Original Research Article

Biochemical analysis of fruiting bodies of *Cordyceps militaris* grown on brown rice

Abstract: *Cordyceps militaris* is a medicinal fungus known for its diverse biofunctional properties. This subject has been extensively analyzed over the past decade. The diverse pharmacological potential of *C. militaris* has garnered significant global interest recently. This research aims to determine the proximate composition of in vitro cultivated *C. militaris* fruit bodies. The analysis of proximate composition included measurements of crude protein, fat, crude fiber, ash, total carbohydrate, and moisture content. The research focused on quantifying the overall nutritional value of the fruit bodies and evaluating biological efficiency, in addition to key bioactive compounds including cordycepin, ergosterol, and Vitamin D2. The results demonstrated that the carbohydrate content constituted 47.67% of dry weight, the protein content was 30.41%, and the fiber content accounted for 15%. The fruit bodies contained 39.82 mg/kg of cordycepin and 61.73 mg/100g of ergosterol, whereas vitamin D2 was absent in the cordyceps mushroom sample. The findings demonstrate that in-vitro-cultivated *C. militaris* fruit bodies exhibit considerable nutritional and therapeutic potential.

Keywords: *Cordyceps militaris*, bioactive compounds, cordycepin, ergosterol, vitamin D2.

Introduction: The genus Cordyceps, belonging to the class Ascomycota, is a species of fungus that attacks insect larvae. The current classification of this fungus consists of three different families: Cordycipitaceae, Ophiocordycipitaceae, and Clavicipitaceae[1]. The genus Cordyceps is known to contain about 680 different species. However, *Cordyceps militaris* and *Ophiocordyceps sinensis* are particularly common in traditional and oriental medicine in India, China, Korea, Japan, and other Asian countries [2,3].*Cordyceps militaris* was approved asthe first novel food of the Cordyceps species byChina's Ministry of Public Health in 2009. Recent advances

have shown that the nutrients and bioactive substances present in the fruiting body of *Cordyceps militaris* are comparable to those of the traditional Chinese tonic *Ophiocordyceps sinensis*[4].The edible fungi of the genus Cordyceps have long been prized for their nutritional value and biological substances with potential medical applications. The primary biologically active components are cordycepin (3-deoxyadenosine) and adenosine, which are nucleoside analogs[5].This mushroom exhibits notable properties including immunological modulation, antioxidant activity, anticancer efficacy, anti-inflammatory action, antidiabetic effects, antihyperlipidemic effects, antithrombotic properties, antiviral activities, antibacterial effects, pest-repellent qualities, and skin whitening effects[6,7,8]. Mushrooms are edible and commonly used in soups, salads, and as an accompaniment to dishes. The nutritional makeup of dried mushrooms is contingent upon the particular sort of organic matter utilized in the manufacturing process [9].*Cordyceps militaris* is the species of Cordyceps that has been most successfully grown and thoroughly studied out of all the others. The fruiting bodies of cultivated *C. militaris* are used to create the majority of Cordyceps products in the market. A chemical analysis indicates that Cordycepin, Adenosine, Ergosterol, Mannitol, Trehalose, Polysaccharide are all present in *C. militaris*[10,11].Cordycepin, also known as 3'-deoxyadenosine ($C_{10}H_{13}N_5O_3$) is a nucleoside analogue of adenosine ($C_{10}H_{13}N_5O_4$) and a secondary metabolite produced by the *Cordyceps militaris*. Among the various bioactive compounds, Ergosterol stands out as a key sterol within the cell membrane, where it is firmly associated with fungi. It has the capability to activate the expression of several defense genes, thereby enhancing plant resistance to pathogens [12]. Cordyceps mushrooms serve as a valuable source of pro-vitamin D, specifically in the form of ergosterol. Insufficient vitamin D levels can result in conditions such as rickets and osteomalacia, stemming from disturbances in calcium and phosphate metabolism. Incorporating mushrooms into one's diet, especially those subjected to ultraviolet light, may aid in preventing vitamin D deficiency, given their status as a natural source of vitamin D2. Nonetheless, although vitamin D2 derived from mushrooms can assist in preventing deficiency, vitamin D3 is generally more efficient in increasing blood levels of vitamin D.The Collection of natural Cordyceps fruiting bodies is sporadic and expensive, too. Furthermore, over-collection is causing the natural populations of important Cordyceps species to decline quickly, necessitating a rise in *in-vitro* cultivation of Cordyceps on artificial media. Out of all

