Natural Products Analysis
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Article

Protein-rich Isolate Extracted from *Cordyceps militaris* Fruiting Body and Anticancer Activities on Cancer Cells

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How To Cite: Radapong, S.; Sahad, T.; Harnkit, N.; et al. Protein-rich Isolate Extracted from *Cordyceps militaris* Fruiting Body and Anticancer Activities on Cancer Cells. *Natural Products Analysis* **2026**, *2*(1), 100006. https://doi.org/10.53941/npa.2026.100006

Received: 9 March 2026
Revised: 17 April 2026
Accepted: 22 April 2026
Published: 30 April 2026

Abstract: Cancer remains a global health challenge due to the limitations of conventional therapies, including severe side effects and drug resistance. While *Cordyceps militaris* (L.) Fr. is well-regarded in traditional medicine for its antitumor effects, research has primarily focused on small molecules like cordycepin. The anticancer potential of its total protein-rich isolate remains less characterized. This study aimed to extract, characterize, and evaluate the in vitro cytotoxic activities of the protein-rich isolate from *C. militaris* fruiting bodies against various human cancer cell lines. Proteins were extracted from dried fruiting bodies using an alkaline extraction method (pH 12.0) followed by isoelectric precipitation (pH 7.0) and lyophilization. The isolate was analyzed for yield, protein content, water activity, and moisture. Bioactive markers (cordycepin and adenosine) were quantified using UHPLC. Cytotoxicity was assessed via MTT assay against MCF-7 (breast), HT-29 and Caco-2 (colorectal), and HepG2 (liver) cancer cells, with HDFn (normal fibroblasts) used to determine selectivity. Results showed that the extraction process achieved a high yield of 72.61% with a protein content of 41.90 ± 0.19%. The isolate demonstrated excellent stability with a low water activity (a_w) of 0.22 ± 0.01. Notably, cordycepin was significantly enriched in the isolate, increasing tenfold to 1048 mg/100 g compared to the raw powder. The protein-rich isolate showed dose-dependent cytotoxicity, with the highest potency against MCF-7 cells (IC_{50} = 8.68 μg/mL). Crucially, the isolate exhibited promising selectivity in this model, being nearly 55 times less toxic to normal HDFn cells (IC_{50} = 475.56 μg/mL). It is concluded that *C. militaris* protein-rich isolate is a stable, bioactive-rich fraction with potent and selective anticancer activity. The significant enrichment of cordycepin and the wide therapeutic window suggest its potential as a safe candidate for functional foods or adjuvant cancer therapies.

Keywords: *Cordyceps militaris*; protein-rich isolate; cordycepin; anticancer activity; cytotoxicity

1. Introduction

Cancer remains one of the leading causes of mortality and morbidity worldwide, presenting a formidable challenge to global public health systems. Despite significant advancements in conventional therapeutic modalities, such as chemotherapy, radiotherapy, and surgical intervention, the prognosis for many malignancies remains poor. Furthermore, standard treatments are often associated with severe adverse effects, multidrug

resistance, and non-specificity toward normal cells [1]. Consequently, there is an urgent and continuous demand for the discovery of novel antineoplastic agents that offer high efficacy with reduced toxicity. In recent decades, natural products have emerged as a prolific source of bioactive compounds, with a significant proportion of modern clinical drugs being derived from identifying active ingredients in traditional medicines [2].

Among natural sources, medicinal mushrooms have garnered considerable attention for their pharmacological potential. *Cordyceps militaris* (L.) Fr., an entomopathogenic fungus belonging to the Clavicipitaceae family, has been utilized for centuries in Traditional Chinese Medicine (TCM) as a tonic for longevity and endurance [3]. Unlike its rarer counterpart, *Ophiocordyceps sinensis*, *C. militaris* can be successfully cultivated on a large scale using artificial media, making it a sustainable and economically viable source of bioactive metabolites. Extensive research has documented various pharmacological activities of *C. militaris*, including immunomodulatory, anti-inflammatory, antioxidant, and antitumor effects [4,5].

To date, the majority of research on the anticancer properties of *C. militaris* has focused on its small molecule compounds, such as cordycepin (3′-deoxyadenosine), adenosine, and various polysaccharides [6]. However, the bioactive potential of fungal proteins and peptides is an emerging field of interest. Mushroom proteins, including lectins, laccases, and fungal immunomodulatory proteins (FIPs), have demonstrated promising antitumor capabilities through mechanisms such as the induction of apoptosis, cell cycle arrest, and inhibition of angiogenesis [7]. Despite this, the specific anticancer activities of the total protein-rich isolate extracted specifically from the fruiting body of *C. militaris* remain less characterized compared to its polysaccharide or nucleoside counterparts.

