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Comparative Analysis of the Antioxidant and Antibacterial Activity of Methanolic Mycelium Extract from *Cordyceps militaris*: A Comprehensive Study

Niketan Deshmukh[1*] and Lakshmi Bhaskaran[2]

[1]LJ School of Applied Sciences, LJ University, Ahmedabad (Gujarat), India.
[2]Department of Biotechnology, Kadi Sarva Vishwavidyalaya, Gandhinagar (Gujarat), India.

(Corresponding author: Niketan Deshmukh)*
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ABSTRACT: Many synthetic molecules with antioxidant and antimicrobial potential have been designed and manufactured over the last several decades. However, the potential toxicity associated with these synthetic compounds remains a major concern. Some compounds that initially exhibit promising antioxidant or antimicrobial properties in vitro or animal studies may prove to be toxic or lead to unfavorable side effects when subjected to human trials. Additionally, the issue of antimicrobial resistance is a significant hurdle in developing effective synthetic antimicrobial agents. Over time, microorganisms can develop resistance mechanisms against synthetic compounds, rendering them less effective or completely ineffective. Due to such toxicity and resistance, it did not result in a beneficial therapeutic outcome. One alternate approach to this is the use of herbal remedies. *Cordyceps* was used as an ancient remedy to treat several diseases in the eastern part of Asia. This study examined the antioxidant and antimicrobial properties of the methanolic mycelium extract of *Cordyceps militaris*. The findings revealed that the extract of the mycelium has DPPH scavenging activity with an EC_{50} value of 2.33 mg/ml. The antimicrobial results also showed that the methanolic extract has broad-spectrum antimicrobial activity. It is most effective against *Escherichia coli* (15.96 ± 0.68) as well as *Pseudomonas aeruginosa* (14.08 ± 0.44). These findings suggested that the methanolic mycelium extract of *Cordyceps militaris* could serve as a valuable antioxidant and antimicrobial natural supplement, which would eventually reduce the dependency on synthetic molecules.

Keywords: *Cordyceps militaris,* Antioxidant, Antibacterial, MIC.

INTRODUCTION

Synthetic antibacterial and antioxidant drugs have been extensively used for the treatment of various infectious and inflammatory diseases. However, the use of these drugs can have serious adverse effects on human health. One common side effect is gastrointestinal disturbances. These drugs can disrupt the natural balance of bacteria in the gastrointestinal tract, leading to temporary symptoms such as diarrhea, nausea, vomiting, and abdominal pain (Ramirez *et al.*, 2020; Rochegüe *et al.*, 2021). Another potential side effect of synthetic antibacterial drugs is the development of allergic reactions. Some individuals may experience allergic responses to these medications, characterized by symptoms such as rashes, itching, hives, swelling, and in severe cases, difficulty breathing or anaphylaxis (Han *et al.*, 2022).The prolonged or inappropriate use of synthetic antibacterial drugs can also contribute to the development of antibiotic resistance (Frieri *et al.*, 2017). This phenomenon happens when bacteria mutate or acquire genetic changes that make them less susceptible to the effects of the drug. Consequently, the antibiotic becomes less effective or even ineffective in treating future infections. Synthetic drugs can also disrupt the natural microbial balance in the body, potentially leading to superinfections (Sencio *et al.*, 2021). This occurs when opportunistic pathogens, such as yeast or *Clostridium difficile* bacteria, overgrow in response to the disruption caused by the medication. Superinfections may present themselves as yeast infections, such as thrush or vaginal yeast infections, or as antibiotic-associated diarrhea caused by *Clostridium difficile* bacteria. Furthermore, some synthetic drugs, particularly those with potent or broad-spectrum activity, carry the risk of organ toxicity. In rare cases, certain antibiotics can cause damage to specific organs like the liver or kidneys, especially when used in high doses or for extended periods (Jalan *et al.*, 2012). They can also interfere with the absorption of essential nutrients, leading to nutritional deficiencies. Therefore, it is essential to use synthetic antibacterial and antioxidant drugs carefully and only when necessary. It is also crucial to monitor patients for adverse effects and adjust dosage and treatment duration accordingly. In addition, exploring natural remedies with antibacterial and antioxidant properties may provide a safer and healthier alternative to synthetic drugs.