the identified species of Cordyceps, a very small percentage have been successfully cultivated in artificial media [13].The current investigation focused on analyzing the nutritional profile of *in-vitro*-cultivated *Cordyceps militaris* through proximate analysis and the assessment of bioactive compounds such as Cordycepin, ergosterol, and vitamin D2.

1. Materials and Methods:

Pure culture: Pure culture of *Cordyceps militaris* was procured from JAM Bio Foods, Bahadurgarh, Haryana andmaintained on PDA (potato dextrose agar) slantsaccording to the procedure of Lin et al. (2017)[14].

2. Chemicals:

All chemicals and reagents utilized were of analytical grade and obtained from Sigma Aldrich Chemicals Co.

3. In Vitro cultivation of *C. militaris:*

3.1.1. Liquid spawnpreparation:12gm of Potato Dextrose Broth (PDB), 2.5gm yeast extract, 2.5gm peptone, 0.25gm $MgSO_4$, and 500ml water were taken into 1000ml beaker autoclaved for 40 minutes at 121°C and 15Psi. After autoclaving, the color of the media will be light brownish. Inoculate 1cm piece from pure culture slants and put the inoculated flasks on a rotary shaker for 5-7 days under lab conditions. After 5-7 days small fibres can be seen and the color will change to brownish yellow**(Picture).**

3.1.2. Substrate preparation: 20gm of brown rice was supplemented in each different bottle with 40 ml of nutritional broth (12g PDB, 2.5gm Peptone, 1.5gm Yeast Extract, 12.5mg Vitamin B1, 5mg Vitamin B12, 0.5gm Tri Ammonium Citrate, 0.5gm KH_2PO_4, 0.25gm MgSO4 and 500 ml distilled water), autoclaved for 40 minutes at 121°C and 15Psi [3].

3.1.3. Inoculation of liquid spawn into bottles: The substrate was inoculated with 7 ml liquid spawn of *C. militaris* and the bottles were closed using a HEPA filter in their caps**(Fig.1a**). The bottles were incubated at 21ºC for the spawn run, with humidity of 60–70% under dark conditions for tendays. After 10 days, white mycelium can be seen on the substrate**(Fig.1b).** After the completion of the dark period, the bottles were exposed to light conditions for color change, fruiting body formation, and development at 22ºC and humidity 85–90%**(Fig.1c).**It needs to provide 12 hours of light (700-1000 lux) and 12 hours of dark till harvesting. In light

conditions, the white color mycelium changed its color to yellowish-orange**(Fig.1d).** Small pinheads appeared in around the next 10 days **(Fig.1e).**The mycelium started to grow like matchsticks soon**(Fig.1f).**After 50-55 days, the mycelium was converted to fully grown *Cordyceps militaris* and ready to harvest**(Fig.1g).**The biological efficiency (B.E.%) was calculated using the formula given by Do et al., 2019 [15].

$$\text{B.E. (\%)} = \frac{\text{Fresh weight of mushroom (gm)}}{\text{dry weight of substrate (gm)}} \times 100$$

The freshly harvested cordyceps mushrooms were analyzed for physical properties, moisture ash, crude fat, crude fiber, protein,and carbohydrate contents.