The protein fraction of medicinal mushrooms represents a complex pool of macromolecules that may exert cytotoxic effects via distinct molecular pathways. Investigating these protein-rich isolates is crucial for a holistic understanding of the mushroom's therapeutic value and for the potential development of protein-based functional foods or nutraceuticals.

Therefore, the objective of this study was to extract and analyze the protein-rich isolate from the fruiting bodies of *C. militaris* and to evaluate its in vitro anticancer activities against selected human cancer cell lines. We assessed the cytotoxicity of the protein-rich isolate providing scientific evidence to support the potential application of *C. militaris* proteins as novel alternative agents in cancer management.

2. Materials and Methods

2.1. Chemicals and Reagents

Adenosine (purity ≥ 99%) was purchased from Sigma Aldrich (Wuxi, China). Cordycepin (purity ≥ 99.18%) was obtained from Fermentek (Jerusalem, Israel). HPLC-grade methanol was purchased from Fisher Scientific (Seoul, Republic of Korea). Ultrapure water was obtained using a Millipore water purification system (Elix and Synergy, Merck Millipore). All other chemicals and reagents used were of analytical grade.

2.2. Mushroom Materials

Dried fruiting bodies of *C. militaris* were obtained from VIP Inter Health Co., Ltd. (Songkhla, Thailand). The samples were cultivated under controlled conditions and certified by the International Federation of Organic Agriculture Movements (IFOAM). The dried samples (moisture content 5.87%) were packaged in aluminum zip-lock bags (approximately 1 kg per bag). The samples were authenticated by Mr. Sumate Sanchoo, Songkhla, Thailand, October 2024.

2.3. Preparation of Protein-Rich Isolate

The dried *C. militaris* fruiting bodies were ground into a fine powder and passed through a 100-mesh sieve. Protein extraction was performed using an alkaline extraction method. Briefly, the mushroom powder (100 g) was mixed with distilled water at a ratio of 1:20 (*w*/*v*). The mixture was homogenized using an overhead stirrer, and the pH of the solution was adjusted to 12.0 measured using a digital pH meter (Mettler Toledo, Greifensee, Switzerland) using appropriate alkaline reagents to facilitate protein solubilization. Elevating the pH to 12 impart a strong negative charge to the proteins, enhancing their solubility and facilitating their separation from insoluble cell wall components. The mixture was then centrifuged to separate insoluble debris. The supernatant containing the solubilized proteins was collected, and its pH was adjusted to neutral (pH 7.0) using 1 N hydrochloric acid (HCl). The resulting solution was subjected to freeze-drying (lyophilization) to yield the final *C. militaris* protein-rich isolate powder.

2.4. Physicochemical Characterization of Protein-Rich Isolate

The physicochemical properties of the obtained protein-rich isolate were evaluated in triplicate. The extraction yield was calculated as a percentage of the weight of the freeze-dried protein-rich isolate relative to the initial weight of the mushroom powder. Water activity (a_w) was determined using a dew point water activity meter (Model 4TE, AQUA LAB, Bangkok, Thailand) at 25 °C. Moisture content was analyzed using a moisture analyzer (Model IR-35M, Denver Instrument, Bangkok, Thailand). Total protein content was quantified based on the Dumas combustion principle using an automated nitrogen/protein analyzer (Model FP-528, LECO, Bangkok, Thailand).

2.5. Phytochemical Analysis

2.5.1. Sample Extraction for UHPLC

To quantify bioactive markers, 1 g of the finely ground sample (each of the mushroom powder and the isolated protein powder) was weighed into a 50 mL polypropylene copolymer (PPCO) oak ridge centrifuge tube. Precisely 25 mL of a water: methanol solvent mixture (95:5, *v*/*v*) was added, and the tube was tightly capped. The mixture was vortexed for 1 min and subsequently extracted via ultrasonication for 30 min. After cooling to room temperature, the mixture was centrifuged at 4000 rpm for 10 min at 4 °C. The supernatant was filtered through a 0.2 μm nylon filter with a glass microfiber membrane (GMF) prior to Ultra High-Performance Liquid Chromatography (UHPLC) analysis.

2.5.2. Liquid Chromatography Instrumentation and Conditions

Chromatographic analysis was performed using a Waters ACQUITY UPLC H-class system equipped with a quaternary solvent manager, a sample manager (flow-through needle, SM-FTN), a column heater, and an eλPDA photodiode array detector. Separation was achieved on an ACQUITY UPLC BEH C18 column (50 mm × 2.1 mm i.d., 1.7 μm particle size). Data acquisition and processing were controlled by Empower software (Waters).

