 mg/L methylcobalamin) and sterilized at 121 °C for 30 minutes in an autoclave. The containers were inoculated with 3-5 mL of liquid culture with a sterilized micropipette after cooling it to room temperature. After, inoculation the containers were placed in a SS rack with white light and timer set up. Relative humidity was maintained at 75% and temperature of 18 °C. The culture was covered with white mycelia after one week of dark incubation, the cultivation was continued for 45 days with 12 hours of led light supply. The pin heads appeared on 5-7 days after the light supply and the fruiting bodies was harvested after full maturity. The fruiting bodies obtained from different varieties of rice substrate was freeze dried and stored in a zip lock bag for further analysis.

3.5 DNA isolation: Fungal & Host:

DNA isolation from Sample was carried out with Qiagen DNeasy plant genomic purification kit following manufactures protocol with some minor modifications-

1. CBUS1-S4 2. CBUAP1 3. CBUB1-B4 4. CBUCM 5. CBUS3-Mycelia

Reagents:

Supplied Reagents of the kit with

1. 0.5 M EDTA, pH 8
2. Nuclei Lysis Buffer

Procedure:

a. 20 mg dry sample was measured and crushed using liquid nitrogen in a motor and pestle.
b. 400 µL nuclei Lysis buffer, 50 µL of 0.5 M EDTA was added with the sample and transferred into a 2 mL centrifuge tubes.
c. The sample-mixture was incubated at 65°C for 1.5 hours.
d. 150 µL of buffer AP1 added and centrifuged at 14000 rpm.

e. The aqueous layer was transferred to a QIAshredder spin column and subjected to centrifugation at a speed of 14000 revolutions per minute for a duration of 2 minutes.
f. A fresh 2 mL tube was used to hold the flow-through.
g. 1.5 volume of buffer AW1 was added, 650 µL of the mixture was transferred into a Mini Spin column and centrifuged at 8000 rpm for 1 minutes.
h. The Mini Spin column was placed on a new collection tube, 500 µL of buffer AW2 was added and centrifuged minutes at 8000 rpm for 1 minutes.
i. Again 500 µL buffer AW2 was added and centrifuged at 14000 rpm for 2 minutes.
j. The column was placed into a new 2 mL tube, 100 µL of AE buffer was added and incubated at room temperature for 5 minutes and centrifuged at 10,000 rpm for 2 minutes
k. The Mini column was discarded and the sample was stored at -20°C for further use.

3.6 Electrophoresis:

The isolated genomic DNA was visualized in a 0.8 % agarose gel

3.7 DNA Quantification:

DNA quantification was performed by Qubit 4 Fluorometer (Invitrogen) following the manufacturer's protocol.

3.8 Target Gene Amplification:

- The PCR amplification of genomic DNA of samples (CBUS1- S4, CBUS3-Mycelia, CBUB1-B4, CBUAP1 & CBUCM) was performed using primers listed below in an Applied Biosystem Thermal Cycler.
- Primers were ordered and purchased from Eurofins Genomic, Bangalore.

Table 3.2. Details of primer used in the study

Sl. No.	Primer	Sequence	Code
1	*ITS-5*	5′-GGAAGTAAAAGTCGTAACAAGG-3′	BU-BT-SC-2019-107
2	*ITS-4*	5′TCCTCCGCTTATTGATATGC-3′	BU-BT-SC-2019-108
3	*COI-F*	5′-GGTCAACAAATCATAAAGATATTG-3′	BU-BT-SC-2019-109
4	*COI-R*	5′-TAAACTTCAGGGTGACCAAAAAAT-3′	BU-BT-SC-2019-110
5	*Cytb-1*	5′-TATGTACTACCATGAGGACAAATATC-3′	BU-BT-SC-2019-105
6	*Cytb-2*	5′-ATTACACCTCCTAATTTATTAGGAAT-3′	BU-BT-SC-2019-106