4. Determination of moisture: The moisture content % reflects the proportion of water present after this period. An aluminum dish containing a 5g sample of fresh mushrooms was used to dehydrate it in an air oven at 45ºC for 24 hours, or until a consistent weight was reached. The subsequent formula was employed to determine the moisture percentage (AOAC, 2003)[16].

$$\text{Moisture(\%)} = \frac{[\text{weight of original sample (gm)} - \text{weight of oven-dried sample (gm)}]}{\text{weight of original sample (gm)}} \times 100$$

5. Estimation of ash: A 3gm sample of dried mushroom powder was placed in a crucible and heated on an oxidizing flame until the smoke cleared. The crucible underwent a controlled heating process within a muffle furnace set at 550 °C for a duration of 6 hours. The sample underwent cooling in a desiccator prior to being weighed. The method employed for determining the ash content in the sample was as follows [17].

$$\text{Ash (\%)} = \frac{\text{Weight of the sample (g)}}{\text{Weight of ash in sample (g)}} \times 100$$

6. Estimation of crude fat (ether extract):

5gm of desiccated mushroom samples were placed in a thimble and injected into the extraction tube of a Soxhlet apparatus for crude fat analysis. The heater's

temperature was adjusted to permit a constant flow of ether droplets onto the sample in the extraction tube. The extraction process employed petroleum ether (B.P. 40-60 ºC) over a duration of sixteen hours. The sample was subjected to extraction, allowing the solvent to evaporate in the fume hood. The extract was subjected to rigorous dehydration in an air oven for 30 minutes at a temperature of 105 ºC. The extract's weight was documented following its chilling in a desiccator (AOAC, 2012). Crude fat was quantified utilizing the following formula.

$$\text{Crude fat (\%)} = \frac{\text{Weight of sample (g)} \times 100}{\text{Weight of fat in sample (gm)}}$$

7. Estimation of Crude fiber: The determination of crude fiber in the samples was conducted using dilute acid and alkali hydrolysis as outlined by AOAC (2012) [18]and calculated as follows:

$$\text{Crude fiber (\%)} = \frac{[\text{Weight of crucible with dry residue} - \text{Weight of crucible with ash}] \times 100}{\text{Weight of the sample}}$$

8. Protein Estimation: Total protein was determined by using Lowry method [19].

9. Carbohydrate estimation: Totalcarbohydrates were calculated using the formula:

Total Carbohydrates (%) =100 – (moisture (%) + protein (%) + fat (%) + crude fibre (%) + ash (%))

10. Bioactive compounds: The primary bioactive components, cordycepin, ergosterol, and vitamin D2, were identified using high-performance liquid chromatography (HPLC) [20].

HPLC Analysis: Non-alkaline (methanol) protocol for ergosterol and vitamin D2extraction:Methanol was utilized to extract ergosterol according to a modified protocol established by Millie-Lindblom et al.[21]. One gram of cordyceps mushroom powder was placed into a 50 mililitre centrifuge tube. 6 mililitre of HPLC-grade

methanol was added to the centrifuge tube, followed by sonication for thirty minutes in an ultrasonic water bath. Following that, they underwent incubation at 80°C for a duration of 30 minutes. Upon reaching room temperature, 1 mililitre of Milli-Q H_2O was added, and the samples were whirlpooled at the vortexerat maximum velocity for one minute. The sample got spun in the centrifuge at 10,000 rpm for a solid 10 minutes.After the supernatant was carefully moved to a different tube, it was kept in a water bath at a steady temperature of 40°C until all of the methanol had evaporated. Each tube received 1 milliliter of methanol of HPLC quality. Following a 15-minute incubation at 40°C, the mixture underwent filtration using 0.2 µm-pore nylon membrane syringe filters, after which it was transferred into amber glass HPLC vials for subsequent analysis.To achieve a more accurate comparison of the extractions, standards of ergosterol and vitamin D2 were employed for their evaluative assessment.

10.1. Ultrasonic extraction method for cordycepin extraction:250 milligrams of cordyceps mushroom powder was measured into a 10 ml volumetric flask to quantify Cordycepin via HPLC. Add 10 ml of 20% ethanol and proceed with extraction for 2 hours utilizing an ultrasonic water bath. The sample underwent centrifugation at 10,000 rpm for ten minutes, after which the supernatant was transferred to a separate tube and filtered using a 0.45-µm syringe filter for injection. To enable a more accurate comparison of the extractions, standards for ergosterol and Vitamin D2 were assessed [22].

