Morpho-Genetic Characterization and Bioactivity Evaluation of *Cordyceps militaris* cultivated on Various Substrates

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Abstract

Cordyceps militaris, a well-known medicinal mushroom, possesses diverse pharmacological properties attributed to its rich repertoire of bioactive compounds such as cordycepin, polysaccharides and phenolics. This study aimed to investigate the morphological and genetic characteristics of C. militaris and to evaluate the antimicrobial and antioxidant potential of its fruiting bodies cultivated on five different low-cost substrates: brown rice, foxtail millet, pearl millet, wheat and corn. Solid-state fermentation was employed for cultivation and morphological traits such as fruiting body length, weight and coloration were recorded.

Brown rice substrate supported the highest yield in terms of fruiting body size and biomass, while foxtail millet showed the least productivity. Genetic identification using ITS sequencing confirmed the strain's alignment with C. militaris and its close phylogenetic relationship with other Cordyceps species. Ethanolic extracts exhibited the strongest antimicrobial activity, particularly those derived from foxtail millet and brown rice substrates, with inhibition zones up to 25.76 mm against S. aureus. Methanolic extracts showed moderate activity, while aqueous extracts were the least effective.

The antioxidant activity was assessed using the DPPH free radical scavenging assay. Ethanolic extracts from fruiting bodies grown on foxtail millet exhibited the highest antioxidant activity with 91% radical scavenging and an IC_{50} of 0.324 mg/mL, outperforming other substrate-derived extracts. This indicates that substrate composition significantly influences the production of bioactive compounds. Overall, this research underscores the importance of substrate selection in optimizing both the yield and medicinal value of Cordyceps militaris. The findings support the potential of C. militaris as a natural source of antimicrobial and antioxidant agents, paving the way for its application in nutraceutical and pharmaceutical industries. Further exploration of genetic mechanisms governing bioactive compound synthesis may enhance production efficiency and therapeutic efficacy.

Keywords: *Cordyceps militaris*, antioxidant, antimicrobial.

Introduction

Mushrooms have been a part of human diet from centuries as functional foods (edible mushrooms) as well as therapeutic agents (medicinal mushrooms)[25]. Many mushrooms have their unique nutritional and pharmaceutical properties[3,18,35]. Medicinal mushrooms have been well documented and reported to have potent and unique compounds that have various medicinal actions such as antitumor, immuno-modulating, antioxidant, cardiovascular, antiviral, antibacterial, anti-parasitic, anti-fungal, detoxification, hepato-protective and antidiabetic effects[28]. Many health benefits including improved heart health and improved athletic performance, have been linked to *Cordyceps*. Furthermore, some herbalists believe that *Cordyceps* promotes libido[37], delays the ageing process[30] and prevents cancer[15,24].

Cordyceps contain a chemical called cordycepin, which has been associated with several health benefits. It might be helpful in the management or avoidance of hyperlipidemia[17] or high blood fat. Benefits have also been demonstrated by the dietary fibre, or *Cordyceps* polysaccharides. Polysaccharides and polysaccharide-protein complexes with anticancer and immunostimulating effects are abundant in medicinal mushrooms. These mushrooms are used to lessen the negative effects of therapy used for treatment of cancer[11,15]. The two species of *Cordyceps* that have been utilised for traditional therapeutic reasons are *Cordyceps militaris* and *Cordyceps sinensis*[1,36]. The majority of *Cordyceps sinensis* grows naturally at higher altitudes and is a wild type.

In contrast to *Cordyceps sinensis*, *C. militaris* is more readily grown in both liquid and solid media that include different sources of carbon and nitrogen. The medicinal qualities and chemical capacity of both *Cordyceps* species are comparable. Cordycepin, ergosterol, mannitol and polysaccharides are only a few of the active ingredients in *C. militaris*, which also has a variety of pharmacological uses and diverse therapeutic purposes. The fruiting bodies of Cordyceps militaris are 2 to 8 cm long, club-shaped and vary in colour from orange to golden. Because wild Cordyceps have a specialised host, their fruiting bodies are scarce and costly[7,12].

Cordyceps militaris is a medicinal mushroom that is commonly cultivated on rice. However, other grains, such as

foxtail millet, wheat, corn or pearl millet, can also be used as substrates. Other organic materials like sawdust, maize cobs, or insects have also been used as substrates to mimic natural conditions. Different substrates may be required for different strains of *Cordyceps militaris* in order to improve the amount of bioactive components and product quality[34]. Antimicrobial activity of *Cordyceps militaris* is morphologically and genetically characterised to determine whether any antimicrobial compounds that might be used medicinally, are present.

Traditional Chinese medicine has been using the fungus *Cordyceps militaris* for many years. It is known to possess a wide range of biological properties, such as anti-aging[30], anti-cancer[15], anti-depression[19], antimicrobial[39], immunomodulator[19] and hypolipidemic agents[17]. Numerous bioactive secondary metabolites produced by the fungus have been shown to have potential medical uses.