The mobile phase consisted of water and methanol in a volume ratio of 95:5, delivered via isocratic elution at a flow rate of 0.4 mL/min. The injection volume was set at 1 μL. The analytes (adenosine and cordycepin) were detected at a wavelength of 260 nm.

2.5.3. Method Validation

The analytical method for the determination of bioactive substances was validated in accordance with the Eurachem Guide on method validation [8]. The linearity of the external standard calibration curve was confirmed with a coefficient of determination (R^2) greater than 0.995. The limits of detection (LOD) and quantification (LOQ) for the target analytes are presented in Table 1. Internal quality control measures included the analysis of solvent blanks, duplicate samples, and spiked samples to ensure method reliability.

Table 1. Limit of Detection (LOD) and Limit of Quantification (LOQ) of the analytical method.

Analytes	LOD (mg/100 g)	LOQ (mg/100 g)
Adenosine	10	40
Cordycepin	4	10

2.6. Cell Viability Assay (MTT Assay)

Cytotoxicity was evaluated using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. Cells were seeded at a density of 5×10^3 cells in 100 μL of medium per well in 96–well plates and incubated for 24 h to allow attachment. The culture medium was then removed, and cells were treated with eight different concentrations ranging from 3.91–500 μg/mL of the protein-rich isolate in triplicate. The plates were incubated for an additional 72 h. Following treatment, the medium was replaced with 200 μL of fresh medium containing MTT reagent, and incubation continued for 4 h. The MTT solution was subsequently discarded, and 200 μL of dimethyl sulfoxide (DMSO) was added to each well to dissolve the purple formazan crystals. The absorbance of the dissolved formazan, proportional to the number of viable cells, was measured at 570 nm using a microplate reader. Background absorbance was subtracted using a blank, and the percentage of cell viability was calculated relative to the untreated control [9].

2.7. Statistical Analysis

The half-maximal inhibitory concentration (IC_{50}) was calculated using nonlinear regression analysis (curve fit) with GraphPad Software (La Jolla, CA, United States). Data are expressed as mean of triplicate experiments (n = 3).

3. Results

3.1. Physical Characteristics and Extraction Yield of Protein-Rich Isolate

The physical appearance of the initial *C. militaris* powder and the extracted protein-rich isolate is illustrated in Figure 1. The raw mushroom powder exhibited an agglomerated, granular texture (Figure 1A). This aggregation is likely attributable to the natural composition of the fungal fruiting bodies, which are rich in fibers and polysaccharides that tend to clump upon exposure to atmospheric moisture. In contrast, the protein-rich isolate obtained after extraction appeared as a fine, dark yellow powder (Figure 1B). This textural refinement is a result of the lyophilization (freeze-drying) process. Lyophilization involves the sublimation of frozen water directly into vapor under reduced pressure, effectively removing moisture without damaging the heat-sensitive protein structure, thereby yielding a fine, non-hygroscopic powder.

(**A**) (**B**)

Figure 1. The physical appearance of the initial *C. militaris* powder (**A**) and the extracted protein-rich isolate (**B**).

The extraction yield and physicochemical properties of the *C. militaris* protein-rich isolate are summarized in Table 2. From an initial starting material of 1008.00 g of dried mushroom powder, 731.94 g of protein-rich isolate was recovered, corresponding to a high extraction yield of 72.61%.

Table 2. Extraction yield and physicochemical properties of *C. militaris* protein-rich isolate.

Physical Characteristics	Value (Mean ± SD)
Initial Mushroom Powder Weight (g)	1008.00
Protein-rich isolate Weight (g)	731.94
Yield (%)	72.61
Protein Content (%)	41.90 ± 0.19
Water Activity (a_w)	0.22 ± 0.01
Moisture Content (% dry basis)	5.18 ± 0.36