10.2. HPLC conditions: Ergosterol, cordycepin, and Vitamin D2 were quantitatively analyzed using a reversed-phase HPLC system according to the Millie-Lindblommethod [21] with some minor modifications. An analytical column C18 (250 x 4.6mm, 5 um pore size) was used for the chromatographic separation. Water: methanol (HPLC grade) in an 85:15 ratio was used as the mobile phase for ergosterol and vitamin D2 quantification and cordycepin quantification and for vitamin d2 quantification 50% methanol (HPLC grade):50% ACN (Acetonitrile) was used in a gradient elution system analysis. The column's temperature was kept at 25ºC, and 1.0 mL/min flow rate was established. The detector wavelength was set to 260 nm, and the injection volume was 20 µL.

11. Results:

11.1. In vitro cultivation of *C. militaris*: In order to cultivate *Cordyceps militaris*, 20g of wheat and 40ml of nutrient broth were combined in bottles. Liquid spawn

was added, and the mixture was incubated at 21°C with 85–90% relative humidity in the dark until the spawn run was completed. The flasks were exposed to light for fruit body initiation after 15 days, with a temperature of 21°C and a relative humidity of 85–90%. After 20 to 25 days, primordial dia begin to form, and in 50 to 60 days, they have fully matured into vertical stromata.

11.2. Morphological studies: The erect, frequently clavate-shaped fruiting body has a length of up to 10 cm and a diameter of 0–2 cm. Its fertile head is orange to bright orange, stuffed, and covered in tiny granules that give it a powdery appearance (Figure1*h*).