For instance, metabolite beauvericin from *Cordyceps* has demonstrated antibacterial action against *Staphylococcus aureus* and *Bacillus subtilis*[2]. *Cordyceps militaris* is rich in bioactive compounds such as polysaccharides, nucleosides (like cordycepin) and phenolic compounds that exhibit strong antioxidant properties. *C. militaris* has been shown to possess antimicrobial effects against various bacterial strains including *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa*. Its extracts demonstrate the ability to inhibit the growth of these pathogens, making it a candidate for natural antimicrobial agents[37]. It has been demonstrated that cordycepin, another metabolite of *Cordyceps militaris*, damages the genomic DNA and cell membranes of Gram-positive and Gram-negative bacterial pathogens, preventing their growth[16].

As members of the Ascomycota group, *Cordyceps spp*. mushrooms are used as natural agents in Asian ethno-medicine for a long time due to their adaptogenic and tonic properties, which include lowering weariness and boosting immunity in humans[15]. The majority of scientific studies on *Cordyceps*, of which there are over 500 species, have focused on *Cordyceps sinensis*, which is listed in the Chinese Pharmacopoeia (2015) and whose adenosine content is regarded as the primary quality indicator. In light of many advantages pertaining to *C. sinensis*, scientists and industry players have recently focused more on the species *Cordyceps militaris*[7,13].

Despite the extensive research on the pharmacological properties of *Cordyceps militaris*, there remains a significant knowledge gap regarding the optimization of cultivation substrates to enhance the yield of bioactive compounds such as adenosine, cordycepin and pentostatin and their associated health benefits[30]. Most studies have primarily focused on *Cordyceps sinensis*, leaving *C. militaris*, which can be more easily cultivated, underexplored in terms of its full potential and therapeutic applications. Additionally, while the antimicrobial and antioxidant properties of *C. militaris* have been documented, there is a need for comprehensive studies that investigate the effects of different substrates on its morphological characteristics and bioactive metabolite production.

The objective of this study was to investigate the bioactive properties of *Cordyceps militaris* cultivated on various low cost substrates such as brown rice, foxtail millet, pearl millet, wheat and corn. The study aims to assess the impact of these substrates on the morphology, antioxidant and antimicrobial activity of *Cordyceps militaris* fruiting bodies. Additionally, molecular characterization was performed to explore genetic diversity. By evaluating the antimicrobial efficiency against bacterial strains and antioxidant potential, the study sought to identify the most effective substrate for increasing the production of bioactive compounds, contributing to efficient nutraceutical and pharmaceutical applications.

Material and Methods

The medicinal mushroom *Cordyceps militaris* (DMRO-1163) and bacterial strains (*Staphylococcus aureus*, *Klebsiella* sp., *E. coli*, *Pseudomonas aeruginosa and Salmonella* sp.) were procured from KBRC laboratory, Bilaspur (H.P.). The MTCC code of *Staphylococcus aureus* (MTCC 96) (Gram-positive), *E. coli* (MTCC 1652) (Gram-negative) and *Pseudomonas aeruginosa* (MTCC 741) (Gram-negative). *Cordyceps militaris* were grown on five different grain substrates such as brown rice, foxtail millet, pearl millet, wheat and corn purchased from local market (strain of *Cordyceps militaris* were procured from KBRC laboratory, Bilaspur, H.P).

The glassware (Borosil), peptone (Hi-media), dextrose (Hi-media), yeast extract (Hi-media), potassium dihydrogen phosphate (Hi-media), magnesium sulphate (Hi-media), mueller- hinton agar (Hi-media), vitamin B12 and vitamin B1 were used. The stock culture of *Cordyceps militaris* was maintained on Sabouraud dextrose agar (SDA) plates. To do this, a sample from the original mother culture was transferred onto fresh SDA plates, which were then incubated for 4 to 6 days at a temperature of 20 to 24°C. After incubation, the culture was further transferred from these stock plates to SDA slants to prepare the working culture.

This working culture was then used for producing liquid spawn. The liquid sawn was prepared in Sabouraud dextrose broth (SDB). The solid substrate can include various organic materials such as brown rice, foxtail millet, pearl millet, corn, or wheat. The choice of substrate may affect the yield of bioactive compounds and the overall growth of the fungi. The nutritional solution is essential for providing the necessary nutrients for the growth of *C. militaris*. The nutritional solution contains dextrose (glucose), sucrose, peptone, yeast extract, potassium dihydrogen phosphate, magnesium chloride, vitamin B12 and vitamin B1 which are essential for growth support metabolic processes. Solid-state fermentation was used to culture *Cordyceps militaris* in

glass or plastic (food grade) jars by combining 30g of solid substrate with 60ml of nutritional solution.

The medium bottles underwent a 15-minute, 121°C autoclave. After cooling the medium to room temperature, 5 mL of *Cordyceps militaris* seed culture was added. The media was then incubated at 20 °C for 14 days under dark conditions and then for two months under periods of light and dark. Throughout the cultivation period, the culture temperature was kept at 20 °C, the relative humidity (RH) was kept between 80% and 90% and 500 lx of white light was used for 14 hours each day and dark period for 6 hours. Harvested and dried mushrooms were placed in a hot air oven set at 50°C[38].

Morphological characterisation: The morphological characteristics of *Cordyceps militaris* were evaluated based on fruiting body length(cm), wet weight, dry weight(gm) and cordycepin level(mg/ml) (Fig. 2). The length of the fruiting bodies was measured with a standard scale immediately after harvesting from the culture jars. This measurement, conducted outside the jars to ensure accuracy, provided insights into the morphological diversity of the species. The fruiting body weight was recorded to understand the influence of different grain substrates on *C. militaris* morphology[23].

DNA Extraction and Polymerase chain reaction (PCR) assay: DNA extraction was performed by using SN fungal DNA extraction kit (magnetic-bead based) by Shankaranarayana Life Sciences LLP No.90, Bengaluru. A micro centrifuge tube containing 1.5–2 ml of the mushroom culture was used to extract DNA and the tube was centrifuged for 2 minutes at 15,000 rpm. After seeing the particle that had developed at the tube's bottom, the supernatant was thrown away. This procedure was carried out once more. The pellet was then mixed again with 200µl LS Buffer, 20µl Proteinase K and 10µl RNase, gently pipetting and vortexed for ten minutes. The mixture was incubated at 56˚C. After adding 300µl of Buffer, the pellet was thoroughly vortexed for 30 seconds and then re-suspended.

After the whole lysate was moved to an SN spin column, it was centrifuged for one minute at 15,000 rpm, discarding the flow-through. Subsequently, 500µl of W1 buffer (ethanol 100%) was introduced into the column, centrifuged at 15,000 rpm for 1 minute and the flow-through was eliminated. To dry the column, this procedure was repeated and a one-minute centrifugation was added. To eliminate any remaining ethanol, the SN spin column was transferred to a fresh 1.5 ml centrifuge tube and incubated for 10 minutes at 56˚C with the cap open. 50µl of preheated elution was applied to the membrane centre of the SN spin column after incubation. With the cap closed, the mixture was incubated at 56˚C for 4 minutes. By distributing the buffer over the centre of the membrane and letting it fully absorb, effective elution was guaranteed. In order to extract the DNA, the tube was lastly centrifuged for one minute at 15,000 rpm. This produced a concentrated and pure DNA sample that was prepared for additional research or use. In the PCR experiment, internal transcribed spacer regions 1 and 2 (ITS1 and ITS4) were amplified[27]. In the PCR experiment, the primers used were 18s fungi-ITS1 as the forward primer with the sequence 5': TCCGTAGGTGAACCTGCGG-3' and 18s fungi-ITS4 as the reverse primer with the sequence 5': TCCTCCGCTTATTGATATGC-3'. These primers are specific to fungal DNA, targeting the internal transcribed spacer (ITS) region, which is commonly used for identifying and studying fungi[10].

The template DNA was first denatured at 94°C for 5 minutes. This was followed by 30 cycles of amplification, which included primer annealing at 57°C for 1 minute, denaturation at 94°C for 1 minute and extension step 1 minute at 72°C; ultimate strand extension: 5 minutes at 72°C.

***In vitro* comparative evaluation of antimicrobial and antioxidant activities of fruiting body ethanolic, methanolic and aqueous extracts of *Cordyceps militaris*:** The study involved a comparative analysis of the antimicrobial and antioxidant properties of *C. militaris* fruiting body extracts using ethanol, methanol and water as solvents. The research aimed to evaluate the extracts' effectiveness against common bacterial species (*Staphylococcus aureus, Klebsiella* sp., *E. coli, Pseudomonas aeruginosa and Salmonella* sp.) and their ability to neutralize free radicals. This investigation highlights the potential of *C. militaris* as a natural source of bioactive compounds with therapeutic applications.

Preparation of extracts from fruiting bodies: The fresh fruiting bodies of *Cordyceps militaris* were cleaned and dried in hot air oven at 50°C. The collected fruiting bodies were mechanically ground into a coarse powder and stored at 4°C for subsequent use. For the extraction process, 70% ethanol, 70% methanol and distilled water were employed as solvents using the Soxhlet extraction method. This extraction aimed to evaluate the antimicrobial and antioxidant activities of the fruiting bodies, which were cultivated on various grain substrates, as depicted in figure 5. The dried extracts obtained from this process were then used to prepare stock solutions at a concentration of 50 mg/mL in their respective solvents[9].

Evaluation of Antimicrobial Potential: The ability of the fruiting bodies of *C. militaris* grown on various substrates to withstand pathogenic microbes such as bacteria, was assessed. Utilising the agar well diffusion technique, the extracts' ability to suppress the test bacterial growth was evaluated. Using the agar well diffusion technique, the antimicrobial activity of ethanolic, methanolic and aqueous extracts was assessed[29]. The agar well diffusion technique of antimicrobial testing for extracts of *C. militaris* was

performed using Mueller-Hinton Agar (MHA) plates. The final inoculum of 1.5 × 10^8 CFU/ml was obtained by culturing the test organisms for a whole night at 37°C, following their injection into nutrient broth, until their turbidity reached 0.5 McFarland standards.

MHA plates were adequately cultured using standardised microbial culture broth. Dimethyl sulfoxide (DMSO) was mixed with extracts at a 50 mg/ml concentration. Using a sterile 8 mm cork borer, five 8 mm wells were drilled into the inoculation medium. 100 µl extracts in different concentrations 20 mg/ml, 30 mg/ml, 40 mg/ml and 50 mg/ml were used[8]. The five wells in this investigation were made on MHA. The first well was filled with the appropriate solvent and the next four wells were filled with extracts of *C. militaris* at varying concentrations. Each experiment was done in triplicate.

Evaluation of Antioxidant Potential: The scavenging capability of the 2,2-diphenyl-1-picrylhydrazyl (DPPH) experiment was used to quantify antioxidant activity[5]. The ethanolic, methanolic and aqueous extracts of *C. militaris* were produced as a stock solution containing 50 mg/mL. Methanol was used to dissolve the DPPH stock solution at a concentration of 0.1 mM. Ascorbic acid was used as a standard. Ascorbic acid dilutions in the range of 0.5 to 10 µg/mL were also made. The spectrophotometer was used to calculate the absorbance at 520nm following a 30-minute dark incubation period. Three duplicates of these trials were conducted. The following formula was used to determine the percentage of radical scavenging activity[26]:

RSA (%) = [(OD control – OD concentration)/ OD control] x 100.

Results and Discussion

Morphological characterisation: In the morphological characterisation, the fruiting body, length up to 5.12-6.94 cm long, often clavate shaped, fertile head, orange to bright orange, was covered with tiny granules giving powdery appearance, stipe orange and stuffed. In present study, the higher wet, dry weight and length of fruiting body were observed on *Cordyceps* cultivated on brown rice substrate. The minimum wet and dry weight of fruiting body were observed on *Cordyceps* cultivated on foxtail millet substrate. The minimum length of fruiting body was observed on *Cordyceps* cultivated on pearl millet substrate described in figure 1 and figure 2.

DNA Extraction and Polymerase chain reaction (PCR) assay: The ITS sequence was amplified using the universal flanking primers ITS1 and ITS4 shown in figure 3. The sequencing was outsourced through Shankaranarayana Life Sciences LLP No.90, Bengaluru. Phylogenetic relationships with the nearest sequences were formed using the neighbor-joining technique and the resultant sequence was examined on the NCBI using BLAST-n[31]. A total of 39 nucleotide sequences were analysed. First, second, third and noncoding codon locations were covered. Every position with incomplete data and gaps was removed. The final dataset had 293 locations in total. MEGA6 was used for evolutionary analysis[32,33]. In this phylogenetic tree, the 423 is *C. militaris* (DMRO-1163) strain used in the present study.

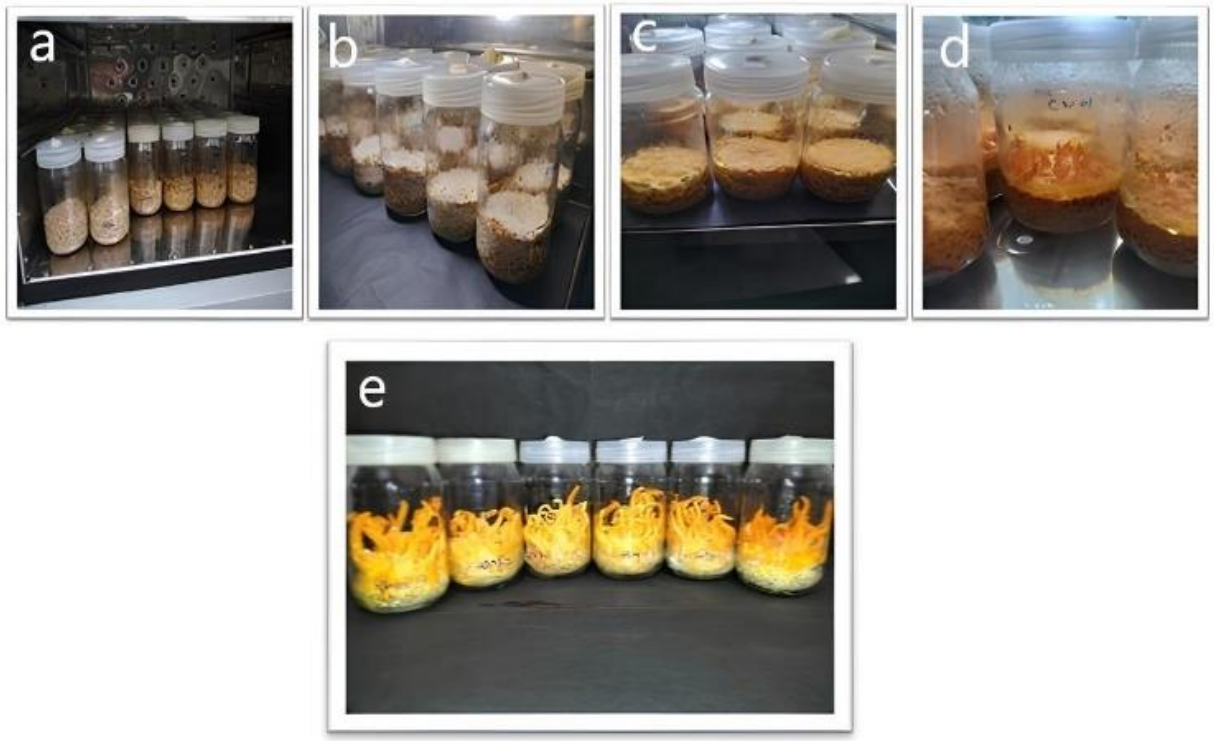
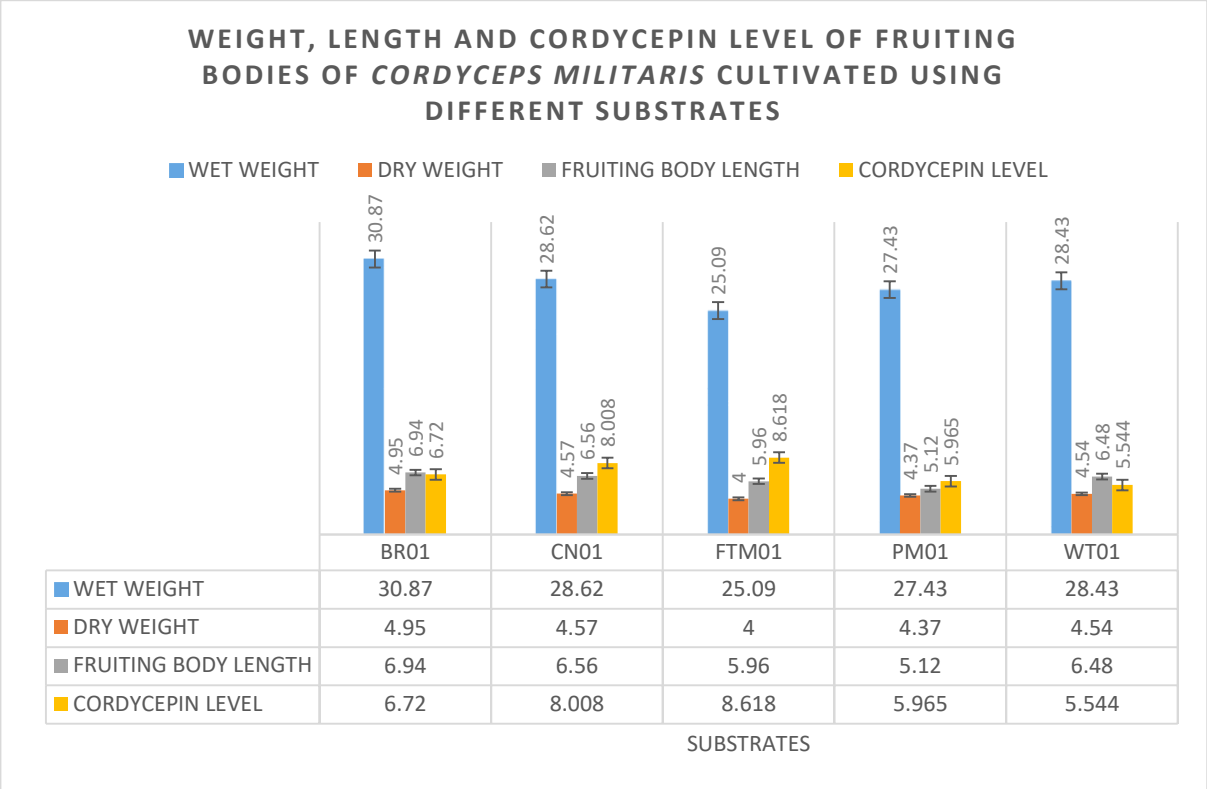


Fig. 1: (a) Incubation after inoculation of culture (b) Mycelial mat formation after ten days (c) Mycelial colour change from white to golden after 14th day (d) Fruiting bodies grown after 30th day (e) Fully mature fruiting bodies after 60th day



BR01-brown rice, CN01-corn, PM01-pearl millet, FTM01-foxtail millet, WT01-wheat

Fig. 2: Chart shows the wet, dry weight (grams), fruiting body length (centimeter) and cordycepin level (mg/g) of the *Cordyceps militaris* cultivated on different substrates.

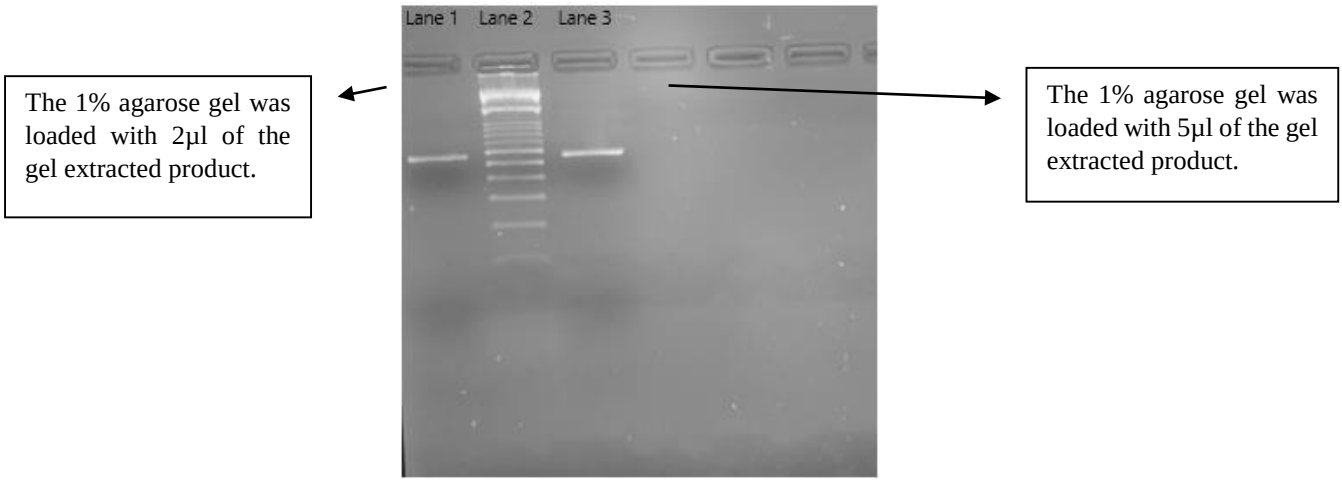


Fig. 3: (Lane 1) 2µl of gel extracted product. (Lane 2) 100+bp ladder. (Lane 3) 5 µl of gel extracted product

Phylogenetic tree Analysis: Based on genetic data, the offered graphic shows a phylogenetic tree that illustrates the connections between different species within the genus *Cordyceps* and allied genera shown in figure 4. Each branch in this tree represents a distinct species or strain and the distance between branches shows how varied their genetic makeup is from one another. Many species of *Cordyceps*, *Isaria*, *Ophiocordyceps* and other related fungi are included in the tree; each species is identified with a unique number such as a GenBank accession number (e.g. AF315049 *Cordyceps hepalidicola*, MW582828 *Cordyceps tsishanensis*). Of special note is strain 423, which is grouped with other *Cordyceps* species and has strong genetic similarities with *Cordyceps militaris* (JN048525) and *Cordyceps kyusyuensis* (EF602907).

Longer branches indicate higher genetic distances and the tree's branching structure shows the evolutionary history and divergence of these species. *Hepatovirus hominis* (AB245627) and *Leptodora kindtii* (AY294177), two outgroups at the base of the tree, act as a reference to establish the root of the tree and offer a standard against which to compare. Researchers may forecast the behaviour, secondary metabolites and possible applications of strain 423 based on its genetic similarity to other well-studied species. Studies on the development of pathogenicity,

symbiosis and other important features can be informed by an understanding of strain 423's place within the phylogenetic tree which sheds light on the species' evolutionary history and divergence from other *Cordyceps species.*

Evaluation of antimicrobial potential: In our study, the extracts of *C. militaris* grown on various substrates (FTM01, CN01, BR01, PM01, WT01) are ethanolic (E), water-based (W) and methanolic (M). The zone diameters (in millimetres) represent the inhibitory effects at different doses (20, 30, 40 and 50 mg/ml) against *Salmonella sp.*, *Escherichia coli (E. coli), Klebsiella sp.* and *Pseudomonas aeruginosa*. For every test organism, the control values stay at 8 mm. In contrast to 11.0 ± 0.20 mm and 10.9 ± 0.10 mm from PM01 and WT01 respectively, *S. aureus* demonstrated inhibitory zones of 25.76 ± 0.70 mm at 50 mg/ml with ethanol extract from FTM01 and 23.8 ± 0.15 mm from CN01.

Although often less effective than ethanol extracts, methanol extracts have also shown noteworthy antibacterial properties; for example, *S. aureus* at 50 mg/ml showed 19.97 ± 0.02 mm. On the other hand, water extracts showed the least antibacterial activity when grown on all substrates; the maximum inhibition zones were rarely larger than 16 mm. According to these results, *C. militaris* ethanol extracts are the most potent antibacterial agents. The potency of these extracts can be affected by differences in substrate with BR01 and FTM01 substrates exhibiting the highest overall efficacy showed.

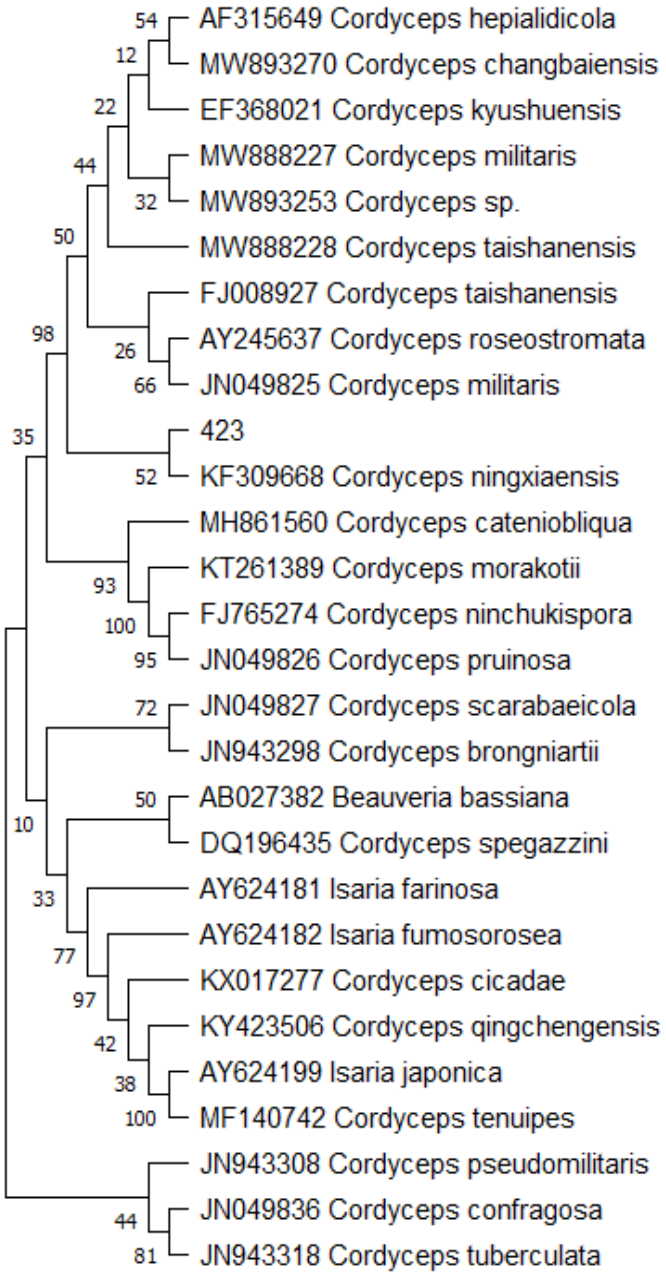


Fig. 4: Phylogenetic tree of the fungal strain

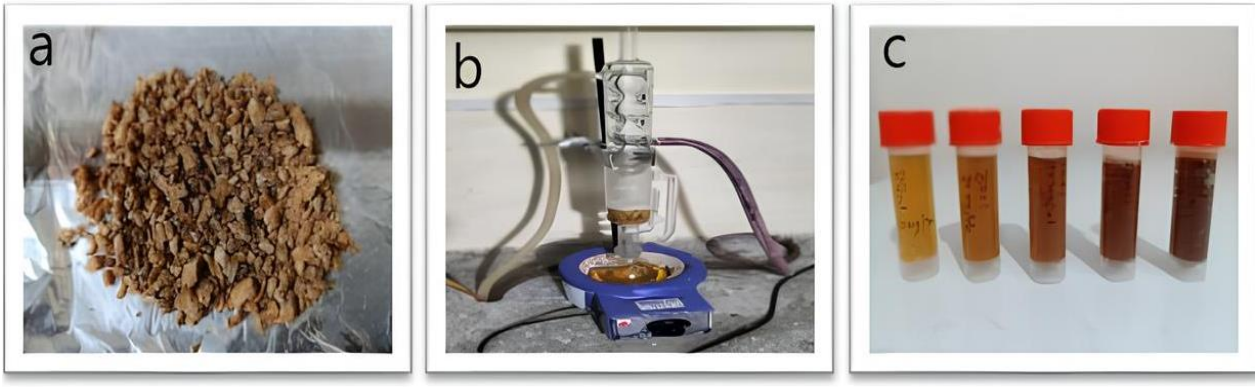


Fig. 5: (a) Grounded *Cordyceps militaris* dried fruiting bodies (b) Extract preparation using soxhlet extraction method (c) Extracts of *Cordyceps militaris* cultivated on different substrates

Evaluation of antioxidant potential: In our study, the extracts of *C. militaris* grown on various substrates (FTM01, CN01, BR01, PM01, WT01) are ethanolic (E), water-based (W) and methanolic (M). Fruiting bodies cultivated on foxtail millet substrate showed significant RAS% (91%) in ethanolic extract, (58.82%) in aqueous extract and (72%) activity in methanolic extract. IC50 value for the antioxidant activity was 0.324mg/ml. The other fruiting bodies cultivated on remaining substrates showed less activity than foxtail millets. All the experiments were done in triplicate.

Previous studies have highlighted the impact of using different grain substrates on the morphological characteristics of *C. militaris*. These investigations revealed significant variations in traits such as growth rate, fruiting body size and yield. Such findings offer valuable insights into the morphological diversity of Cordyceps militaris, emphasizing how substrate selection can influence the development and quality of the fungi. This knowledge contributes to optimizing cultivation practices by selecting appropriate substrates to enhance the morphological and bioactive properties of the species[23]. Various cultivation methods including solid-state fermentation (SSF) and liquid-state fermentation (LSF), have been reported in the literature.

SSF was selected for this study due to its cost-effectiveness, simplicity and compatibility with readily available agricultural substrates like brown rice, foxtail millet and wheat. These substrates mimic the natural growing conditions of C. *militaris* and promote efficient fruiting body production. SSF was preferred for its higher bioactive compound yield compared to LSF, as supported by previous studies. Substrate selection was based on accessibility, nutrient profiles and regional relevance, with brown rice prioritized for its high yield and foxtail millet for its antioxidant potential.

While alternative methods using agricultural waste or synthetic media show promise, our focus was on scalable, cost-effective strategies suitable for local growers and nutraceutical applications. Liang and co-workers[21] in their studies on *Cordyceps militaris* cultivation revealed that combination of pearl barley with yeast extract substrate was optimal for mycelial growth, yielding high fruiting body production. Combination of brown rice with peptone substrates showed superior yield and efficiency. Variable bioactive compound contents highlight substrate-specific preferences for cordycepin, mannitol and adenosine in different strains[21].

In a different research, Lin and colleagues[22] evaluated the viability of using agricultural wastes as substitute materials for the economically viable culture of *C. militaris*. These waste materials include corn cob particles, cottonseed shells, Italian poplar sawdust and substrates wasted by *Flammulina velutipes*. Rice was used as the control media[22]. However, in our study, the maximum yield of fruiting body was observed with brown rice substrate. Antibacterial properties of an ethanol extract made from *C. militaris* fruiting bodies were evaluated using agar well diffusion method. In a study, the ethanolic extract exhibited sensitive action against *Staphylococcus aureus* (ZOI = 30.33 ± 2.08 mm) at 100 mg/mL concentration and moderate activity against *Salmonella spp.* (ZOI = 16.33 ± 1.52 mm), *Escherichia coli* (ZOI = 9.33 ± 1.15 mm) and *Aspergillus niger* (ZOI = 7.83 ± 0.76 mm)[26].

Using the agar well diffusion method, the study demonstrated that the ethanol extract of *Cordyceps militaris* exhibited significant antibacterial activity. This suggests that the extract holds promise as a potential ingredient for developing herbal supplements and pharmaceutical products, owing to its strong antimicrobial properties. An additional investigation determines the antioxidant activity while assessing various drying techniques for *C. militaris*. In our study, the maximum antimicrobial activity was observed against *Staphylococcus aureus* (ZOI = 25.76 ± 0.70 mm) at 50 mg/mL concentration. The dried *C. militaris* extracts may possess strong antioxidant activity[20]. Antioxidant properties of *C. militaris* extracts showed a moderate inhibition on the DPPH assay which was 54.3% (IC50 = 2.95 mg/ml)[4].

Another research revealed that the polysaccharide-iron (II) that was isolated from *C. militaris*, has notable ability to scavenge radicals, namely superoxide, hydroxyl and DPPH

radicals. The polysaccharide-iron (II) at 2.5 mg/mL had a 74.02% DPPH clearance rate; however, it had no discernible effect on the clearance of superoxide and hydroxyl radicals[6]. Our study showed RAS% (91%) in ethanolic extract, (58.82%) in aqueous extract and (72%) activity in methanolic extract. The medicinal mushroom *C. militaris* is gaining popularity because of its many health advantages, which are mainly linked to cordycepin, a bioactive metabolite of the medicinal mushroom. This study assessed the morphological, molecular, antioxidant and antibacterial capabilities of *C. militaris* and investigated its culture on a variety of substrates to maximise cost-effectiveness.

The study also showed how crucial substrate selection is to maximise *C. militaris* bioactive component concentrations and medicinal effects. All these findings, broaden our knowledge about low cost cultivation of *C. militaris* and its possible uses in the nutraceutical and pharmaceutical sectors. In order to fully realise its medicinal potential and increased bioactivity, more investigation and optimisation are required.

This study has some limitations including a lack of comprehensive investigation into the genetic pathways and mechanisms responsible for the production of bioactive compounds in *C. militaris*. Additionally, the potential of *C. militaris* extracts as natural antimicrobial agents was not fully explored, warranting further research in these areas.

Conclusion

In conclusion, this study provides a comprehensive morphological, genetic and biochemical characterization of *C. militaris* cultivated on various grain substrates. Among the tested substrates, brown rice proved to be the most favourable for producing larger and heavier fruiting bodies while foxtail millet yielded the least in terms of wet and dry weight. The phylogenetic analysis further confirmed the genetic alignment of the strain used in this study with other closely related *Cordyceps* species. The antimicrobial and antioxidant evaluations revealed that the ethanol extracts of *C. militaris*, particularly those cultivated on foxtail millet and brown rice, exhibited potent antibacterial and antioxidant activities.

These findings underscore the influence of cultivation substrate on the morphological traits and bioactive potential of *C. militaris*, suggesting that substrate optimization could enhance its therapeutic applications. Further research is recommended to explore the underlying mechanisms that contribute to these variations and to expand the potential uses of *C. militaris* in various biotechnological and pharmaceutical fields. Future research could explore the use of these extracts as alternatives to synthetic antibiotics. This holds promise for developing new natural antimicrobial to combat drug-resistant bacterial strains.

The strong antioxidant properties of C. *militaris* could pave the way for its use in the development of dietary supplements targeting oxidative stress-related conditions such as cardiovascular disease and neurodegenerative disorder. Further molecular studies could search deeper into the genetic pathways responsible for bioactive compound synthesis in *C. militaris.* Understanding these pathways could enable the enhancement of cordycepin and other metabolites through genetic engineering and optimization of growth conditions.

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