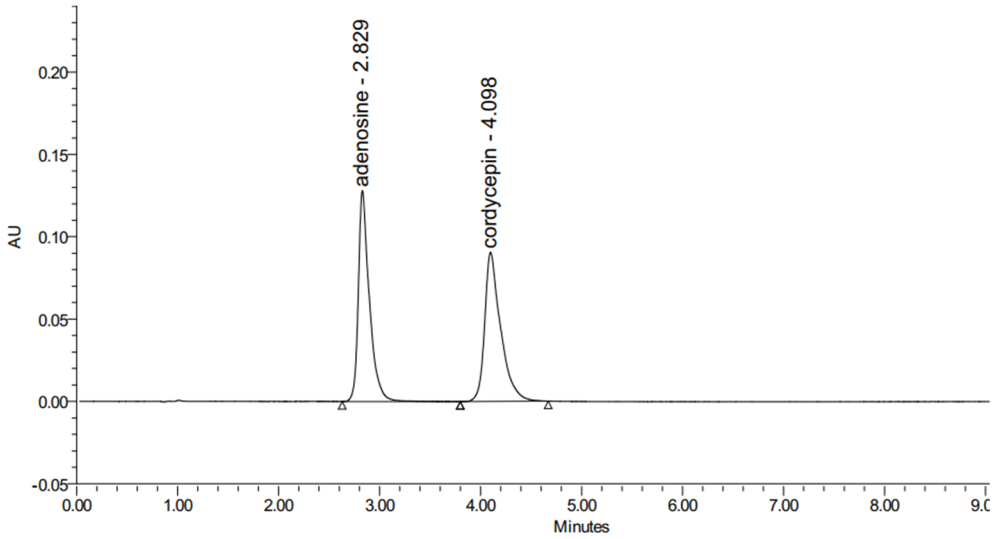
Note: Values are expressed as mean ± standard deviation (n = 3).

The analysis revealed that the protein-rich isolate contained a substantial protein content of 41.90 ± 0.19%. In terms of stability, the moisture content was found to be 5.18 ± 0.36% (dry basis). This value falls well within the acceptable range for dried food products (typically 10–20%), suggesting good storage stability. Furthermore, the water activity (a_w) was determined to be 0.22 ± 0.01. This low water activity is critical for preventing the proliferation of spoilage microorganisms, particularly yeasts and molds, thereby ensuring the microbiological safety and extended shelf-life of the protein-rich isolate.

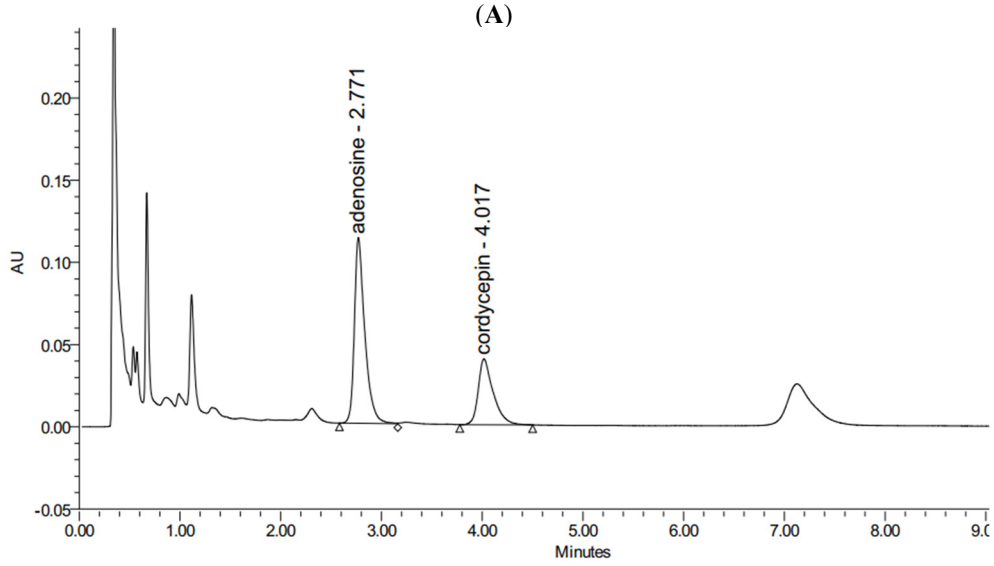
3.2. Quantification of Bioactive Markers (Cordycepin and Adenosine)

To verify the quality and chemical composition of the samples, the mushroom powder and protein-rich isolate were analyzed for its content of two primary bioactive markers found in *Cordyceps militaris*: cordycepin and