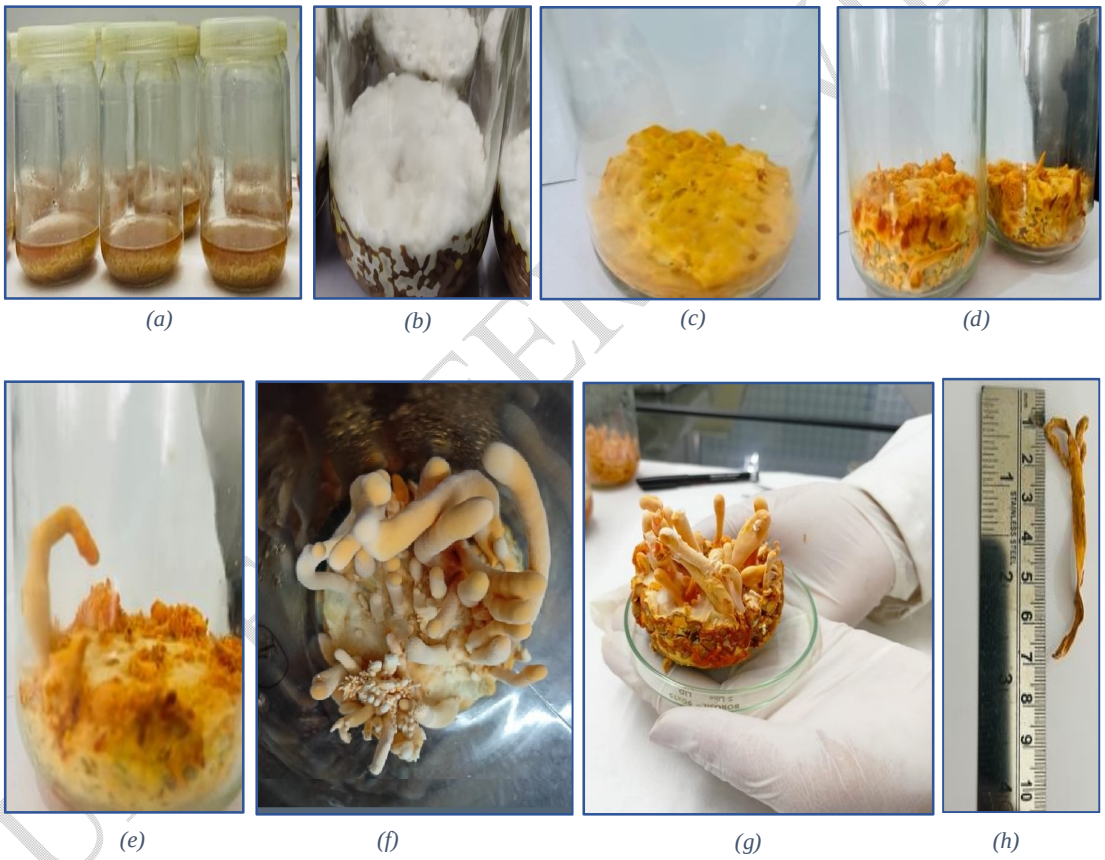

Fig .1(a-h)Inoculation of liquid spawn into bottles

11.3. Drying of fruiting bodies: Hot air drying was performed using a hot-wind circulation oven. Fresh samples of *Cordyceps militaris* (100 g) underwent drying at a temperature of 45 °C, utilizing an air velocity of 1.0 m/s. The moisture content of

Cordyceps militaris was found to be below 9.0% (wet basis). The overall duration for drying was approximately 14 hours.

11.4. Proximate composition of Cordyceps mushroom: The Association of Official Analytical Chemists (2012) methods were followed in determining the proximate composition of substrates and dried mushrooms. The content of moisture, ash, fat, crude fiber, crude protein, and total carbohydrate are listed in the table below:

S.No.	Proximate analysis	Unit	Value
1.	Moisture	%	85.27±1.89
2.	Ash	%DW	3.14±0.25
3.	Fat	%DW	3.38±0.65
4.	Crude fiber	%DW	15.3±0.57
5.	Crude protein	%DW	30.25±0.59
6.	Carbohydrate	%DW	48.05±0.82
7.	B.E.	%	82.33±2.51

Table 1: Proximate composition of dried cordyceps mushroom powder.

Table 2 liststhe amountsof cordycepin, ergosterol, and vitamin D2 in the dried powdered cordyceps mushrooms. The standard chromatogram (Figure 2) displays the retention times for the peak of cordycepin, ergosterol, and vitamin D2 at 8.683, 8.654, and 8.863, respectively. The cordycepin and ergosterol peaks in the sample chromatogram (Table 2) have good resolution, but vitamin D2 is absent. The results showed thatergosteroland cordycepin contents were 61.7355 mg/100gm and 39.821 mg/kg, respectively. It was found that cordyceps mushroom powder did not contain vitamin D2.

S.No.	Parameter	Retention time	values	unit
1	Cordycepin std.	8.683	1.0358	mg/kg
2	Ergosterol std.	8.654	1.0351	mg/100gm
3	Vitamin D2 std.	8.863	1.095	mg/100gm

4	Cordycepin	8.646	39.821	mg/kg
5	Ergosterol	8.695	61.735	mg/100gm
6	Vitamin D2	8.682	nil	mg/100gm

Table2: Amount of Cordycepin, ergosterol, and vitamin d2 in dried Cordyceps mushroom powder.