Herbal remedies have been used for centuries to treat various ailments and diseases. Many natural compounds found in mushrooms and herbs have been shown to have medicinal properties, including antibacterial and antioxidant effects (Gyawali & Ibrahim, 2014; Mau *et al.*, 2002). In recent years, there has been a growing interest in the use of herbal remedies as an alternative to synthetic drugs. Some of the commonly used mushrooms and plants as remedies are listed in Table 1.

Table 1: Commonly used Mushrooms and plants as remedies.

Mushrooms			
Common name	**Scientific name**	**Use**	**Reference**
Reishi	*Ganoderma lucidum*	Fatigue, insomnia, and various respiratory conditions.	(Wasser, 2004)
Shiitake	*Lentinula edodes*	High cholesterol, liver disease, and infections.	(Soroko-Dubrovina *et al.*, 2022)
Turkey Tail	*Trametes versicolor*	Cancer	(Benson *et al*., 2019)
Cordyceps	*Cordyceps militaris*	Respiratory conditions and kidney disease, Boost the immune system	(Paterson, 2008)
Lion's Mane	*Hericium erinaceus*	Cognitive function and treat nerve damage	(Mori *et al*., 2008)
Maitake	*Grifola frondosa*	Immune boosting	(Nishihira *et al*., 2017)
Chaga	*Inonotus obliquus*	Immune function, reduce inflammation	(Kim *et al.*, 2005; Mishra *et al*., 2012)
Tremella	*Tremella fuciformis*	Skin health related conditions	(Shen *et al*., 2017)
Porcini	*Boletus edulis*	Oxidative damage	(Vidović *et al*., 2010)
Oyster	*Pleurotus ostreatus*	Immune support and cardiovascular health.	(Alam *et al.*, 2011)
Plants			
Echinacea	*Echinacea purpurea*	Boost the immune system	(Woelkart *et al.*, 2006)
Ginger	*Zingiber officinale*	Nausea, vomiting, and indigestion	(Shahrajabian *et al.*, 2019)
Turmeric	*Curcuma longa*	Inflammation conditions	(Jurenka, 2009)
Milk thistle	*Silybum marianum*	Liver problems	(Flora *et al.*, 1998)
St. John's Wort	*Hypericum perforatum*	Depression and Anxiety	(Husain *et al.*, 2011)
Garlic	*Allium sativum*	Cardiovascular health and immune support	(Londhe *et al*., 2011)
Peppermint	*Mentha × piperita*	Digestive disorders	(Shah & D'Mello, 2004)
Valerian	*Valeriana officinalis*	Sleep disorders and anxiety	(Shinjyo *et al.*, 2020)
Chamomile	*Matricaria chamomilla*	Relaxation and stress relief	(Saki, 2018)
Saw Palmetto	*Serenoa repens*	Benign prostatic hyperplasia (BPH) symptoms	(Cicero *et al.*, 2019)

Cordyceps is a genus of ascomycete fungi that has been used in traditional Chinese medicine for centuries (Dong *et al.*, 2015). It is known for its numerous health benefits, particularly its immunomodulatory (Liu *et al.*, 2022), anti-inflammatory (Park *et al.*, 2015), and anti-tumor properties (Ng & Wang 2005). The most popular species of *Cordyceps* are *Cordyceps sinensis* and *Cordyceps militaris*, which are found in the high-altitude regions of the Himalayas. It has been used for centuries to treat a wide range of conditions, including fatigue, respiratory infections, kidney and liver disease, and even sexual dysfunction (Panicker, 2017).