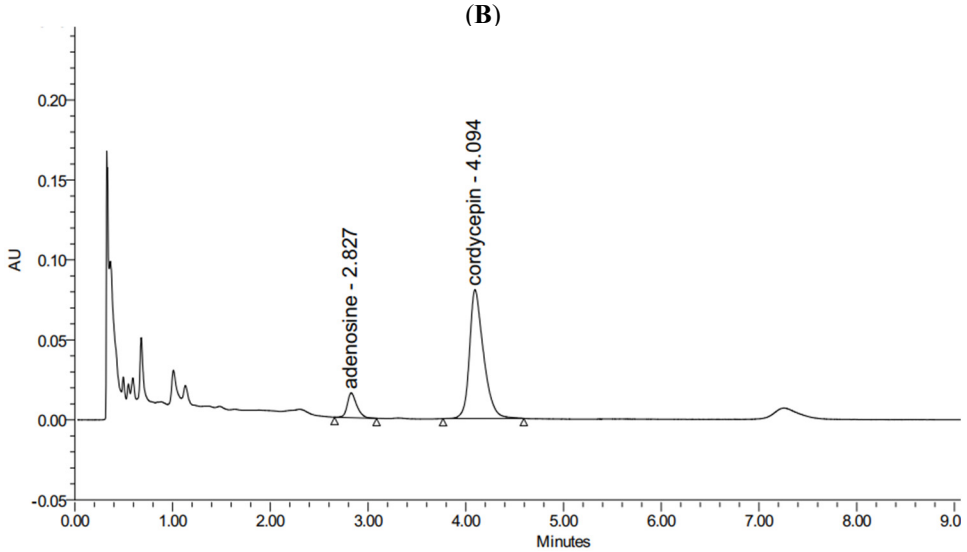
adenosine. The chromatograms of the standards and those in the mushroom powder and protein-rich isolate are shown in Figure 2. The quantitative results, comparing the initial mushroom powder and the protein-rich isolate, are presented in Table 3.



(**A**)



(**B**)



(**C**)

Figure 2. Chromatograms of 100 µg/mL cordycepin and 100 µg/mL adenosine (**A**), 40 mg/mL mushroom powder (**B**) and 40 mg/mL protein-rich isolate(**C**), inj. vol. of 1 µL, monitored at 260 nm.

The analysis revealed a marked enrichment of cordycepin in the protein-rich isolate in Table 3. The concentration of cordycepin increased significantly from 103 mg/100 g in the raw mushroom powder to 1048 mg/100 g in the protein-rich isolate. Conversely, the adenosine content showed a reduction, decreasing from 216 mg/100 g in the raw powder to 104 mg/100 g in the protein-rich isolate. This shift suggests that the alkaline extraction and precipitation process utilized in this study may selectively concentrate cordycepin while potentially removing adenosine or retaining it in the discarded supernatant fraction.

Table 3. Content of Cordycepin and Adenosine in *C. militaris* mushroom powder and protein-rich isolate.

Sample	Cordycepin (mg/100 g)	Adenosine (mg/100 g)
C. militaris Mushroom Powder	103	216
C. militaris Protein-rich isolate	1048	104

3.3. In Vitro Cytotoxicity of Protein-Rich Isolate

The anticancer potential of the *C. militaris* protein-rich isolate was evaluated by determining its cytotoxicity against a panel of human cancer cell lines, including breast adenocarcinoma (MCF-7), colorectal adenocarcinoma (HT-29 and Caco-2), and hepatoblastoma (HepG2). Additionally, the selectivity of the protein-rich isolate was assessed by testing its toxicity on normal Human Dermal Fibroblasts (HDFn).

The results, expressed as half-maximal inhibitory concentrations (IC_{50}), are summarized in Table 4. The protein-rich isolate demonstrated a dose-dependent inhibitory effect on the proliferation of all tested cancer cell lines. Notably, the isolate exhibited the highest potency against the MCF-7 breast cancer cell line, achieving the lowest IC_{50} value of 8.68 μg/mL. Moderate cytotoxicity was observed in HT-29 and HepG2 cells, with IC_{50} values of 17.42 μg/mL and 34.05 μg/mL, respectively. In contrast, the Caco-2 cell line showed significantly lower sensitivity, with an IC_{50} value of 159.01 μg/mL.

Crucially, the protein-rich isolate showed very low toxicity toward normal human fibroblast cells (HDFn), with an IC_{50} value of 475.56 μg/mL. This high IC_{50} value in normal cells, compared to the much lower values in cancer cells (particularly MCF-7), indicates a high degree of selectivity. This finding suggests that the *C. militaris* protein-rich isolate targets cancer cells preferentially while sparing normal healthy cells, a desirable characteristic for potential therapeutic agents.

Table 4. IC_{50} values of *C. militaris* protein-rich isolate against selected human cancer and normal cell lines.

Cell Lines	Cell Type	IC_{50} (μg/mL)
MCF-7	Human breast adenocarcinoma	8.68
HT-29	Human colorectal adenocarcinoma	17.42
HepG2	Human hepatoblastoma	34.05
Caco-2	Human colorectal adenocarcinoma	159.01
HDFn	Human Dermal Fibroblasts (Normal)	475.56

4. