

Nutritional composition of *in-vitro* grown fruit bodies of *Cordyceps militaris* for health and wellness

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ABSTRACT

Cordyceps militaris an edible and medicinal mushroom has been extensively investigated in the past decade. This health food has now gained global attention due to its immense therapeutic and pharmacological benefits. The present study aims at determining the proximate composition of *in vitro* grown *C. militaris* fruit bodies. Proximate composition including crude protein, crude fat, crude fiber, total ash, total carbohydrate and moisture content was determined. Total energy value of the fruit bodies and biological efficiency was estimated during the study along with the principle bioactive compounds like cordycepin, adenosine and cordycepic acid. The results showed the carbohydrate content as 60.04±0.07% of dry weight, whereas protein 23.02±0.10% and fibre 11.06±0.04%. The fruit bodies showed 9.24±0.02 mg/g of cordycepin while 1.05±0.03 mg/g adenosine and 14.36 mg/g of cordycepic acid. The results of indicates that *in-vitro* grown *C. militaris* fruit bodies holds a good nutritional and therputic potential.

Keyword: *Cordyceps militaris*, cultivation, nutritional composition, proximate composition, cordycepin, adenosine, cordycepic acid

Mushrooms are being consumed from many centuries by human being. Other than being culinary delight, mushrooms possess several medicinal potential also. Carbohydrates, proteins and fats are the major constituent of human diet that gives energy for day-to-day activities. Mushrooms are considered as excellent source for proteins and are often consumed as an alternative to animal protein. It is reported that mushroom consist of around 16.5% dry matter, of which approximately 7.4% is crude fiber, 4.48% is fat and oil, and 14.6% crude protein. Mushrooms are also excellent source of minerals, vitamins and several essential amino acids (Adedayo, 2011).

Wild mushrooms are widely collected by mushroom gatherers to compensate the increasing

demand of mushroom. However, the availability of wild form of mushrooms is decreasing day by day hence focus is now shifting towards cultivated mushrooms. Moreover, the cases of mushroom poisoning from wild collection is also reported. Many nutritionally important and medicinally valuable mushrooms have now been cultivated *in-vitro*. *Cordyceps militaris*, known with different names such as Dong Chong Xia Cao, Yartsa gunbu or keera jadi is well known medicinal mushroom and is in traditional use by folk healers across many Asian countries. It is an entomopathogenic fungus that parasitizes insects (*Lepidoptera* larvae or pupae) and takes them to final stages of its life. After the death of the insect orange red or orange brown fruit bodies emerges from the body of the insect (Chan *et al.*,

2015). *Cordyceps militaris* consists many different bioactive ingredients like cordycepin, cordycepic acid, ergosterol, α -aminobutyric acid (GABA), etc. with therapeutic potential (Liu *et al.*, 2015). In today's world the consumers demand not only restrict to the food that meet nutritional requirements but also the food that boost health by giving additional benefits like antioxidants, anticancer, anti-inflammatory etc.

Cordyceps militaris is known to have anti-microbial (Xiao *et al.*, 2009), anticancer (Park *et al.*, 2009), anti-diabetic, anti-nephritic and anti-aging (Li *et al.*, 2010) properties. These properties make it a suitable candidate to study and cultivate. Several novel proteins and polysaccharides present in *C. militaris* play different therapeutic role. Cordycepin (3'-deoxyadenosine), a nucleotide precursor, play immensely important biological roles. Similarly, cordycepic acid (D-mannitol) is a carbohydrate reserve found in *Cordyceps militaris* that play role in osmoregulation and control of metabolic pathways (Jedrejko *et al.*, 2021). Adenosine, another major nucleoside in *Cordyceps militaris*, is an important signal transduction molecule, which provide cytoprotection and help prevent tissue damage (Liu *et al.*, 2016). In the present study, the nutritional profile of *in-vitro* grown *Cordyceps militaris* was analysed via its proximate analysis and bioactive compounds like cordycepin, adenosine and cordycepic acid.

MATERIALS AND METHODS

Source of study material

The culture of *Cordyceps militaris* (DMRO116) was obtained from the ICAR-Directorate of Mushroom Research (DMR), Solan, HP, India. The culture was maintained in Potato dextrose agar (PDA) plates.