Cordyceps militaris is closely related to *Cordyceps sinensis*. Both species have similar medicinal properties, there are several advantages of using *Cordyceps militaris* over *Cordyceps sinensis*. One advantage is that *Cordyceps militaris* can be grown in a controlled environment, which ensures consistent quality and purity of the product (Lu *et al.*, 2020). In contrast, *Cordyceps sinensis* is typically harvested from the wild, which makes it difficult to control contaminants and variations in potency. Another advantage of *Cordyceps militaris* is that it has a higher yield of bioactive compounds compared to *Cordyceps* sinensis. However, Cultivation of *Cordyceps* fruiting bodies is a time-consuming process that can take several months, which makes it unfeasible for large-scale production. However, an efficient alternative is the submerged fermentation of *Cordyceps* mycelial biomass. This method enables the production of a higher quantity of mycelial biomass in a shorter time and within a smaller space, while also reducing the risk of contamination. In this direction the present study investigates the antioxidant and antimicrobial properties of the methanolic mycelium extract of *Cordyceps militaris.*

MATERIAL AND METHODS

Sample Preparation. *Cordyceps militaris* was grown in a 250ml Erlenmeyer flask containing 100ml of basal media (peptone 0.5%, glucose 1.5%, K_2HPO_4 0.1%, KH_2PO_4 0.3%, NaCl 0.05%, $MgSO_4$ 0.05%). The flasks were incubated for 10 days in a static condition at 20°C. After incubation, Whatman #4 filter paper was used to filter out the culture media containing mycelium. The collected mycelia were washed thrice with autoclaved double distilled water and oven dried at 45°C until a constant dry weight was obtained.

Preparation of extracts. Dried samples of the mycelia (5g) were separately extracted using 100 ml of methanol using Soxhlet apparatus. The extract was then dried at 45°C and re-dissolved in methanol to achieve a

concentration of 100 mg/mL. This prepared extract was subsequently utilized to assess antioxidant and antibacterial properties.

Scavenging assay. The antioxidant potential of the methanolic extract was determined using the 2,2-diphenyl-picrylhydrazyl (DPPH) assay. The extract's ability to scavenge free radicals was assessed by observing the decolorization of the methanolic solution of DPPH reagent. The absorbance of the solution was measured using spectrophotometer at 517 nm. Vitamin C (Vc) used as a positive control. The percentage of scavenging activity was calculated using the following equation:

% Scavenging activity = $\{(A_0-A_1)/A_0\} \times 100$

Where A_0 is the absorbance of the DPPH solution and A_1 is the absorbance of the sample.

Antimicrobial assay. The methanolic extract was subjected to antimicrobial activity testing against *Enterobacter aerogenes, Bacillus megaterium, Bacillus subtilis, Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus* obtained from L J University, using well diffusion method. The test microorganism was cultured overnight until it reached a concentration of 10^8 CFU/mL. Following that, 100 µL of the microbial suspension was spread evenly over the surface of MHA plates. Wells of 6 mm are punched aseptically by using sterile cork-borer.100 µL of the methanolic extract of concentration 1mg/ml are introduced into a well. The agar plates were refrigerated for a duration of 30 minutes to allow for optimal diffusion of the extracts into the agar. Subsequently, the plates were incubated for a period of 24 hours at a temperature of 37°C.The antimicrobial activity was assessed by measuring the diameter of the zone of inhibition. including the wells, that appeared after the incubation period. Streptomycin and sterile methanol are used as a standard control.

Preparation of resazurin solutions. In order to prepare the resazurin solution, strict aseptic procedures were implemented. Initially, 337.5 mg of resazurin powder was dissolved in 50 ml of sterile doubled distilled autoclaved water. To ensure a consistent mixture, the solution underwent vigorous mixing using a sterile vortex mixer for a duration of 1 hour. It is noteworthy that all steps of the preparation were meticulously conducted in a dark condition to minimize any potential impact from light. Subsequently, the Resazurin solution was carefully transferred to a ambered-colored bottle to avoid light exposure, as the solution is highly sensitive to light.

