

In-Vitro Cultivation Optimization of Cordyceps Militaris and Analytical profiling: Implications for Nutraceutical and Functional Food Development

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Received: 10th Sep, 2025; Revised: 10th Oct 2025; Accepted: 6th Nov, 2025; Available Online: 1st December, 2025

ABSTRACT

The current study focused on the optimization of a rapid in-vitro-culturing protocol and analytical characterization of the Cordyceps militaris (L.) Link fruiting bodies. The main intension of the study is to increase the yield and bioactive metabolite content in the Cordyceps militaris. The tissue engineering process, the solid-state fermentation technique, used in the culturing of the Cordyceps militaris (L.) Link. The different cultural conditions were optimized to increase the growth and metabolite production. The main parameters were optimized with respect to physicochemical condition and to growing media. The temperature, pH, and shaking speed were systematically optimized to maximize growth and production of metabolite the temperature at 19–21°C, pH 5.5–6.0, and 120–150 rpm established the uniform and good growth of the mycelium. The Potato Dextrose Broth (PDB) confirmed the uniform growth and yield of the Cordyceps militaris fruiting bodies. The nutrient broth supplemented with brown rice used as substrate in the process of mycelial growth and taken 50–55 days, achieving a biological efficiency of 90–110%.

The phytochemical screening of the cordyceps militaries shows the presence of major bioactive components, including phenolics, flavonoids, saponins, and proteins. The UV- spectroscopic analysis revealed the assay of total phenolic and flavonoid contents of 14.36% and 0.81% (w/w), respectively. The sequential solvent extraction demonstrated maximum recovery with ethyl acetate and hydroalcoholic solvents. The HPLC findings identified adenosine (1.20%) and cordycepin (3.73%) with retention times of 4.5 and 5.3 minutes, respectively, indicating efficient extraction of pharmacologically relevant nucleosides. The cordyceps militaries extract was subjected to GC–MS and LC-MS analysis and GC-MS revealed 80 volatile and semi-volatile compounds, including esters, fatty acids, and nitrogenous derivatives. The LC–MS confirmed the presence of inosine and 5-methyluridine as key polar metabolites. The study confirms the in-vitro culture of Cordyceps militaris can serve as a reproducible and sustainable system for producing high-quality fruiting bodies rich in therapeutic and nutritional constituents comparable to previous cultivation studies.

Keywords: Cordyceps militaris, solid-state fermentation, optimization, cordycepin, adenosine, HPLC, GC–MS, LC–MS, phytochemical profiling, nutraceutical potential.

How to cite this article: Sukanya P, Chandan S, Shivaprasad K Chandan D and Pradeep S; In-Vitro Cultivation Optimization of Cordyceps militaris and Analytical profiling: Implications for Nutraceutical and Functional Food Development. Int J Drug Deliv Technol. 2026;16(1): 321-336. DOI: 10.25258/ijddt.16.1.35

Source of support: Nil.

Conflict of interest: None

1. INTRODUCTION

The cordyceps species has long been used as traditional medicine for its efficient therapeutic potential and nutritional values. In modern world research has redefined the production and cultivation of the pharmacologically active natural species. The *Cordyceps militaris* (L.) Link, a parasitic ascomycete within the family *Cordycipitaceae* recognized for producing a wide range of secondary metabolites with significant pharmacological activity. The main active compound in

Cordyceps militaris are cordycepin, adenosine, ergosterol, and complex polysaccharides. These bioactive compounds

are proven for the antioxidant, immunostimulatory, anti-inflammatory, and anticancer activity [1-4]. The active components in *Cordyceps militaris* contribute to the broad therapeutic range and in positioning it as a remarkable species in modern mycological biotechnology [5]. The natural propagation of *Cordyceps militaris* remains limited due to its host specific and parasitic dependence on lepidopteran larvae, particularly *Bombyx mori*.

Ecological fragility of wild population and expense of collection cost have underscored the need for controlled artificial cultivation [6]. From past two decades, there has been substantial efforts made to reproduce the insect-based

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life cycle using invitro defined substrates suitable environmental condition. The tissue engineered process shows promising yield and uniform growth when cultivated in submerged fermentation and solid-state fermentation approaches. Some cases the metabolite consistency is the constrain in the process. [2,7]. The successful invitro cultivation morphogenesis and metabolite biosynthesis of the *cordyceps militaries* depends on the nutrient composition, pH, temperature, humidity, and photo period all which influence [8, 9]. Recent reports have pointed to the critical need for empirical optimization of the cordyceps invitro cultivation. Sibounnavong et al. demonstrated that fine-tuning substrate formulations can markedly alter fruiting initiation and yield and Deshmukh and Bhaskaran applied statistical modeling to identify critical variables enhancing mycelial biomass productivity [10, 11]. Chou et al. and Park H.J. in their findings have shown that nutrient modulation and environmental control directly regulate cordycepin biosynthesis [12, 13]. Simultaneously in-vitro grown *cordyceps militaries* attract interest due to their nutritional and aesthetic use. The lab grown fruiting bodies and rich in nutrients like proteins, carbohydrates, and essential minerals, along with remarkable concentrations of bioactive nucleosides [14]. This nutraceutical and therapeutic potential of the cordyceps species opens the alternative source for the functional food ingredients source. The proper systematic profiling and growth optimization with detailed nutritional and chemical profiling are still lacking, retreating a critical gap between the artificial cultivation and functional assessment. The present research focused on to bridge this gap, to establish robust and reproducible procedure for in-vitro cultivation of *Cordyceps militaris* fruiting bodies through optimization physicochemical factor. The study also emphasizes complete phytochemical and nutritional profiling and derives the industrial scale up protocol. The study provides the standardized framework for the sustainable cultivation of *Cordyceps militaris* suitable to biotechnology and industrial applications.

2. MATERIAL AND METHODS

2.1 Materials Used

The analytical grade chemicals and reagents the solvents such as methanol, ethanol, chloroform, and hexane were used for extraction and phytochemical analysis, all conforming to analytical standards were procured for this study from authentic suppliers (HiMedia, India and Merck, Germany). The required culture medium used for fungal growth and maintenance the Potato Dextrose Agar (PDA) and Potato Dextrose Broth (PDB), Yeast extract, peptone, malt extract, glucose, dextrose, starch, and mineral salts ($MgSO_4$, KH_2PO_4) were procured from HiMedia, India. The analytical instruments rotary shaker incubator, laminar airflow cabinet, autoclave, hot-air oven, UV-Visible spectrophotometer (Shimadzu UV-1700), HPLC system (Waters 2695 with PDA detector), and GC-MS apparatus was used in the study.

The fungal strain *Cordyceps militaris* RPIMKA0319 was obtained from Jam Bio Foods, Haryana, India. The strain was originally developed through a controlled hybridization program in Yesan, Chungnam, Republic of Korea, in 2022, involving sexual crossing between two single-ascospore isolates (C0601S05 and C0601S33) to enhance genetic stability and fruiting performance.

2.2. Collection and Maintenance of Fungal Strain

The *Cordyceps militaris* RPIMKA0319 strain was maintained on PDA slants at 4°C and sub-cultured monthly to preserve viability and genetic consistency. For routine culture, mycelial plugs were inoculated on fresh PDA plates and incubated at 22°C for 7 days. All glassware and media were sterilized by autoclaving at 121°C for 20 minutes at 15 psi pressure. The working inoculum was prepared under aseptic conditions within a laminar airflow cabinet to prevent contamination.

2.3. Preparation and Inoculation for Mushroom Fungi (*Cordyceps militaries*)

2.3.1. Culture and Spawn Preparation

Liquid spawn was produced by transferring two 3 mm mycelial discs from actively growing PDA cultures (3-day-old) into 250 mL Erlenmeyer flasks containing 100 mL of sterilized nutrient broth (dextrose 20 g/L, peptone 10 g/L, yeast extract 10 g/L). The flasks were incubated at 19°C on a rotary shaker at 150 rpm for seven days to achieve a homogeneous mycelial suspension. This liquid inoculum served as the starter culture for subsequent solid-state fermentation trials.

2.4. In- Vitro Cultivation and Harvesting of *Cordyceps militaris*

2.4.1 Substrate and Media Preparation

A nutrient-enriched fruiting medium was formulated by boiling a mixture containing 5 g peptone, 5 g yeast extract, 200 g potato, 20 g glucose, 20 g baby corn, and 10 g silkworms in 1 L of distilled water. The polypropylene (PP) jar of 750mL volume supplemented with 65–70 mL of the prepared broth and filled with 30–32 g of brown rice. The jars were capped with polyfoil to allow sufficient aeration. The jars were subjected to sterilization at 121°C for 40 minutes under 15 psi pressure, after the sterilization process jars were allowed to be exposed to UV light for 30 minutes cool to room temperature prior to inoculation.