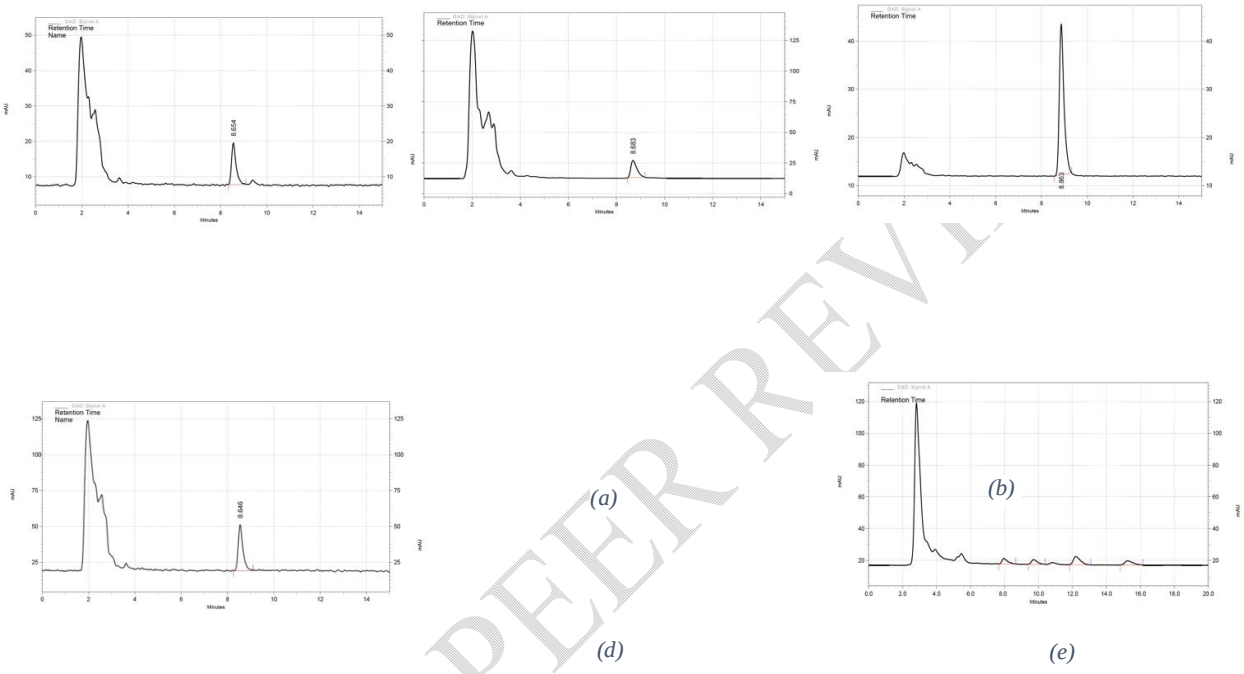

Fig .2 HPLC Chromatogramsof (a) Ergosterol std. (RT=8.654), (b) cordycepin std. (RT=8.683), (c)vitamin d2 std. (RT=8.863), (d) cordycepin sample (RT=8.646), (e) Ergosterol sample (RT=8.695), (f) Vitamin D2 sample.

Discussion: The proximity analysis of the fruit body confirmed that the primary components of *Cordyceps militaris* are fiber, protein, and carbohydrates. Srikram et al. [23] demonstrated in their study that mushrooms contain elevated levels of proteins, carbohydrates, and fiber. The shelf life of mushrooms is reduced due to their elevated moisture content. Mushrooms frequently deteriorate post-harvest if not preserved correctly [24]. The study indicates that the fruit body exhibited a moisture content of approximately 85.21%. The dried fruit body exhibited a carbohydrate content of 47.67%. The primary element in energy metabolism is carbohydrates. Over the past decade, there has been an increased interest in polysaccharides

derived from fungi due to their potential therapeutic benefits, which encompass immune stimulation, antioxidant activity, and antimicrobial properties [25,26,27]. The protein content was determined to be 30.41%, whereas a lower amount of 23.02% DW was reported in a study conducted by Pavleen et al.[28]. The contents of ash, crude fibre, and fat were determined to be 3.4%, 15%, and 3.52%, respectively. Fibre constitutes a significant component of *C. militaris* and is frequently regarded by dieticians and nutritionists as essential for sustaining a healthy diet.

Conclusion: The current study encompasses the proximate analysis of the *C. militaris* fruit body, along with the quantification of principal secondary metabolites, namely Cordycepin, and Ergosterol. It was found that there is absence of Vitamin D2 The findings indicated that *Cordyceps militaris* fruit bodies are abundant in carbs, protein, and fiber. A reduced fat level renders it an excellent choice for individuals seeking low-calorie options. The results indicated a substantial presence of cordycepin, and ergosterol in the Cordyceps fruit bodies, rendering it a beneficial option for healthy food with nutritional and medicinal potential.

Disclaimer (Artificial intelligence):

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc.) and text-to-image generators have been used during the writing or editing of this manuscript.

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