Determination of Minimum Inhibitory Concentration MIC. To evaluate the inhibitory effects of mycelium extracts, the resazurin-based turbidimetric (TB) assay was utilized. In a 96-well round-bottom microtiter plate, first (A) and last (H) rows are used for positive and sterility control respectively. The rest of the rows from B to G were utilized for MIC determination. In each vertical column, a volume of 100 µl of Mueller-Hinton broth (MHB) was added to all eight wells. The first well of each row is added with 100ul streptomycin antibiotic (positive control), mycelium extract (100mg/ mL) and MHA broth (sterility control). The contents of the first well of each row were mixed properly using sterile pipette and transfer 100 µl into second well followed by proper mixing. In the similar fashion dilution are made up to eighth well. Finally, 100 µl of the solution was discarded from the eighth well. Next, a volume of 5 µl of a diluted bacterial suspension containing 1.5×10^6 cells/ml was carefully added to each well (except broth sterility column), ensuring that all wells received an equal amount of the bacterial suspension. After overnight incubation of the microtiter plate at 37 °C add 5 µl of resazurin (6.75 mg/mL) to all the wells and again incubate the microtiter plate for 4 hours at 37 °C.

A review of cultivation strategies to ensure reproducibility, all experiments were conducted in triplicate. The data generated from all the experiments are analyzed using analysis of variance (ANOVA) to calculate their significant differences.

RESULTS AND DISCUSSION

Antioxidant potential of mycelium extract. The evaluation of the antioxidant activity of the methanolic mycelium extract of *Cordyceps militaris* against the DPPH radical was conducted. This assay is a commonly employed method for assessing the antioxidant potential of herbal extracts. The principle of this assay revolves around the reaction between bioactive molecules possessing antioxidant properties and the DPPH radical.

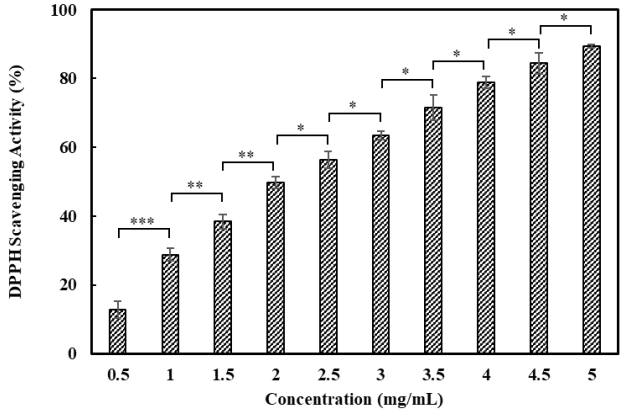

Fig. 1. Effect of mycelium extract on DPPH scavenging activity. Results are expressed as Mean ± SD (n=3). Significant difference calculated with ANOVA (ns = non-significant, * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$)

This reaction involves the transfer of either an electron or a hydrogen atom, resulting in the neutralization of the radical which can measured using spectrophotometer at 517 nm. The study demonstrated that the scavenging activity of the methanolic extract exhibited an increase with the rise in the concentration of the mycelium extract, ranging from 0.5 to 5 mg/mL.

The results obtained from the assay are visually represented in Fig. 1. The scavenging activity of mycelium extracts exhibited significant variation at different concentrations ($P<0.05$). The EC50 value of the methanolic extract was determined to be 2.33 mg/mL, indicating its antioxidant potential. However, it should be noted that the EC50 value of ascorbic acid (0.1 mg/mL) was lower than that of the mycelium extract, suggesting greater antioxidant potential of V_c in comparison. The scavenging activity of any extract is influenced by several factors, including the method of extraction, extraction conditions, nature of solvent, drying conditions and methods, as well as the species of the mushroom. "Previous research has highlighted the impact of different extraction conditions, such as temperature, nature of solvent, and extraction time on the antioxidant activity of extracts. Notably, the study revealed that 80% acetone, 40% methanol, and 40% ethanol were particularly effective in extracting polyphenolic compounds"(Wissam *et al.*, 2016). "Another study examined the impact of different methods of drying on the antioxidant activity of mycelium extracts. The study compared three different methods: sun drying, freeze drying, and microwave drying. The objective was to assess how each drying conditions, method affected the antioxidant potential of the extracts. The results of the study demonstrated that the drying method significantly influenced the total antioxidant potential of the mycelium extracts. Among the three drying techniques, microwave drying exhibited the highest antioxidant activity. This finding suggests that microwave drying preserved the antioxidant compounds present in the mycelium extracts more effectively compared to sun drying and freeze drying"(Piskov *et al*., 2020). The extraction method used significantly impacts the antioxidant activity of extracts. Numerous extraction methods are available, including Soxhlet extraction, Ultrasound-assisted extraction, accelerated solvent extraction, Microwave-assisted extraction, Supercritical fluid extraction, Enzyme-assisted extraction, and Pressurized hot water extraction. The selection of an appropriate extraction technique is essential as it directly influences the dependability and integrity of subsequent analytical processes. The primary objective of the extraction process is to ensure economic viability, sustainability, less time consuming, and improved yields of bioactive compounds, while also preserving their biological activities. These aspects play a crucial role in evaluating the overall effectiveness of the extraction technique and the subsequent antioxidant capacity of the acquired extracts. By optimizing these parameters, it becomes possible to obtain extracts that contain higher levels of bioactive compounds and exhibit stronger antioxidant properties (Bitwell *et al*., 2023)

Antimicrobial Activity and MIC of mycelium extract. The antimicrobial activity of the methanolic mycelium extract of *Cordyceps militaris* was evaluated using the agar well diffusion method. The findings of the study demonstrated the effective inhibition of test organisms by the methanolic extract of *Cordyceps militaris* mycelium. Table 1 provides specific information regarding the zone of inhibition observed.

The results are presented as means ± standard deviation (SD) for triplicate samples. Different letters assigned to means within the same row indicate statistically significant differences ($P < 0.05$).

The study demonstrated that the mycelium methanolic extract exhibited antimicrobial activity against both gram-positive and gram-negative microorganisms, although the effectiveness varied. The extract exhibited the largest zone of inhibition against *Escherichia coli*, with a diameter of 15.96 ± 0.68, while the smallest zone was observed against *Bacillus subtilis*, with a diameter of 11.02 ± 0.39. The results of the study suggest that the mycelium methanolic extract of *Cordyceps militaris* exhibits slightly higher efficacy against gram-negative bacteria when compared to gram-positive bacteria.

Table 1: Antimicrobial activity of extract.

Sr. No.	Test microorganism	Zone of Inhibition (mm)	
		Methanolic extracts (50 mg/mL)	Streptomycin (1 mg/mL)
1.	*Enterobacter aerogenes*	13.19 ± 0.62[a]	20.25 ± 0.17[b]
2.	*Pseudomonas aeruginosa*	14.08 ± 0.44[a]	18.98 ± 0.42[b]
3.	*Escherichia coli*	15.96 ± 0.68[a]	29.42 ± 0.56[c]
4.	*Bacillus subtilis*	11.02 ± 0.39[a]	26.52 ± 0.39[c]
5.	*Bacillus megaterium*	12.18 ± 0.55[a]	25.72 ± 0.33[c]
6.	*Staphylococcus aureus*	12.06 ± 0.35[a]	28.53 ± 0.49[c]

Table 2: Minimum inhibitory concentration of the extract.

Sr. No.	Test microorganism	MIC (mg/mL)
1.	*Enterobacter aerogenes*	12.50[a]
2.	*Pseudomonas aeruginosa*	12.50[a]
3.	*Escherichia coli*	6.25[b]
4.	*Bacillus subtilis*	25.00[c]
5.	*Bacillus megaterium*	25.00[c]
6.	*Staphylococcus aureus*	12.50[a]

Statistical significance ($P < 0.05$) was determined by comparing the means across the same column using different letters.

The resazurin-based turbidimetric (TB) assay was employed to determine the minimum inhibitory concentration (MIC). The study noted that the wells representing the sterility control remained blue in color after incubation. However, the color of the wells containing the antibiotic changed from blue to pink.

A study observed *Escherichia coli* exhibited the lowest MIC value of 6.25 mg/mL. The significantly lower MIC value suggests that *Escherichia coli* is highly susceptible to the extract compared to the other microorganisms. *Pseudomonas aeruginosa*, *Enterobacter aerogenes*, and *Staphylococcus aureus* have the same MIC value of 12.50 mg/mL. Although they displayed higher MIC values than *Escherichia coli*, they still exhibited relatively good susceptibility to the tested substance. *Bacillus subtilis* and *Bacillus megaterium*, both microorganisms had the highest MIC values of 25.00 mg/mL. Similar MIC values suggest that they may have comparable levels of resistance to the extract compared to the other microorganisms.

In a previous study, the antimicrobial activity of different fractions of *Cordyceps militaris 3936* was investigated. These fractions, obtained using hexane, chloroform, n-butanol, and water as extraction solvents, were tested against pathogenic microorganisms including *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Aspergillus niger*, and *Trichophyton rubrum* (Tuli *et al.,* 2014). Another study revealed that certain species of *Cordyceps* produce an antibacterial protein in their mycelia. This protein possesses a unique hydrophobic N-terminal sequence, namely N-ALATQHGAP. Additionally, the research demonstrated that this protein displayed inhibitory effects on the growth of both Gram-positive and Gram-negative bacteria. The MIC observed are *Bacillus subtilis* (>100 g/l), *Escherichia coli* (75-100 g/l), *Proteus vulgaris* (75 g/l), *Staphylococcus aureus* (75 g/l) and for *Salmonella typhi* (50 g/l). However, it did not show significant inhibitory effects against fungi or yeasts (Hu *et al*., 2006). Another investigation reported the antimicrobial activity of the novel silver nano particles synthesized with the help of *Cordyceps militaris* mycelium. The study specifically used four typical aquatic pathogens including *Vibrio anguillarum*, *Vibrio alginolyticus*, *Aeromonas punctata* and *Vibrio parahaemolyticus.* Study also performed antimicrobial test on gram-negative *Pseudomonas aeruginosa* and *Escherichia coli*, and gram-positive *Bacillus subtilis* and *Staphylococcus aureus* and report significant results (Wang *et al.,* 2016).

CONCLUSIONS

In conclusion, the results of this study demonstrate the antioxidant potential and antimicrobial activity of the methanolic mycelium extract of *Cordyceps militaris*. The antioxidant activity was evaluated using the DPPH scavenging assay, and the extract exhibited concentration-dependent scavenging activity. The EC50 value of the extract was determined to be 2.33 mg/mL, indicating its antioxidant potential. The antimicrobial activity of the extract was assessed using the agar well diffusion method. The extract exhibited antimicrobial activity against both gram-positive and gram-negative microorganisms, although the effectiveness varied. *Escherichia coli* showed the highest susceptibility to the extract, while *Bacillus subtilis* exhibited the lowest susceptibility. The minimum inhibitory concentration (MIC) values further confirmed the antimicrobial activity of the extract, with *Enterobacter aerogenes* showing the lowest MIC value. The findings of this study highlight the potential applications of *Cordyceps militaris* as a natural antioxidant and antimicrobial agent.

FUTURE SCOPE

The mycelium extract of *Cordyceps militaris* could be further explored and developed as a functional ingredient in the food, pharmaceutical, and cosmetic industries.

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Conflict of Interest. None.

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