2.4.2. Solid State Fermentation and Fruiting

The inoculum of about 10 mL was aseptically transferred into each sterilized culture jar using sterile pipettes in a laminar airflow cabinet to maintain the aseptic condition. After the inoculation the jars were incubated in a dark chamber at 19–22°C with 60–75% relative humidity until complete mycelial colonization of the substrate occurred. The jars were exposed to 12-hour light/dark photoperiod with a light intensity of 800–1000 lux to induce the fruiting body formation. The complete formation of mycelial fruiting body takes nearly 55–60 days after the inoculation after complete vertical growth the mycelium can be harvested. Yang et al. and Das et al. described the

biological efficiency (B.E.) of the invitro cultivation [15-16].

Table 1. biological efficiency (B.E.) calculation formula

$$BE (\%) = \frac{\text{Fresh weight of fruiting bodies (g)}}{\text{Dry weight of substrate (g)}} \times 100$$

2.4.3. Post Harvesting Processing and Storage

The complete grown mycelium can be harvested in a sterile clean perforated stainless-steel tray and dried in a hot-air tray dryer at 60°C until moisture-free (“bone dry”). The dried mushrooms were weighed and packed in an airtight container for further use. The dried fruiting body can also be powdered and stored at -20°C in airtight containers for subsequent analysis.



Figure 1. Preparatory stages of Inoculum

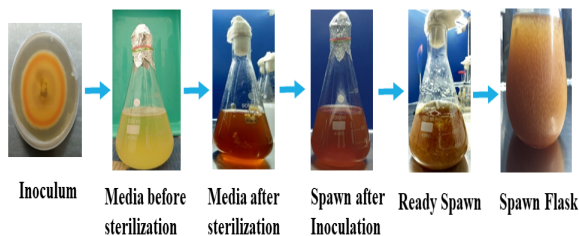


Figure 2. Culture Stages of *Cordyceps militaris*

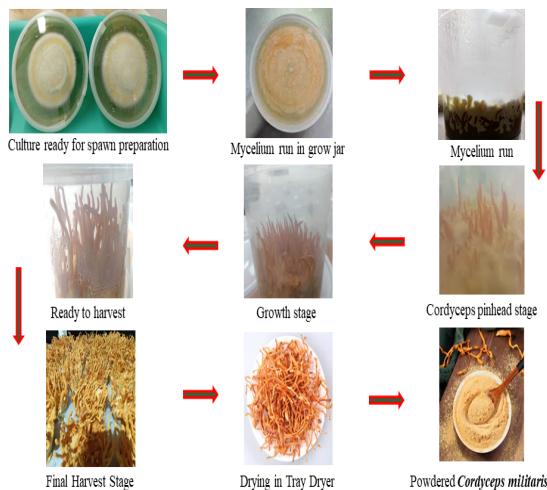


Figure 3. Growth Stages of *Cordyceps militaris*

2.5. Optimization of Culture Medium

2.5.1. Optimization of Temperature, pH, and Shaking Speed on growth of the fruiting bodies

The growth and the functional physiological traits and secondary metabolites production in the *cordyceps* species are strongly influenced by the key environmental factors including the temperature, pH, agitation rate, and light illumination. The growth optimization for *Cordyceps militaris* strain RPIMKA0319 was conducted under controlled in-vitro conditions. The pH optimization the initial pH values were adjusted to 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, and 9.0. and the mycelial discs were inoculated on Dextrose Agar (PDA) plates whose initial pH were adjusted. The initial pH adjustments achieved prior to sterilization by using acid (1 mol L⁻¹ hydrochloric acid) and base (1 mol L⁻¹ sodium hydroxide) solutions. The temperature also plays an important role in the uniform growth and development of mycelial production. The inoculated cultures were incubated in the temperature-controlled growth chamber, and the set temperature range from 5, 10, 15, 20, 25, and 30 °C. The effect of agitation on the mycelium growth and secondary metabolite yield was studied at 21°C for six days using shaking speeds of 100, 150, and 200 rpm in shaking incubator. The optimization was carried out in triplicate trails to ensure the reproducibility and repeatability of each parameter. After the incubation the mean growth rate of the colony diameters were calculated in three perpendicular directions on each plate. The light illumination was examined under the controlled light conditions with illumination-controller incubator. The subsequent replicate culture was incubated either in dark or under fluorescent light (14.3 W m⁻²) at 21°C.

2.5.2. Carbon and Nitrogen Sources

Carbon and nitrogen play a very vital role in the uniform growth and development of the mycelial mass. The basal medium consisting of 2% water agar was used in assessing the effect of carbon and nitrogen sources on mycelial growth. The medium was supplemented with the concentration of 2% (w/v) twelve carbon sources and twelve nitrogen sources. The evaluated carbon sources include fructose (C₁), galactose (C₂), glucose (C₃), mannitol (C₄), dextrose (C₅), lactose (C₆), maltose (C₇), sucrose (C₈), sorbitol (C₉), dextrin (C₁₀), xylose (C₁₁), and starch (C₁₂). The nitrogen sources encompassed ammonium phosphate (N₁), calcium nitrate (N₂), glycine (N₃), potassium nitrate (N₄), yeast extract (N₅), malt extract (N₆), peptone (N₇), tryptone (N₈), ammonium chloride (N₉), ammonium sulfate (N₁₀), sodium nitrate (N₁₁), and ammonium nitrate (N₁₂). The media was adjusted with pH to 6.0 sterilized at 121 °C for 20 minutes and poured in to the sterilized petri plates in aseptic condition. 5 mm diameter of mycelial plugs were excised from the PDA cultures and transferred to the center of each plate containing the respective carbon or nitrogen source. After the inoculation the plates were incubated at 24°C for mycelial growth and expansion. The colony

growth was recorded periodically to evaluate the impact of each nutrient source on mycelial growth and radical extension.

2.5.3. Determination of Optimum C: N Ratio

To determine the effect of nutrient balance on fungal metabolism the carbon-to-nitrogen (C: N) ratio was systematically varied. The sucrose was selected as primary carbon source and the peptone was selected as the sole nitrogen source. For examining the carbon-to-nitrogen (C: N) ratio 10:1, 20:1, 30:1, and 40:1 was chosen. The carbon content of sucrose (C₁₂H₂₂O₁₁) was calculated based on the molecular composition 42% for sucrose and the nitrogen in peptone was determined by the Kjeldahl method to be approximately 12%. The peptone concentration of each formulation kept 5 g L⁻¹ strength and the sucrose concentration was varied to attain the desired C:N ratio. The growth rate was quantified after the incubation of the cultures at 24°C

2.6. Extraction and Optimization of *Cordyceps Militaries* Extract

Extraction plays vital role in the enrichment and isolation of the bioactive compounds. The extraction process involves the isolation of the active components from the complex matrices. The process involves different solvent mixtures at different temperatures and extraction time and method of extraction. Sequential solvent extraction was adopted in the extraction process for better results. The extraction techniques provide the enriched bioactive ingredients to nutraceutical and pharmaceutical research the main intention is to achieve purity and reproducibility in the standardization process.

2.6.1. Sequential Solvent Extraction (Maceration)

The shade dried and finely powdered *Cordyceps militaris* powder was taken for the sequential solvent extraction by maceration technique. The solvent was employed based on a polarity gradient ranging from non-polar to polar solvents—hexane, chloroform, ethyl acetate, ethanol, and water. 50 g of the dried powder was immersed in 500 mL of each solvent with intermittent stirring to facilitate diffusion of soluble components for about 24 hours. After the completion of the extraction the extract was filtered and concentrated under reduced pressure using a rotary evaporator to obtain dry residue. The yield of the extracts was recorded based on the initial weight and all the extracts were stored at 4°C in airtight containers without any microbial contamination until further study.



Figure 4. Extraction of *Cordyceps militaries* Extract

2.6.2. Hydroalcoholic Extraction (Soxhlet Method)

Hydroalcoholic extraction ensures the recovery of both polar and moderately polar compounds.

Ethanol: water, 60:40 v/v ratio was taken for hydroalcoholic extraction was employed by using Soxhlet extraction technique. About 10 grams of the dried *Cordyceps militaris* powder were placed in a thimble and extracted with 100 mL of a hydroalcoholic solvent mixture (ethanol: water, 60:40 v/v) at 80 °C for 30 minutes. Exhaustive extraction was done by using fresh portions of 25 mL of the same solvent until the siphon liquid became nearly colorless. The extracts were collected and

Dried to evaporate on a water bath to get concentrated extract and remove residual solvent. The concentrated extract was dried in a hot-air oven at 60 - 80°C for two hours to obtain a fine, dry extract. The extracted powder was stored in desiccator for further analytical characterization and formulation studies.

2.7. Nutritional profiling and Analytical characterization and of *Cordyceps militaris* (L.)

Cordyceps militaris is recognized as the costliest mushroom for its extensive therapeutic potential as well as nutritional values. The active compounds in *Cordyceps militaris* shows antioxidant, anticancer, anti-inflammatory, immunomodulatory, and anti-fatigue activities. Analytical standardization and characterization ensure repeatability and reproducibility in its bioactive efficiency. The standardization of the extract is imperative for maintaining uniformity in phytochemical composition, quality as well as regulatory prospective. Optimization and standardization are essential to validate the commercialization of the *Cordyceps militaris*, as nutraceutical ingredients. Analytical characterization ensures the diverse application of analytical and bioassay techniques to assess the chemical constituents and biological functionality. The final standardized extract was obtained as fine golden yellow colored extract powder with characteristic taste and odor.

2.7.1. Qualitative Phytochemical Screening

The AOAC standard methodologies were used for the qualitative phytochemical screening of the *Cordyceps militaris* RPIMKA0319 fruiting bodies. The gravimetric and proximate analysis were conducted for the estimation of moisture, fat, protein, and carbohydrate contents.

Moisture Estimation by Gravimetry

The moisture content was measured by taking approximately 2 g of powdered sample in a pre-dried, stoppered weighing bottle and dried in a hot-air oven maintained at 105 ± 2 °C. The loss on drying was calculated based on the initial and final constant weight. The pre calibrated and validated Metler MB 45 (OHAUS, USA) moisture analyzer was used under automated thermal conditions to achieve the cross-validate the results.

Ash Content Estimation

The estimation of ash was performed in a muffle furnace by igniting 2–4 g of the air-dried, powdered sample in a pre-weighed silica crucible at 500–600°C. Allow the crucible till the residue become carbon-free once the cooling occurs take out the crucible and place in desiccator to avoid the moisture absorption. Once cooling in a desiccator, the crucible was reweighed, and the percentage of ash was estimated.

Fat Content Estimation

The total fat content was estimated by using Soxhlet apparatus by continuous solvent extraction process, as per the method described by James et. al. [17]. Accurately weighed about 5 grams of sample in thimble and extracted with 200 mL of petroleum ether for 4 hours. The extracted solvent fraction was collected and evaporated to dryness at 60°–80°C. The lipid layer was cooled in a desiccator, and the total results were reported as percentage of dry weight.

Protein Content Estimation

The protein content in the *cordyceps* species gives the more projection as the dietary ingredient in nutraceutical prospective. The total protein content was estimate by the advanced Micro-Kjeldahl method [18]. The sample 1.75 gram was digested with the concentrated sulfuric acid in the presence of mercuric oxide. Sodium sulfate was also used in the process to convert organic nitrogen into ammonium sulfate. The reaction mixture was alkalized and distilled during the digestion process to liberate ammonia, and the liberated ammonia was trapped in standard hydrochloric acid. To calculate the total nitrogen the excess acid was titrated with sodium hydroxide solution. By using the conversion factor 6.25, total nitrogen can be converted into crude protein content.

Carbohydrate Content Estimation

Carbohydrates act as both structural polymers and reserve materials in most of the macro fungus including *Cordyceps militaries*. Carbohydrates occurring in simple sugar from as well as complex polysaccharides form in the *Cordyceps militaries* fruiting bodies. The anthrone method was used in the estimation of carbohydrates the sample was hydrolyzed under acidic conditions to yield monosaccharides. The glucose molecules in the

carbohydrates get converted into hydroxy-methyl furfural, in the presence of concentrated acid and heat, as precursor. The hydroxy-methyl furfural consequently reacts with anthrone reagent to form a chromogenic, green-colored complex which detects at wavelengths between 620 and 630 nm. The total carbohydrate different aliquots of glucose standard solution were mixed with anthrone reagent. The reaction mixture containing test tubes were heated in a water bath at 100°C for 15 minutes to complete the reaction. The sample and standard readings were recorded to estimate the total carbohydrate content at 620nm using a UV-spectrophotometer.

2.7.2. Quantitative Estimation of Bioactive Compounds

Flavonoid Content Estimation by Spectrophotometry

The Flavonoids content in *Cordyceps militaris* extracts was quantified using a UV-Visible spectrophotometric method (Shimadzu, Model UV-1700, Japan). Flavonoids produce yellow colored complex with aluminum chloride reagent, and this yellow color can be read at 410nm. 1 gm of the finally powdered *cordyceps* powder was taken in the round bottomed flask extracted in methanol reflux at 80 °C for 30 minutes and filtered through Whatman No. 1 paper. Rutin used as standard; 1% (w/v) rutin solution in methanol was taken for the analysis.

This method was adapted from Chang et al., and Siddique et al. conforms to the analytical standards outlined in the European Pharmacopoeia (*European Pharmacopoeia PH Eur. monograph 1174*) [19-21].

Estimation of Total polyphenols by Folin-Ciocalteu Method

Total phenolic compounds were estimated by folin-ciocalteu method by using spectrophotometer at the absorbance of 765nm (Shimadzu, Model UV-1700, Japan). The reaction mixture produces the blue chromophore when the sample was treated with folin-ciocalteu and sodium carbonate. Quantification was achieved by comparison with a gallic acid calibration curve, and results were expressed as milligrams of gallic acid equivalents (GAE) per gram of dry extract. This widely accepted method provides a reliable measure of total polyphenols in plant-derived materials [22, 23].

Saponin Content Estimation by Gravimetric method

Saponins were quantified gravimetrically. Five grams of powdered *Cordyceps militaris* sample were extracted with 50 mL of 90% methanol under reflux at 70–80°C for 30 minutes. The residue was extracted several times with 25 mL portions of the same solvent until the filtrate became nearly colorless. All methanolic extracts were combined and concentrated on a water bath, followed by drying in a hot-air oven at 6°C for 2 hours. The dried extract was then treated sequentially with 25 mL each of petroleum ether, chloroform, and ethyl alcohol to remove non-saponin impurities. The remaining residue was dissolved in 25 mL of 90% methanol, filtered, and the filtrate was added dropwise with continuous stirring into 125 mL of acetone to precipitate the saponins. The precipitate was allowed to settle, decanted, and dried at 105°C to constant weight.

Saponin content was calculated gravimetrically as a percentage of dry weight based on the mass difference before and after precipitation.

Bitter Estimation by Gravimetric method

The total bitter constituents were determined by reflux extraction. Approximately 5 g of finely powdered *Cordyceps militaris* sample were extracted with methanol on a water bath maintained at 80 ± 2 °C for 30 minutes. The combined methanolic extracts were concentrated to a thick paste. This concentrate was dissolved in hot water; if incomplete dissolution occurred, 25 mL of ethyl acetate was added, and the mixture was sociable for 5 minutes to achieve uniformity. The aqueous layer was subjected to liquid–liquid extraction using ethyl acetate in a separatory funnel. The ethyl acetate residue was dried at 105°C and taken in pre-weighed chain dish and the final residue was recorded. This is the widely accepted method follows WHO Quality Control Methods [24].

2.8. Adenosine & Cordycepin Content by HPLC Method

Quantification of adenosine and cordycepin was performed using a reverse-phase HPLC system (Waters 2695, USA) equipped with a C18 analytical column and UV–PDA detector. Standard solutions of cordycepin and adenosine (0.1 mg mL^{-1}) and sample extracts (1 mg mL^{-1}) were prepared in analytical-grade methanol. The mobile phase consisted of 10% acetonitrile in water, delivered at a flow rate of 0.8 mL min^{-1} . Chromatographic separation was achieved at 25 °C with a total run time of 15 minutes. Peaks corresponding to adenosine and cordycepin were detected at retention times of 4.5 min and 5.3 min, respectively, consistent with standard references [25, 26]. The concentrations were calculated from calibration curves and expressed as % w/w of the dry extract.

2.9. Gas-Chromatography- Mass Spectroscopy (GC-MS) Analysis

GC–MS analysis was conducted to identify volatile and semi-volatile constituents in the *Cordyceps militaris* extract, following USP General Chapter <621>. The dried extract was vaporized and injected into a capillary GC column, and the separated compounds were detected by mass spectrometry based on their mass-to-charge ratios. The acquired spectra were matched against NIST and Wiley library databases for compound identification. The identification of the active elements in *Cordyceps militaris* obtained chromatographic peak were assessed for spectral fidelity to reconfirm precise identification.

3. RESULT AND DISCUSSION

3.1. Culture conditions Optimization of *Cordyceps militaris*

The in-vitro culturing and the secondary metabolite production in the *Cordyceps militaris* RPIMKA0319 were strongly dependent on the physicochemical factors. The optimization of medium, temperature, pH, and shaking speed are the key parameters for the high-quality mycelial biomass formation.

3.1.1 Effect of Growth Medium

The five different culture media with varied nutritional components was used to assess the growth of *Cordyceps militaris* RPIMKA0319. The culture media includes Oatmeal Agar (OMA), Potato Dextrose Agar (PDA), Wheatmeal Agar (WMA), Malt Extract Agar (MEA), and Yeast Potato Dextrose Agar (YPDA). The media optimization was done based on the optimal yield of biomass and mycelial proliferation. In all the tested media the yield of biomass and mycelial density and morphology were observed. (Figure 5). The Potato Dextrose Agar (PDA) demonstrated the highest mycelial growth, with a score of 4 (good growth) on the 0–5 scale compared to all other media. The PDA medium derived from potato starch and glucose with balanced carbon–nitrogen composition. Which enhance the production of cottony mycelia dense colonization and rapid radial expansion of cordyceps growth. The moderate growth (score 3), on the 0–5 scale was observed with the Yeast Potato Dextrose Agar (YPDA). The media was supplemented with the yeast extract, which enhances nitrogen and vitamin availability. The other media shown very limited growth score of 1 on the scale of 0-5. There was no visible growth (score 0) was observed with the other media, while Oatmeal Agar (OMA) and Malt Extract Agar (MEA). The down performance of these media was the limitation with the nutrient solubility and limited sugar availability. PDA was considered as the basal medium for *Cordyceps militaris* RPIMKA0319 due to the good growth score. The ingredients of the PDA medium reside with the free availability of the nutrient molecule for the mycelial growth, especially the carbon, glucose sources and balanced nutrient profile. These conclusions are consistent with earlier studies by Trung et al. and Jędrejko et al. These two researchers observed that in PDA-based cultures the active compound the cordycepin accumulation and mycelial growth was rapid [1, 8]. The same was also observed by Das et al., the potato-derived nutrients cultures particularly with glucose molecule improve fungal morphogenesis and biomass yield [16]. The PDA based medium was selected as the standard medium for *Cordyceps militaris* RPIMKA0319 in-vitro cultivation, on the experimental results as well as previous data. The PDA proven with the repeatability and reproducibility with ensuing optimization studies involving temperature, pH, and agitation speed.

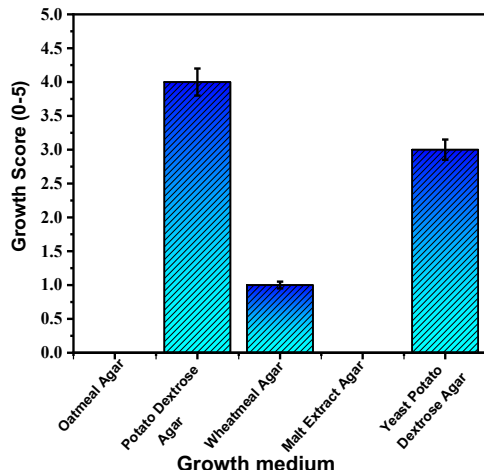


Figure 5. Comparative evaluation of *Cordyceps militaris* RPIMKA0319 mycelial growth on different culture media. Growth was scored on a 0–5 scale, where 0 = no growth, 1–3 = moderate growth, and 4–5 = good growth

3.1.2. Effect of Initial pH

The pH acts as the determination factor in enzymatic activity, nutrient solubility and availability of ions during fungal metabolism. To comprehend the pattern of initial pH on the mycelial growth the pH range was set with the range from 5.0 to 9.0 (Figure 6) with the nutrient medium PDA. There was the maximum growth, and colony density was found with the high score at the pH range 6.5–7.0 (score 4), signifying its preference for a near-neutral condition.

There was a decline in the growth observed at the pH values below 6.0 and there was less growth observed at the pH 5.0. No visible above visible mycelial growth above pH 7.5, and with 9.0. It's a clear indication that the pH values play vital role in enzymatic activity, carbohydrate and nitrogen metabolism. The extreme acidic or alkaline conditions may inhibit nutrient uptake and spore germination. Adnan et. al. who proven optimal cordycepin production and biomass accumulation at pH 6.0–7.0 for *Cordyceps militaris* strains [27]. Based on the previous literature, the consequent experiments were conducted at an initial pH of 6.5.

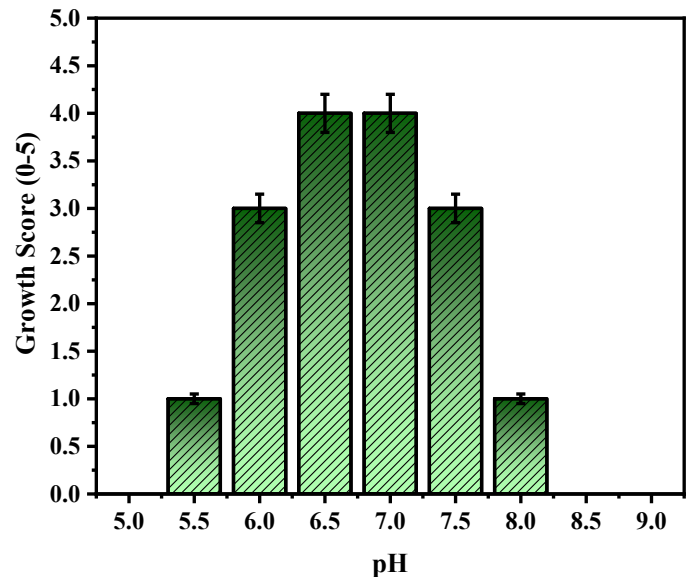


Figure 6. Effect of initial pH on the mycelial growth of *Cordyceps militaris*RPIMKA0319.

3.1.3. Effect of Temperature

The main aspects for the *Cordyceps militaries* growth initiation and proliferation are the temperature which influences the enzyme kinetics, nutrient assimilation, and mycelial morphology. The development of *Cordyceps militaris* (RPIMKA0319) was estimated at temperatures ranging from 10°C to 28°C (Figure 7). The ideal growth occurred at 22°C (score 5), with good growth also observed the accumulation of the active phytocomponents. There was the moderate irregular growth observed at 18°C and 20°C (score 4) and the growth diminished sharply behind 24°C. At low temperatures at 10°C and high temperature 28°C there was no visible growth observed. Exactly at 22°C, the growth not only was dense, but it was also uniform, and pigmented slightly creamy white, suggesting efficient metabolic activity.

The findings from the scientists reported in their study that the *Cordyceps militaris* confirm maximal mycelial extension and secondary metabolite production between 20°C and 23°C. There was a decline in the mycelium growth while higher temperatures may denature metabolic enzymes or alter membrane integrity [28, 29]. From the trial and the reported data confirms the optimal temperature for the *Cordyceps militaris* uniform growth and metabolite accumulation should considered as, 22°C.

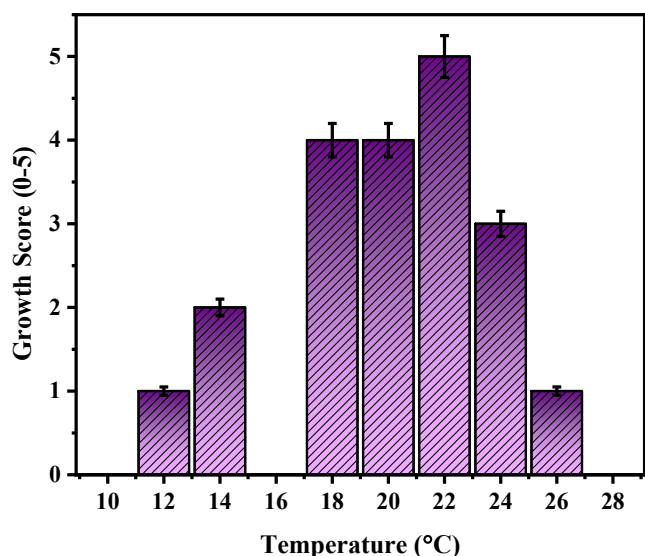


Figure 6. Effect of temperature on the mycelial growth of *Cordyceps militaris* RPIMKA0319.