The primers used for rDNA internal transcribed spacer region (ITS) amplification are listed in the table 3.2. forward primer *ITS-5* (5'-GGAAGTAAAGTCGTAACAAGG-3') and reverse primer ITS-4 (5'-TCCTCCGCTTATTGATATGC-3') (Wu et al., 2016). The amplification of the cytochrome region from the genomic DNA of host larva was carried using forward primer *Cytb-1* (5'-TATGTACTACCATGAGGACAAATATC-3') and reverse primer *Cytb-2* (5'-ATTACACCTCCTAATTTATTAGGAAT-3') (Biswa et al., 2021) and (Wu et al., 2016). The *COI* gene amplification was performed using the forward primer *COI-F* (5'-GGTCAACAAATCATAAAGATATTG-3') and the reverse primer *COI-R* (5'-TAAACTTCAGGGTGACCAAAAAAT3'). The polymerase chain reaction (PCR) was performed on the genomic DNA from the stroma (fruiting body) using the *ITS* region. The PCR protocol consisted of an initial denaturation step at 95 °C for 5 minutes, followed by 35 cycles of denaturation at 95 °C for 30 seconds, annealing at 51 °C for 2 minutes, and extension at 72 °C for 1 minute. Finally, there was a final extension step at 72 °C for 10 minutes (Biswa et al., 2021). The polymerase chain reaction

(PCR) settings used to amplify the *Cytb* gene of the host larva involved an initial denaturation stage at 95 °C for 5 minutes, followed by 35 cycles of denaturation at 95 °C for 30 seconds, annealing at 50 °C for 2 minutes, and extension at 72 °C for 1 minute. The amplification process concluded with a final extension step at 72 °C for 10 minutes. The PCR amplification of the *COI* gene in the host larva involved many steps. First, there was an initial denaturation step at a temperature of 95 °C for a duration of 5 minutes. This was followed by 35 cycles of denaturation at 95 °C for 30 seconds, annealing at 48 °C for 2 minutes, and extension at 72 °C for 1 minute. Finally, there was a final extension phase at 72 °C for 10 minutes. These details were reported by Wu et al., in 2016. The amplification products were purified using the Qiaquick PCR purification kit (Qiagen, Germany) and confirmed on 1.2 % agarose gel electrophoresis with a 100-bp ladder. The fragments were excised and purified using QIAEX gel extraction kit (Qiagen), and the concentration was measured on a Qubit 4 Fluorometer (Invitrogen, USA) (Biswa et al., 2021).

3.9 Sequencing:

The amplified target gene was used for sequencing in a AB13730XL, Applied Biosystem Sequencer following sanger sequencing method.

3.10 Phylogenetic analysis:

The raw sequences were edited using BioEdit version 7.2.5. and subjected to nucleotide blast in NCBI database (https://www.ncbi.nlm.nih.gov). The reference sequences were downloaded from gene bank. Multiple sequence alignment was generated with MAFFT online database (http://mafft.cbrc.jp/alignment/server). Model testing was performed using model testing tool in Mega X version 11.0.13 and best model was selected for construction of phylogenetic tree. Phylogenetic analysis was performed using Mega X version 11.0.13. The phylogenetic tree was visualized in Figtree v1.4.4 and illustrated in Microsoft word document.

3.11 Protein Quantification:

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 + 10 mL Conc. H_2SO_4 for digestion @ 250-450ºC for 2-3.5 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, 2-3 drops of methyl red and bromocresol green was added. For Titration 0.1 N H_2SO_4 was used.

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

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.

3.12 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 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 aluminum 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 reaches 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 reached 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}-PBL_{ANK}-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)

3.13 DPPH Scavenging Activity:

The antioxidant activity of the aqueous- methanolic (30:70) extracts of *Ophiocordyceps sinensis* (CBUS1, CBUS2, CBUS3, CBUS3- Mycelia, CBUS4), *Ophiocordyceps liangshanensis* (CBUAP1), *Cordyceps militaris* CBUCM grown on different varieties of rice (brown rice, black rice, basmati rice, barni rice and joha rice) as substrate was carried out following the method described by Molyneux (2004). Approximately 1 mL of 100 micromolar (μM) 2,2-diphenyl-1-picrylhydrazyl (DPPH) solution in methanol was combined with an equal volume of the extract in methanol of various concentrations. The mixture was incubated in darkness for 30 minutes while a solution of pure methanol served as the control. The change in color was analyzed through absorbance measurements at 517 nm using a spectrophotometer (Shimadzu,1900i series). Ascorbic acid was used as a reference compound at different concentrations. The inhibition percentage of free radical by the sample was used to express radical scavenging activity and was calculated with the following formula.