Solid state fermentation

The liquid culture was prepared by inoculating two 3 mm disc from three days old petri plates containing the pure culture in 250 ml Erlenmeyer flask in the broth medium (dextrose 20g/l, peptone 10 g/l and yeast extract 10 g/l). The flasks were incubated at 19°C for 7 days on a shaker at 150 rpm. For the cultivation of the mushroom, 10 ml of nutrient media (dextrose 30 g/l, starch 19 g/l, yeast extract 3 g/l, malt extract 9 g/l, peptone 5 g/l, MgSO₄ 5 g/l and KH₂PO₄ 2g/l) was added to culture bottles containing 10 g brown rice substrate. The culture bottles were autoclaved and inoculated using 5 ml liquid culture. The culture bottles were than incubated in dark at 22°C with more than 70% relative humidity. The spawn run culture bottles were given light exposure (700-1000 lux) for 12 hours/day for fruit body initiation (Fig. 1). Fruit bodies were harvested after 50 days of inoculation, dried at 50°C until the constant weight was obtained. The dried fruit bodies were powdered and stored at -20°C for further use. The percent biological efficiency (B.E) was calculated by the formula given by Do *et al.* (2019).

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



Fig. 1. Strain DMRO 1164 cultivated on brown rice substrate

Moisture determination

The fresh fruit bodies were taken in the aluminium can and weighed. They were then dried in oven at

70°C for 48 h. The difference in weight of fresh fruit body (w) and dry fruit body (d) was measured to analyse moisture content using the formula:

$$\text{Moisture content (\%)} = \frac{w-d}{w} \times 100$$

Proximate composition

To determine proximate composition the dried fruit bodies (moisture free) were crushed to fine powder and analysed for following parameters:

Estimation of crude fat

Crude fat analyses were performed using Soxhlet apparatus as per the method described by AOAC (1984). 2 g dried sample was placed in a cellulose thimble and extracted using petroleum ether at 80°C for 6 hours. After extracting the thimble was removed and dried in oven overnight (60°C) until constant weight was achieved. Crude Fat content was estimated using the following formula:

$$\text{Fat (\%)} = \frac{W2-W3}{W2-W1} \times 100$$

W1= Weight of thimble

W2= Weight of thimble + sample

W3= Weight of thimble + defatted sample

Estimation of crude protein

Crude protein was estimated using Kjehdahl method according to the method given by AOAC (1984). A total of 0.5 g of sample was digested with 3 g catalyst (CuSO₄ and K₂SO₄ in ratio 10:1) and 10 ml H₂SO₄ for 3-4 hour at 420°C. The digested samples were cooled and kept for distillation. 15 ml boric acid (4% solution with 2-3 drops of methyl red indicator) was loaded per sample in a conical flask. 40% NaOH was then added drop wise until the colour of the mixture changes to brown. The nitrogen released during the process in the form of ammonia

was collected in conical flask as ammonium borate which makes the solution colourless. The titration was than performed using 0.1N HCl until the colour of solution turns pink again. The final and initial readings were noted and the nitrogen in sample was calculated using the formula:

$$N\% = \frac{(TR-B) \times \text{Normality of HCl} \times 14}{1000 \times \text{weight of sample in gram}} \times 100$$

TR= ml HCl used (final reading-initial reading)

B= ml HCl in blank

The content of crude protein was determined by multiplying nitrogen content by the factor of 4.38 (Mattila *et al.*, 2002).

$$\text{Crude protein (\%)} = \text{Nitrogen content} \times 4.38$$

Estimation of crude fibre

1.5 g of fat free sample (W) was used for estimation of crude fibre using the method determined by AOAC (1990). The sample was first boiled with acid using 0.25N H₂SO₄ for 30-40 minutes. The extract was then filtered and washed with boiling water until extract is acid free. The extracted sample was than subjected to alkali boiling using 0.3N NaOH for 30 minutes. The sample was again collected again and thoroughly washed and kept at 100°C overnight. The crucibles with the samples were weighed (W1) and ashed in muffle furnace at 550°C. The crucibles were weighed and cooled (W2). The crude fibre was calculated using following equation:

$$\text{Crude fiber (\%)} = \frac{W1-W2}{W} \times 100$$

Estimation of total ash content

1.5 g moisture free and ether extracted sample (W) was used for ash determination as per the procedure described by AOAC (1984). Crucible were ignited with flame along with the samples until they

were carbonized. The crucibles were then kept in muffle furnace at 600°C for 4-6 hours. The crucibles were then cooled and weighed (W1). The ash content was estimated using the formula given below:

$$\text{Total ash (\%)} = \frac{W1}{W} \times 100$$

Estimation of Carbohydrate content

Total Carbohydrate content was determined by applying the formula (Srikram *et al.*, 2016)

$$\% \text{ Carbohydrate} = 100 - (\text{ash content} + \text{fat content} + \text{fibre content} + \text{protein content})$$