3.1.4. Effect of Shaking Speed (Aeration Rate)

Aeration rate is very significant in the submerged cultures which influences oxygen transfer, nutrient distribution, and shear stress managing within culture growth environment. Mycelial growth was studied linear incremental order under the shaking speeds ranging from 10 to 200 rpm (Figure 4). The maximum growth of *Cordyceps militaris* RPIMKA0319 was occurred at 150 rpm (score 5), followed by good growth at 125 rpm (score 4) and moderate growth at 100 rpm (score 3). The culture growth was weak or negligible below 75 rpm, due to insufficient aeration; there was the reduced growth observed at higher agitation at 200 rpm growth retarded may be due to higher mechanical stress and disturbance in the mycelial network. Park et al., in his findings noted that the optimal aeration speeds favors oxygen transfer and mycelial yield and, where excessive agitation disrupts hyphal integrity and disruption in the cell wall may occur [30]. From the findings it's confirmed that 150 rpm was standardized as the optimal agitation speed for maintaining high biomass yield and homogeneous mycelial distribution.

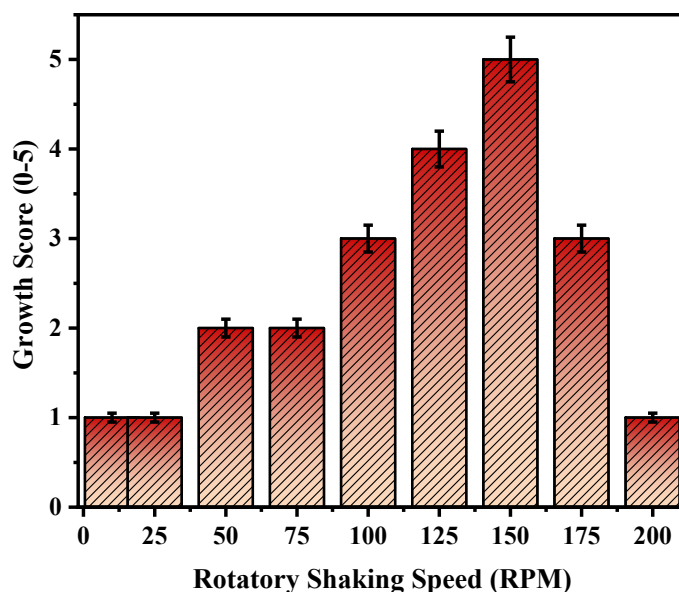


Figure 7. Effect of shaking speeds on the mycelial growth of *Cordyceps militaris* RPIMKA0319

3.1.5. Overall Optimization Outcome

From the experimental observations, the following culture conditions were identified as optimal for *Cordyceps militaris* RPIMKA0319 growth as shown in table 1:

Table 1. Optimized culture conditions for *Cordyceps militaris* RPIMKA0319 growth.

S. No.	Parameter	Optimal Condition	Growth Response
1.	Growth Medium	Potato Dextrose Agar (PDA)	Good growth (score 4)
2.	pH	6.5–7.0	Good growth (score 4)
3.	Temperature	22°C	Maximum growth (score 5)
4.	Shaking Speed	150 rpm	Maximum growth (score 5)

The findings from the experimental trails proven that the *Cordyceps militaris* RPIMKA0319 exhibited greater mycelial proliferation, and improved fruiting body development and higher cordycepin yield in ensuing cultivation stages.

3.2. Fruiting body development and Solid-State fermentation

In the optimization of seed culture, the standardized parameters were applied with solid-state fermentation (SSF), to better understand the effectiveness of the variation of physicochemical parameters in the study with respect to culture growth. The nutritionally balanced nutrient broth was selected for mycelial colonization and fruiting body proliferation, and which was supplemented with brown rice. The cultures were incubated at 22°C in dark condition to promote rapid substrate colonization and

mycelial initiation. Following exposure to light (800–1000 lux, 12 h/day) triggered synchronized fruit body initiation. To complete the cycle of fruiting body development and with mature stromata displaying characteristic orange pigmentation and cylindrical morphology takes 50–55 days. The biological efficiency (B.E.) of cultivation ranged from 90–110%, positioning with earlier findings [7, 8]. The findings revealed that the solid-state fermentation (SSF) not only enhances mycelial and metabolite yield but also improves reliability in morphology and uniform growth. To prevent the contamination and assuring the uniformity in the cultural condition the controlled humidity (70–75%) and moderate aeration were essential factors. The optimized conditions and methods can be considered as the rapid protocol or standard operating procedure for the culturing of *Cordyceps militaris*. The protocol helpful in industrial production of cordyceps since its proven the validation parameters while ensuring, reproducibility, scalable approach to sustainable in in-vitro cultivation of *cordyceps militaries*.

3.3. Phytochemical screening of In-Vitro of Cultivated *Cordyceps militaris*

3.3.1. Proximate and Physicochemical Analysis

The nutritional factors were estimated by proximate analysis of the cultivated *Cordyceps militaris* RPIMKA0319. The proximate analysis provided insight into its nutritional quality and suitability as a functional food ingredient. By using thermogravimetric method the ash content was quantified, total ash content was 3.81% w/w, water-soluble ash 2.90% and acid-insoluble ash was 0.56% w/w. The ash content established that low contamination with inorganic residues, and moderate mineral content confirming the overall purity of the in-vitro cultured *Cordyceps militaris*. The loss drying was below 5% both in advanced halogen moisture analysis 2.80% w/w and conventional oven drying methods 3.06% w/w confirm the low residual moisture and good shelf stability. Comparable observations have been reported in existing researchers ensure the low moisture content and controlled ash values confirm to boost the biochemical stability of *Cordyceps militaris* fruiting bodies during storage and processing [8, 14]. Proximate analysis confirms the amount of nutrients found in the *Cordyceps militaris* the protein content was found 59.55% w/w, carbohydrates 37.65% w/w and lipids 7.57% w/w. This specifies that the *Cordyceps militaris* is a protein-rich and low-fat functional ingredient, suitable for nutraceutical and applications. Liu et al. and Johri et al. also reported that the high protein content is consistent with the metabolic accumulation of enzymatic and structural proteins during mycelial growth [5, 7]. The carbohydrate content reflects both structural polysaccharides and soluble sugars, which serve as energy reserves for metabolite formation and mycelial development.

3.3.2. Qualitative Phytochemical Screening

The Qualitative Phytochemical Screening of the *Cordyceps militaris* RPIMKA0319 extract revealed the presence of different bioactive compounds which proven

curative benefits as shown in Table 2. The qualitative tests confirm the presence of flavonoids, phenolic compounds, amino acids, proteins, carbohydrates, reducing sugars, and terpenoids. The reaction also proven that the high concentration of antioxidant molecules flavonoids and phenolic compounds were shown strong reactions with the alkaline and alcoholic ferric chloride reagent. The wild and invitro cultivated *Cordyceps militaris*, shows identical chemical profiles which protective effects against oxidative stress and inflammation of the fungus [1, 12, 35, 36, 37].

Table 2. Qualitative phytochemical screening of *Cordyceps militaris*

S. No	Test	Sample (Hydro alcoholic extract of 65:35)	Reagent	Observation	Remarks
1	Flavonoids	2ml of extracted sample	Alkaline reagent	Yellow color to colorless	Positive
2	Phenolic compounds	2ml of extracted sample	Alcoholic Ferric chloride	Bluish green	Positive
3	Amino acids	2ml of extracted sample	Ninhydrin reagent	Violet color	Positive
4	Proteins	2ml of extracted sample	Concentrated nitric acid and 40% NaOH	Orange color	Positive
5	Carbohydrates	2ml of extracted sample	Barfoed's reagent	Reddish brown precipitate	Positive
6	Reducing sugars	2ml of extracted sample	Benedict's solution	Red precipitate	Positive
7	Terpenoids	2ml of extracted sample	Chloroform and concentrated sulfuric acid	Reddish brown	Positive

3.3.3. Quantative analysis of bioactive compounds.

The validated quantitative methods were used for the estimation of the bioactive compounds in the in-vitro cultured *Cordyceps militaris*. The total flavonoid assay was found to be 0.81% w/w, and the total phenolic content was 14.36% and total polyphenols assay was 13.61% w/w. The assay content of the tannin c was 0.63% w/w, and total saponin was 0.76% w/w and bitter content ws 0.11%

w/w. From the qualitative analysis the *Cordyceps militaris* ensures a rich phytochemical profile comparable to naturally occurring strains. From the group assay of total flavonoids and phenolic compounds reflects a high antioxidant capacity, which has been associated with enhanced cellular protection and free radical scavenging activity [1, 13]. The gravimetric assay of saponins and bitters confirms the *Cordyceps militaris* fruiting bodies and having the favorable palatability with respect to taste and odor compared with other medicinal fungi this favors food formulators.