$$\text{DPPH radical scavenging \%} = \frac{\text{Abs.of control} - \text{Abs of extract}}{\text{Abs.of control}} \times 100$$

3.14 FRAP (Ferric Reducing Antioxidant Power):

FRAP analysis of the extract of *Ophiocordyceps sinensis* (CBUS1, CBUS2, CBUS3, CBUS3- Mycelia and CBUS4), *Ophiocordyceps liangshanensis* (CBUAP1), *Cordyceps militaris* CBUCM grown on different varieties of rice (brown rice, black rice, basmati rice, barni rice and joha rice) as substrate was carried out following Benzie and Strain, 1996 and Huang, et al., 2005. For preparation of FRAP reagent, 2.5 mL of TPTZ (10 mmol) was added on 40 mmol HCl, 25 mL of acetate buffer (300 mmol) and 2.5 mL of $FeCl_3.H_2O$ (20 mmol). Freshly prepared 3mL FRAP reagent was taken and incubated at 30° C for 10 minutes. Absorbance was taken at 593 nm, 1 mL of the extract was added to 3 mL FRAP solution. The mixture was incubated at 30º C for 10 minutes and absorbance was recorded at 593 nm. 1mg/ mL ferrous sulphate was used for plotting standard curve and acetate buffer served as blank.

FRAP value= *Abs. FRAP+ extract- Abs. FRAP reagent*

3.15 ABTS radical scavenging activity:

ABTS radical scavenging activity of the extract of *Ophiocordyceps sinensis* (CBUS1, CBUS2, CBUS3, CBUS3- Mycelia and CBUS4), *Ophiocordyceps liangshanensis* (CBUAP1), *Cordyceps militaris* CBUCM grown on different varieties of rice (brown rice, black rice, basmati rice, barni rice and joha rice) as substrate was carried out following the method described by Re. et al., 1999. ABTS of 7mM concentration was prepared using Mili Q water, 2.45 mM potassium persulphate was added to the solution. The mixture was then left overnight in dark. The color developed by $ABTS^{*+}$was diluted to get the absorbance of 0.75 OD at 734 nm for which ethanol was used for diluting the solution. 3 mL of the diluted $ABTS^{*+}$ + 1 mL of the extract were mixed and incubated for 10 minutes at room temperature and the absorbance was recorded at 734 nm.

$ABTS^{*+}$ radical scavenging % = $\frac{Abs.of\ control - Abs.of\ extract}{Abs.of\ extract} \times 100$

3.16 Antimicrobial Activity

3.16.1. Microbial cultures:

Escherichia coli (MTCC 40), *Bacillus cereus* (MTCC 430), *Staphylococcus aureus* (MTCC 3160), *Pseudomonas aeruginosa* (MTCC 2297), *Mycobacterium smegmatis* (MTCC 14468), were purchased from Microbial Type Culture Collection and Gene Bank (MTCC) Chandigarh, India while as *Salmonella typhimurium* (ATCC 14038) was purchased from the American Type Culture Collection (ATCC), The cultures were revived and stored at -80°C in 20% glycerol stock (Borah et al., 2023).

3.16.2 Extraction for antimicrobial Activity:

One gram of dried sample comprising of *O. sinensis, O. liangshanensis* and *Cordyceps militaris* grown on different rice varieties was mechanically ground into coarse powder. Methanol and Mili Q water in a ratio of 70:30 was utilized as solvents for extraction. The mixture was initially subjected to 85°C in water bath for 1 hour, followed by ultrasonication for 30 minutes. The resultant extract was filtered using Whatman filter paper no. 41, and the residue was subjected to a second round of extraction using the same procedure. The filtrate was then lyophilized in a freeze dryer.