Energy value calculation

Total energy value was calculated using the equation given by Barros *et al.*, (2007).

$$\text{Energy (Kcal)} = 4 \times [\text{protein (g)} + \text{carbohydrate (g)}] + 9 \times [\text{fat (g)}]$$

Cordycepin and adenosine estimation

The extraction of cordycepin and adenosine was performed by the method given by Wang *et al.* (2014) with slight modifications. 200 mg of fine powder of *C. militaris* fruit body was extracted using 2 ml of HPLC grade water at 75°C in a hot water bath for 3 hours. Following extraction, the supernatant was obtained by centrifugation at 7500 rpm for 30 min. The supernatant was filtered through 0.45 µm membrane filters and filled in HPLC vials. The experiment employed an HPLC system (Perkin Elmer, USA, LC 200D) equipped with a Photo diode Array (PDA) detector. Separation was conducted using a 150 x 4.6mm, 5 µm C18 reverse phase column. The mobile phase consisted of a mixture of water and methanol (85:15 v/v), with a flow rate of 1 ml/min and a column temperature of 30°C. Injection volume was 20 µl, and detection wavelength was 260 nm. Standard solutions of Cordycepin and Adenosine (procured from Sigma)

were prepared with HPLC grade water. The standard curve was generated by plotting peak area against concentration.

Cordycepic acid estimation

The concentration of cordycepic acid was assessed following the method outlined by Xiao *et al.* (2009). 100 mg of the powdered sample was extracted with double distilled water at room temperature for 4 hours. After centrifugation at 7500 rpm for 30 minutes, the supernatant was collected for analysis. Subsequently, 0.1 ml of the supernatant was diluted in 5 ml distilled water, and 1 ml of the resulting diluted sample were combined with 1 ml of 0.015M periodate potassium, then subjected to a water bath at 53°C for 10 minutes. Following this, 2 ml of L-Rhamnose (0.1%) and 4 ml of freshly prepared Nash reagent were added to the mixture. The resulting mixture was then incubated at 50°C for 15 minutes before being cooled to room temperature. Spectrophotometric measurement of absorbance at 412 nm was performed using spectrophotometer (Shimadzu make). The concentration of cordycepic acid was determined by employing the standard curve equation, utilizing mannitol (Sigma Aldrich) as a standard (procured from sigma).

Statistical analysis

All the experimental was performed in triplicates. The results are represented as mean of three triplicate values. The statistical analysis was performed using R studio software (4.0.2). The statistical significance was determined using Analysis of Variance (ANOVA) along with Fishers Least significant difference (LSD) at p<0.001.

RESULTS AND DISCUSSION

The nutritional value of *in-vitro* cultivated fruit body of *Cordyceps militaris* is shown in Table 1. The

proximity analysis results on fruit body confirmed that major components present in *C. militaris* are carbohydrate, protein and fibre. Previous investigations on mushrooms have also shown that proteins, carbohydrates and fibre are more abundantly found in mushrooms (Srikram *et al.*, 2016; Salamat *et al.*, 2017). The moisture content in the fruit body was around $85.48 \pm 0.56\%$, the higher moisture content in mushrooms account for their shorter shelf life. Thus, mushrooms tend to deteriorate if not properly preserved after harvest (Adedayo, 2011). The carbohydrate content in the fruit body was very high, which was $60.04 \pm 0.07\%$ as per the present study. The study performed by Sethy *et al.* (2016) also reported the carbohydrate content in fruit body as 55.68% . Carbohydrates are considered as principle components in energy metabolism. As a matter of fact, fungus derived polysaccharides have gained attention in past decade due to therapeutic roles such as antioxidant, immune stimulating, antitumour and antimicrobial activities (Chen *et al.*, 2013; Lee *et al.*, 2010; Zhu *et al.*, 2012; Zhang *et al.*, 2017). Crude protein was calculated by multiplying the % Nitrogen with the factor 4.38. The common factor 6.25 was not used because mushrooms contain non protein nitrogen in a significant amount in cell wall chitin and as free amino acids. The amount of crude protein was $23.02 \pm 0.10\%$ was in approximation with the previous studies conducted (Sethy *et al.*, 2016). The ash, crude

fibre and fat content was found to be $2.06 \pm 0.12\%$, $11.06 \pm 0.04\%$ and $3.84 \pm 0.02\%$, respectively. Fibre was also found to be a major component in *C. militaris* and also often considered by dieticians and nutritionists as an important component to maintain healthy diet. The total energy value was estimated to be 366.66 ± 0.49 Kcal/100g.

Table 2 shows the content Cordycepin, Adenosine and Cordycepic acid in the fruit body of *C. militaris*. The chromatogram of the standard (Fig 2) shows the peaks of adenosine and cordycepin with retention time at 4.7 and 5.90, respectively. The sample chromatogram (Fig 3) shows good resolution in the peaks of Cordycepin and adenosine. The Cordycepic acid content was estimated spectrophotometrically. The content of cordycepin, adenosine and cordycepic acid depends on many

Table 2. Amount of cordycepin, adenosine and cordycepic acid in *C. militaris* fruit body.

S. No.	Parameter	Unit	Values
1	Cordycepin	mg/g DW	9.21 ± 0.02^b
2	Adenosine	mg/g DW	1.05 ± 0.03^c
3	Cordycepic acid	mg/g DW	14.36 ± 0.29^a

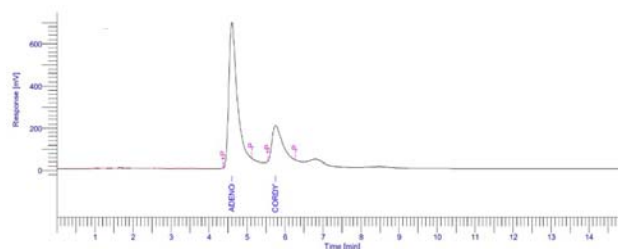


Fig 2. HPLC chromatogram of standard of Adenosine and Cordycepin

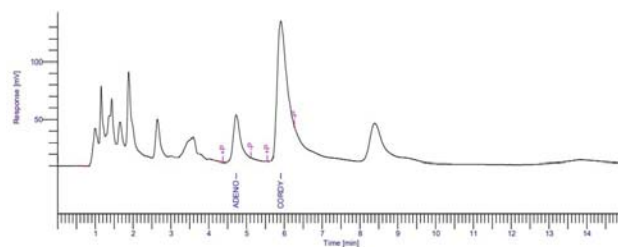


Fig 3. HPLC Chromatogram of *C. militaris* fruit body

Table 1. Proximate composition of fruitbody of *C. militaris*

S. No.	Parameter	Unit	Values
1	Crude fat	% DW	3.84 ± 0.02^e
2	Crude protein	% DW	23.02 ± 0.10^c
3	Ash content	% DW	2.06 ± 0.12^h
4	Crude fibre	% DW	11.06 ± 0.04^f
5	Total carbohydrate	% DW	60.04 ± 0.07^d
6	Moisture content	%	85.48 ± 0.56^b
7	Total energy value	Kcal/100g	366.66 ± 0.49^a
8	B.E	%	84.40 ± 0.20^c

factors like culture conditions, type of substrate and *C. militaris* strain. Our results on the concentration of Cordycepin and adenosine matches with the study previously conducted (Kumar *et al.*, 2023) on the same strain.

CONCLUSION

Present works covers the proximate analysis of *C. militaris* fruit body along with the estimation of major secondary metabolites viz Cordycepin, Cordycepic acid and Adenosine. The results concluded that *Cordyceps militaris* fruit bodies are rich in carbohydrates, protein and fibre. Further lower content of fat makes it a good candidate for people opting for low calorie food. The results showed, the fairly good amount of cordycepin, adenosine and cordycepic acid was present in the *Cordyceps* fruit bodies making it a good choice for good health food with nutritional and therapeutic potential.

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