The wide range of phytochemicals were observed in the cultivated strain of the *cordyceps militaries*; the bioactive compounds underscore the nutritional and therapeutic potential particularly the coexistence of phenolics, flavonoids, proteins, and amino acids. The analytical profile confirms that the in-vitro culture system continued the metabolic efficacy and optimal production of both primary and secondary metabolites essential for its nutraceutical purposes. The complete analytical characterization of *Cordyceps militaris* RPIMKA0319 enriched under optimized solid-state conditions allies closely with the compositional data reported for high-quality commercial strains in East Asia [12, 14]. The reliability relating proximate composition, physicochemical purity and phytochemical richness, authenticates the cultivation and extraction protocols applied in this study. The wide range of phytochemicals have the strong potential use as dietary supplements, functional foods supplements, as well as pharmaceutical formulations. The bioactive compounds of carbohydrates, proteins, flavonoids, and polyphenols reflect a synergistic nutritional as well as therapeutic model. The in-vitro cultivated *Cordyceps militaris* established high nutrient density, exceptional physicochemical stability, and a wide-ranging of bioactive composites attributes that confirm its feasibility as a consistent, high-value substitute to wild-harvested samplings.

3.3.4 Extraction Yield and Standardization

The extraction yields of the *Cordyceps militaris* RPIMKA0319 varied significantly depending on the solvent polarity table 3 tabulated the yield of the different solvent extraction. The yield of the successive solvent extraction tabulated was ranging from 2.9% to 8.3% (w/w), through the hexane extract showing the highest recovery (8.3%), followed by water (7.6%), ethanol (6.2%), acetone (4.44%), ethyl acetate (4.2%), and chloroform (2.9%). The comparatively high yield obtained with hexane implies the presence of non-polar and semi-polar compounds such as sterols, fatty acids, and lipophilic pigments in the mycelium. The substantial yield was observed with water and ethanol, which can be attributed to their capability to dissolve polar components such as polysaccharides, nucleosides, amino acids, and phenolic compounds. The *Cordyceps militaris* requires a wide range of solvents to recovery of metabolite since it contains both water-soluble, and lipid-soluble metabolites [2, 31]. The moderate yield was observed with acetone and ethyl

acetate (4.2–4.44%) specify that mid-polar compounds, with glycosides and flavonoids are found in lower concentrations. There was the low yield observed with the chloroform extraction (2.9%) indicates a limited proportion non-polar compounds in the sample matrix. Chemical heterogeneity was observed with the combined extraction process, and across-the-board varied classes of metabolites distributed across different polarity ranges. The complete extraction yield outline (hexane > water > ethanol > acetone > ethyl acetate > chloroform) associates with previous, confirming that solvent polarity plays a significant role in determining extraction productivity and chemical composition [5, 13]. The hydro-ethanolic extraction (ethanol and water) was effective for recovering the nutraceutical components as they preserve hydrophilic polysaccharides and polar phenolics and without any chemical degradation. The standardization of the extracts determined that all fractions exhibited a uniform physical parameter of color and odor. The color ranges from pale yellow to deep orange, with characteristics of earthy odor. The moisture content of each extract was maintained below 5% to maintain the stability of the extract powder. After all the results, the hydroalcoholic and aqueous fractions were selected for exhaustive analytical characterization due to their abundance of pharmacologically active compounds and higher yields.

The optimized extraction process was indicated in this study ensures the effective recovery of both

polar and non-polar constituents of *Cordyceps militaris*. This optimization strategy enables the complete assessment of its nutritional and therapeutic potential. The optimized solvents system established validated protocols by ensuring the reproducibility for *Cordyceps militaris*-based functional food and pharmaceutical formulations.

Table 3. Percentage yield of different cordyceps solvent extraction

S. No.	Cordyceps Abbreviation	% Yield of Extraction (G)
1.	Hexane Cordyceps Extract	8.3
2.	Chloroform Cordyceps Extract	2.9
3.	Ethyl acetate Cordyceps Extract	4.2
4.	Acetone Cordyceps Extract	4.44
5.	Ethanol Cordyceps Extract	6.2
6.	Water Cordyceps Extract	7.6

3.4. Analytical Characterization of Bioactive molecules in *Cordyceps militaris*.

3.4.1. Quantitative Estimation of Adenosine and Cordycepin by High-Performance Liquid Chromatography (HPLC) Analysis

The different solvent extracts of the *Cordyceps militaris* RPIMKA0319 was analyzed in the High-performance

liquid chromatography (HPLC) for the quantification of the adenosine and cordycepin content.

The HPLC chromatograms shown in Figure 8 showed the separation of Adenosine and Cordycepin molecules in different solvent extracts. The summarized results are tabulated in Table 4. Including all the solvent fractions analyzed, the ethyl acetate extract demonstrated the highest concentrations of both adenosine (1.20%) and cordycepin (3.73%). The moderate amount of cordycepin (1.65%), trace amounts adenosine (0.05%) detected in chloroform extract. In the hexane and acetone extracts the assay content was below detection limit displayed negligible also the compounds indicating their limited polarity and lowered efficacy in extracting nucleoside metabolites. 0.11% adenosine and 0.55% cordycepin were found in the hydroalcoholic (65:35 ethanol: water) extract which shows signifying partial solubility of these polar compounds in mixed solvents. The results confirm that the extraction efficiency and yield depend on the solvent polarity.

Cordycepin and adenosine, being moderately polar, are better extracted in solvents of intermediate polarity, such as ethyl acetate, rather than in nonpolar or highly aqueous systems. The previous findings also reported that the extractive values depend on the variation of solvent and ratio of the solvent mixtures. Compare to the other solvents ethyl acetate and methanol extracts progressively yield higher concentrations nucleosides [32]. The high concentration of cordycepin in ethyl acetate fraction entails better purine metabolism during mycelial development, as cordycepin is biosynthesized through the deamination and ribose modification of adenosine precursors. The low adenosine level was found across the most solvent extracts can also indicate its enzymatic conversion to cordycepin through secondary metabolite development [2, 3]. The HPLC results prove that the ethyl acetate extract is the most pharmacologically enriched fraction with high yield of the assay content. The separate chromatographic peaks of adenosine and cordycepin, observed respectively at 4.5 and 5.3 min, confirms both the accuracy and reproducibility of the established HPLC method.

Figure 8. Representative HPLC chromatograms of *Cordyceps militaris* extracts obtained using different solvents (a) standard, (b) hexane, (c) chloroform, (d) ethyl acetate, (e) acetone, (f) ethanol, (g) hydroalcoholic mixture and (h) water

Table 4. Quantitative estimation of Adenosine and Cordycepin in *Cordyceps militaris* extracts Chromatograms.

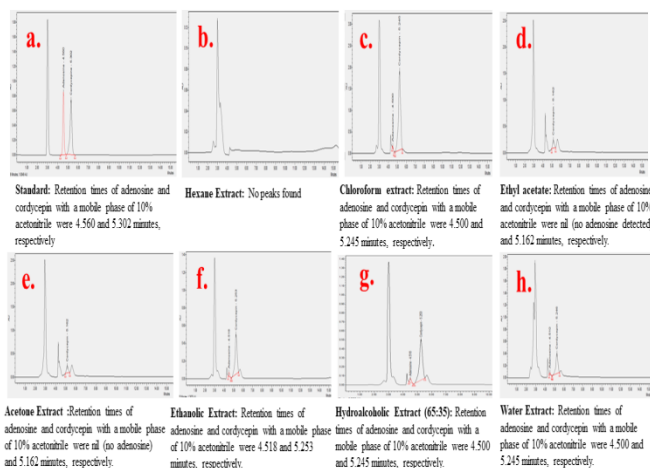
S. No.	Extract	Adenosine (%)	Cordycepin (%)
1.	Hexane	Nil	Nil
2.	Chloroform	0.05	1.65
3.	Ethyl acetate	1.20	3.73
4.	Acetone	Nil	0.10
5.	Ethanol	0.09	0.19
6.	Water	0.07	0.10
7.	Hydroalcoholic (65:35)	0.11	0.55

The overall extraction optimization process resultant in the high yielding of the nucleoside based bioactive compounds and the HPLC methods proven the reproducibility of the assay values. The earlier reports underlined that solvent selection is an essential component for achieving high yields of pharmacologically significant active [33]. The process is completely given the operating procedure about the optimization of the extraction process and analytical characterization with proven validation features. Overall, the study confirmed that the *Cordyceps militaris* extracts can be considered as the nutraceutical and pharmaceutical ingredients. Additionally, the experimental correlation between adenosine and cordycepin levels emphasizes the biosynthetic interrelationship of these compounds and highlights the potential of culture optimization strategies to boost cordycepin yield through metabolic parameters.

3.4.2. GC-MS and LC-MS profiling of Hydroalcoholic Extract of *Cordyceps militaris*

3.4.2.1. GC-MS Analysis

The GC-MS analysis of the hydroalcoholic extract of *Cordyceps militaris* elucidated that the extract contains both volatile and semi-volatile constituents. The GC-MS ion chromatogram as shown in figure 9, showed in around 80 diverse compounds, containing various chemical groups such as esters, amines, fatty acids and their esters, nitrogenous compounds, carboxylic acids, aldehydes, and alcohols. Previous studies correspondingly described that the *Cordyceps militaris* reflects the complex and varied metabolic profile [1, 34]. The vivid range of therapeutically active compounds were distinguished, with Octodrine, Dihydromorphine, Pregnenolone, Methylene butanedioic acid, and Zidovudine (AZT). Many bioactive compounds known for their anticancer, antimicrobial, and anti-inflammatory properties were identified. Along with sterol derivatives such as Stigmasta-3,5-diene and Androstenediol diacetate were also found, demonstrating



the presence of bioactive steroids which influence anti-aging and immunomodulatory effects. From the findings from the GC-MS analysis summarized in Table 5, while Tables 6 and 7, rich in anti-cancer metabolites and anti-inflammatory and antimicrobial compounds, respectively. These results indicate that *Cordyceps militaris* can be considered as the promising ingredients with multifunctional bioactive molecules which synergistically influence to its pharmaceutical as well as efficiency as reported in traditional medicine systems.

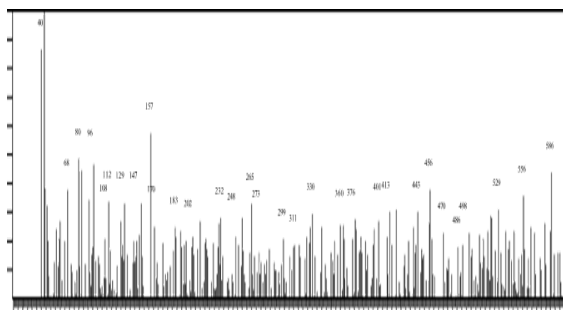


Figure 9. GC-MS chromatogram of hydroalcoholic extract of *Cordyceps militaris*

Table 5. Anticancer-related bioactive compounds identified in *Cordyceps militaris*

Peak No.	Compound Name	Molecular Formula	Molecular Weight (g/mol)	Retention Time (min)	Peak Area (%)	Reported Bioactivity	Reference/Library Match
1	Octodrine	C8H19N	129.24	5.32	3.12	CNS stimulant; anticancer adjuvant	NIST/Wiley
2	Nitro pyrimidine	C4H3N3O2	125.08	6.11	2.65	Antitumor; DNA intercalator	NIST/Wiley
3	Bromopyridopyrimidine	C8H5BrN4	253.06	7.08	1.74	Antiproliferative; cytostatic	Wiley
4	Dihydro morphine	C17H21NO3	287.35	7.21	4.05	Anticancer; analgesic	NIST
5	Bromosuccinic acid	C4H5BrO4	197.98	7.89	2.44	Antimetastatic; detoxifier	NIST
6	Nitro pyrazole	C3H3N3O2	113.07	8.03	2.15	Nitrogenous heterocycle with antiproliferative activity	NIST
7	Chloroacetylpiperidine	C7H6ClNO	155.58	8.36	1.88	Anticancer; enzyme inhibitor	Wiley
8	Dimethyl nitro pyridone	C7H8N2O3	168.15	8.75	3.01	Antioxidant; antimutagenic	NIST
9	Dimethyl acetate	C6H10O3	130.14	9.04	1.72	Cytoprotective; metabolic modulator	NIST
10	MDA acetate	C4H6O3	102.09	9.42	2.56	Antioxidant; apoptosis inducer	Wiley
11	Chloropyridazine	C4H3ClN2	130.53	9.88	1.93	Antitumor; alkylating agent	NIST
12	Dichloronitrothiophene	C4HCl2NO2S	198.02	10.17	2.20	Anticancer; antimicrobial	NIST
13	5-Nitroisophthalic acid	C8H5NO6	211.13	10.64	3.45	Proapoptotic; radical scavenger	Wiley
14	Methylenbutanedioic acid	C5H8O4	132.11	11.03	2.88	Antioxidant; metabolic regulator	NIST
15	HEIDA (Hydroxyethyliminodiacetic acid)	C6H11NO4	161.15	11.37	1.57	Metal chelator; cytoprotective	NIST
16	Diethylaminorhodanine	C7H12N2OS2	204.32	11.59	2.67	Anticancer; antimicrobial	Wiley
17	Pregnenolone	C21H32O2	316.48	11.92	5.14	Anti-inflammatory; antitumor steroid	NIST
18	Androstenediol diacetate	C23H34O4	374.52	12.26	4.02	Cytotoxic; anti-aging	Wiley
19	5-Methylisoxazolidonecarboxylic acid	C5H9NO3	131.13	12.65	2.12	Anticancer; antimutagenic	NIST
20	2-Bromotetracanoic acid	C14H27BrO2	307.27	13.08	3.79	Cytotoxic fatty acid	Wiley

21	Zidovudine (AZT)	C10H13N5O4	267.24	13.52	4.41	Antiviral; anticancer nucleoside	NIST
22	Bisnorcholenic acid	C27H44O2	400.64	13.97	3.20	Anti-angiogenic; steroidal acid	Wiley
23	Acetoacetanilide	C11H13NO4	223.23	14.34	2.08	Antioxidant; antitumor	NIST
24	2'-O-Methylguanosine	C11H15NO5	297.27	14.59	2.93	Anticancer; RNA-modifying nucleoside	NIST
25	(8Z)-Oxacycloheptadec-8-en-2-one	C17H32O2	268.44	15.02	3.18	Cytotoxic macrocyclic lactone	Wiley
26	Phenylthiazolidine carbonylic acid	C10H11NO2S	209.27	15.34	2.41	Antiproliferative; antimicrobial	NIST
27	Imidazoticarboindamide	C2H5N3O2	103.08	15.68	1.69	Cytostatic; nitrogenous heterocycle	NIST
28	Chlorobenzilate	C16H14Cl2O3	325.19	16.12	3.02	Anticancer; anti-oxidative	Wiley
29	Hydroxypyrazine carbonylic acid	C5H4N2O3	140.09	16.47	2.50	Pro-apoptotic; antimetabolic	NIST
30	Pyridolidoneacetamide	C6H12N2O	128.17	16.82	1.93	Anticancer; metabolic inhibitor	NIST
31	Bromacil	C9H13BrN2O2	261.12	17.16	2.22	Cytotoxic; herbicidal analog	Wiley
32	Stigmast-3,5-diene	C29H48	396.68	17.55	6.40	Anticancer; cholesterol modulator	NIST

Table 6. Anti-inflammatory bioactive compounds in *Cordyceps militaris*

Peak No.	Compound Name	Molecular Formula	Molecular Weight (g/mol)	Retention Time (min)	Peak Area (%)	Reported Bioactivity	Reference/Library Match
1	Nitro pyrimidine	C4H3N3O2	125.08	6.11	2.65	Anti-inflammatory; NF- κ B inhibitor	NIST
2	Dihydromorphine	C17H21NO3	287.35	7.21	4.05	Analgasic; suppresses prostaglandin synthesis	Wiley
3	Bromosuccinic acid	C4H5BrO4	197.98	7.89	2.44	Anti-inflammatory; antioxidant	NIST
4	Nitro pyrazole	C3H3N3O2	113.07	8.03	2.15	Inhibits oxidative stress pathways	NIST
5	Chloroacetylpyridine	C7H6ClNO	155.58	8.36	1.88	Cyclooxygenase inhibitor	Wiley
6	Dimethyl nitro pyridinone	C7H8N2O3	168.15	8.75	3.01	Antioxidant and nitric oxide modulator	NIST
7	Dimethyl acetoacetamide	C6H11NO2	129.16	8.89	1.73	Suppresses inflammatory mediator synthesis	NIST
8	MDA acetate	C4H6O3	102.09	9.42	2.56	Lipid peroxidation inhibitor	Wiley
9	Chloropyridazine	C4H5ClN2	130.53	9.88	1.93	Antioxidant; suppresses cytokine release	NIST
10	Dichloro-nitrothiophene	C4HCl2NO2S	198.02	10.17	2.20	Anti-inflammatory; microbial defense molecule	Wiley
11	5-Nitrosophthalic acid	C8H5NO6	211.13	10.64	3.43	Anti-inflammatory; free radical scavenger	NIST
12	Methylene butanedioic acid	C5H8O4	132.11	11.03	2.88	Metabolic anti-inflammatory agent	NIST
13	Diethylamino-rhodanine	C7H12N2OS2	204.32	11.39	2.67	Inhibits TNF- α -mediated inflammation	Wiley
14	5-Methylisoxazolidonecarboxylic acid	C5H9NO3	131.13	12.65	2.12	Antioxidant; suppresses iNOS expression	NIST
15	Phenylthiazolidonecarboxylic acid	C10H11NO2S	209.27	15.34	2.41	COX-2 inhibitor; antipyretic	NIST
16	Hydroxy-pyrazine carboxylic acid	C5H4N2O3	140.09	16.47	2.50	Anti-inflammatory; scavenger of reactive oxygen species	NIST

Table 7. Antimicrobial compounds detected in *Cordyceps militaris*

Peak No.	Compound Name	Molecular Formula	Molecular Weight (g/mol)	Retention Time (min)	Peak Area (%)	Reported Bioactivity	Reference/Library Match
1	Dihydromorphine	C17H21NO3	287.35	7.21	4.05	Antimicrobial; inhibits bacterial quorum sensing	NIST
2	Bromosuccinic acid	C4H5BrO4	197.98	7.89	2.44	Broad-spectrum antibacterial	Wiley
3	Nitro pyrazole	C3H3N3O2	113.07	8.03	2.15	Antibacterial; antifungal agent	NIST
4	Chloroacetylpyridine	C7H6ClNO	155.58	8.36	1.88	Antimicrobial; disrupts microbial cell membrane	Wiley
5	Dimethyl nitro pyridinone	C7H8N2O3	168.15	8.75	3.01	Inhibits bacterial oxidative enzymes	NIST
6	Dimethyl	C6H11NO2	129.16	8.89	1.73	Antifungal;	Wiley

	acetoacetamide					metabolic inhibitor	
7	MDA acetate	C4H6O3	102.09	9.42	2.56	Disrupts bacterial biofilm formation	NI ST
8	Chloropyrazine	C4H3CIN2	130.53	9.88	1.93	Antimicrobial; nitrogen heterocycle	NI ST
9	Dichloro-nitrothiophene	C4HCl2NO2S	198.02	10.17	2.20	Broad-spectrum antimicrobial	Wiley
10	5-Nitroisophthalic acid	C8H5NO6	211.13	10.64	3.45	Antifungal and antibacterial	NI ST
11	Methylbutanedioic acid	C5H8O4	132.11	11.03	2.88	Disrupts bacterial metabolism	NI ST
12	Diethylaminorhodanine	C7H12N2OS2	204.32	11.59	2.67	Antimicrobial; biofilm inhibitor	Wiley
13	5-Methylisoxazolecarboxylic acid	C5H9NO3	131.13	12.65	2.12	Antibacterial; antifungal	NI ST
14	Phenylthiazolidinecarboxylic acid	C10H11NO2S	209.27	15.34	2.41	Antimicrobial; inhibits spore germination	NI ST
15	Hydroxypyrazinecarboxylic acid	C5H4N2O3	140.09	16.47	2.50	Antibacterial; antifungal antioxidant	NI ST

3.4.2.2. LC-MS Analysis

To complement the GC-MS findings, LC-MS profiling was performed to identify non-volatile, thermolabile, and polar constituents of the hydroalcoholic extract. The LC-MS chromatogram depicted in figure revealed 999 distinct molecular features under positive ionization mode, with the most intense peak appearing at RT 1.309 min (m/z 679.527; $[2M+Na]^+$). Most high-intensity peaks eluted between 1.0–1.4 minutes, signifying the dominance of highly polar constituents. Several nucleoside and nucleotide-related compounds characteristic of *Cordyceps* species were observed. Inosine appeared as $[M+Na]^+$ at RT 0.256 min (m/z 291.0710), while 5-methyluridine was

identified at RT 0.825 min (m/z 259.0930). However, adenosine and cordycepinkey pharmacological markers were not distinctly observed under these non-targeted conditions, likely due to ion suppression from sodium adduct formation and the absence of targeted fragmentation (MS/MS). Nevertheless, the LC-MS results aligned with the HPLC quantification data, which indicated that the ethyl acetate fraction contained the highest levels of both adenosine (1.20%) and cordycepin (3.73%). The integration of GC-MS and LC-MS data therefore provides a comprehensive chemical fingerprint of *Cordyceps militaris*, encompassing both volatile and polar metabolite domains. This analytical dual-approach demonstrates the presence of diverse classes of bioactive compounds with potential pharmacological functions supporting earlier reports and highlights the capacity of *Cordyceps militaris* extracts as a potent nutraceutical and functional food ingredient.

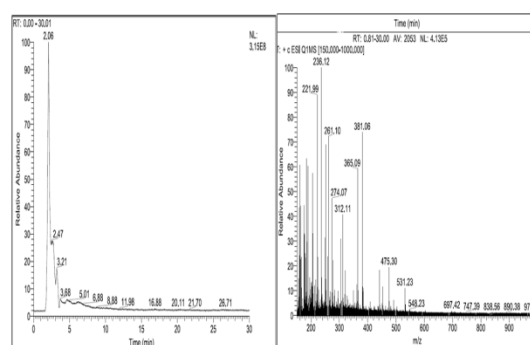


Figure 10. LC-MS chromatogram of hydroalcoholic extract of *Cordyceps militaris*

4. CONCLUSION

The present work confirms that the successful completion of the derivation of the protocol for the in-vitro cultivation of the *Cordyceps militaris* and proves the analytical characterization of the mycelium of the *Cordyceps militaris*. The optimization of the culture condition is done with the different nutrient medium and different conditions and the Potato Dextrose Agar medium at pH 6.0, temperature of 21 °C, and shaking speed of 150 rpm, yielded superior mycelial growth and biomass productivity. To confirm the phytochemicals in the mycelium of the *Cordyceps militaris*, subjected qualitative analysis. The analysis reveals the presence of many active compounds including flavonoid, polyphenols, tannins, and bitters. Proximate analysis *Cordyceps militaris* confirms content of proteins (59.55%), carbohydrates (37.65%), and bioactive secondary metabolites including flavonoids, phenolics, tannins, and saponins. The bio analytical marker was optimized with HPLC the adenosine and cordycepin content was optimized by using different solvent fractions. Out of all the ethyl acetate fraction exhibited the highest amount of adenosine (1.20%) and cordycepin (3.73%). The complete analytical characterization was conducted by using GC-MS and LC-MS. From GC-MS 80 different groups of components were identified and nearly 1000 mass spectral features were confirmed by LC-MS. The hyphenated analytical

techniques confirm the different chemical matrixes of fatty acids, nucleosides, steroids, and alkaloids. The study proven many bio- active components in the *Cordyceps militaris* mushroom are responsible for the anticancer, anti-inflammatory, antimicrobial, and antioxidant activity.

Overall, this study given complete platform for the entrepreneurs, agripreneurs, and pharmaceutical, nutraceutical enthusiasts about the invitro cultivation protocol and confirmation of the bioanalytical marker. The findings further confirm the *Cordyceps militaris* as a biotechnologically viable alternative to wild species of rare mushrooms, supporting its sustainable industrial scale production of high-value functional compounds and active biomarkers.

5. REFERENCES

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