3.16.3 Test for antimicrobial activity:

The antimicrobial activity of *O. sinensis, O. liangshanensis* and *Cordyceps militaris* grown on different rice varieties was carried out following Agar disc diffusion method described by (Borah et al., 2023). 5 agar discs with a diameter of 8 mm was placed in each Petri plate containing nutrient agar, using a sterilized stainless-steel forceps. The disc in each plate was loaded with 25%, 50%, 75%, and 100% concentrations of extracts prepared from *O. sinensis, O. liangshanensis and Cordyceps militaris*. The plates were then incubated at 37 ± 2ºC for 24 hours in a bacteriological incubator. The zone of growth inhibition was determined by measuring the diameter of the inhibition zone surrounding the well (including the disc diameter) in millimeters, Minimum Inhibitory Concentration (MIC) was determined by evaluating the minimum concentration required to inhibit the growth of the microbes in a perpendicular direction. These readings were taken for all plates, and the average values were calculated.

3.17 Anticancer Activity:

3.17.1 Cell Culture:

The MCF-7, HeLa, and SKOV3 cell lines were obtained from ATCC. The cells were cultivated in DMEM supplemented with 10% inactivated Fetal Bovine Serum (FBS), penicillin (100 IU/mL), and streptomycin (100µg/mL). The cells were cultured in a humidified atmosphere with 5% CO_2 at 37°C until they reached confluence. The cells were separated using a 0.05% trypsin solution and then spun at 1000 revolutions per minute for 5 minutes. The growth liquid was discarded and the cell pellet was carefully reconstituted using 2mL of DMEM complete medium. The cell viability was assessed, and a solution containing a concentration of 5.0 x 10^5 cells/mL was generated by creating a single cell suspension (Pandey et al., 2023).

3.17.2 Drug Treatment:

32 mg/mL stocks were generated for cytotoxicity experiments using DMSO. A series of dilutions were made, starting at a concentration of 320 µg/mL and decreasing to 5 µg/mL. The dilutions were prepared using DMEM plain media for treatment. The final control group consisted of plain media with 1% DMSO, as described by Pandey et al., 2023.

Table 3.3 List of samples

Sl. No.	Sample Name	Stock	Solubility
1	CBUAP1	32mg/mL	DMSO
2	CBUS3 (Fruiting)		
3	CBUS3 (Mycelia)		

3.17.3 Determination of Cell Viability and Apoptosis:

The MTT assay was used to evaluate the extracts (CBUAP1, CBUS3-Mycelia, and CBUS3-Fruiting) on the MCF-7, HeLa, and SKOV3 cell lines, following the method reported by Cui et al., in 2018. 100 µL of the produced cell suspension containing 50,000 cells per well was added to each pre-labelled well of the 96-well microtiter plate. The plate was then incubated at a temperature of 37℃ with a CO2 concentration of 5%. Following a 24-hour incubation period, the liquid portion above the solid layer was extracted and the layer was washed with unadulterated medium. 100µL of test medicines at different concentrations were applied to each pre-determined well and incubated for 24 hours. Following incubation, the test solutions in the wells were removed and 100 µL of MTT reagent (5 mg/10 mL of MTT in PBS) was introduced into each well. The plates were placed in a controlled environment with a temperature of 37℃ and a CO2 concentration of 5% for a duration of 4 hours. The liquid portion was extracted and 100 µL of DMSO was introduced, followed by gentle agitation to dissolve the formazan crystals. The measurement of absorbance was conducted using a multimode plate reader, namely the Spectramax i3X model from Molecular Devices. The wavelength used for the measurement was 590 nm. This method was reported by Shirisha et al., in 2023. The percentage growth inhibition was determined by applying the following method. The concentration of the test drug required to inhibit cell growth by 50% (IC50) was obtained from the dose-response curves for each cell line using Graph Pad Prism 5.0 software (Alwashm & Satya, 2019).

Calculating Inhibition: % Inhibition= $\frac{\text{OD of Control} - \text{OD of sample}}{\text{OD of Control}} \times 100$

3.17.4 LDH Assay (Lactate Dehydrogenase Assay):

The LDH analysis of the samples (CBUAP1, CBUS3-Mycelia, and CBUS3-Fruiting) was conducted. Stocks of 32mg/mL and 10mM were created using DMSO. Serial two-fold dilutions were then prepared, ranging from 320µg/mL to 10µg/mL and from 100µM to 3.125µM, using RPMI plain media for treatment. MCF 7, HeLa, and SKOV3 cells were obtained from ATCC. The stock cells were cultivated in DMEM media containing 10% FBS, penicillin (100IU/mL), and streptomycin (100µg/mL). The cells were cultured in a humidified atmosphere with 5% CO_2 at 37℃ until they reached confluence. The cell was dissociated using a cell dissociating solution consisting of 0.2% trypsin, 0.02% EDTA, and 0.05% glucose in PBS. The viability of the cells is assessed and then they are centrifuged. In addition, a quantity of 50,000 cells per well was placed in a 96 well plate and allowed to incubate for 24 hours at a temperature of 37℃ in a CO_2 incubator with a concentration of 5%. The monolayer cell culture was treated with trypsin and the cell count was modified to 1 x 10^5 cells/mL using appropriate medium containing 10% FBS. 100 µL of the diluted cell solution containing 50,000 cells per well was applied to each well of the 96 well microtiter plate. After 24 hours, when a partial layer of cells had developed, the liquid above the cells was removed by flicking, and the cells were rinsed once with a medium. Then, 100 µL of various doses of test medicines were put onto the partial layer of cells in microtiter plates. The plates were thereafter placed in an incubator set at a temperature of 37 ℃ for a duration of 24 hours, within an environment containing 5% carbon dioxide. Following incubation, the test solutions in the wells were collected and subsequently incubated with assay reagents at a temperature of 37 ℃ for a duration of 1 minute. The rate of change in absorbance per minute (OD/Min) was monitored during a period of 3 minutes. The LDH activity was calculated using the formula provided by Smith et al., in 2007.

The change in optical density (ΔOD) is equal to the difference in absorbance between the third minute and the first minute.

$$\text{LDH activity (U/L)} = \frac{(\Delta OD)}{\text{Min}} \times 16030$$

3.18 High Performance liquid Chromatography (HPLC) analysis for Cordycepin and adenosine:

3.18.1 Sample Preparation:

One-gram dried each of the samples was measured and ground to fine powder, the sample was transferred to a 30 mL Borosil glass bottle with screw cap. 30 mL of Milli Q Type I water was measured and transferred into a conical flask with the sample. 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). The sample mixture was filtered through Whatman 41 filter paper. For second extraction 20 mL of solvent was added to the left-over extract and subjected to 30 minutes of ultrasonication, filtration was carried out and both the filtered extract was mixed. The extract was freeze dried (Labfreeze model FD-10MR) for 48 hours and weight were measured for further study.

Materials: Reference standard Cordycepin (3′-Deoxyadenosine) and Adenosine (adenine riboside) was purchased from Merck (USA)

3.18.2 HPLC conditions were as follows:

An analytical HPLC system (Waters 1525) with a 2998 dual wavelength UV detector and Empower analytical software (Waters Corporation, Milford, MA, USA) was used for the detection and analysis of cordycepin and adenosine. Column, C18 (250×4.6 mm, Waters USA); mobile phase, Methanol: Water (15:85, v/v); flow rate, 1 mL/min; UV detection, 254 nm; and injection quantity, 20 µL. The sample was filtered through a 0.45- µm membrane filter prior to injection. Quantitative analysis of cordycepin and adenosine was conducted by evaluating the peak area on the basis of a standard curve. Peaks of cordycepin and other compounds in the sample were identified by their retention times and co-injection tests with corresponding standard compounds.

3.19 GCMS Analysis

3.19.1 Extract Preparation for GCMS- Analysis:

One-gram dried *O. sinensis* (CBUS3 and CBUB1)*, O. liangshanensis* (CBUAP1) 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

3.19.2 GC-MS conditions were as follows:

Shimadzu, gas chromatography – mass spectroscopy GC-MS 2010QP Plus. Zb-5 column capillary column (30 m x 0.25mm x 0.25µm). 50µl of sample with 250µL of mixed solution (Methanol/ Water/$CHCl_3$). 10µL of internal standard (margaric acid + tetracosane) was used and internal standard 2 (tropic acid). The mixture was incubated for 30 min at 37°C and the volume was made to 500 µL with water and then centrifuged at 4°C for 5 min. Sample was completely dried under nitrogen which was then derivatized with 100 µL(BSTF+TMS) and then loaded on to GCMS.

3.20 Statistical Analysis:

All the statistical analysis (Mean, Standard Deviation, One way ANOVA, Turkey test) were done in Origin pro 10.1.0.178 2024.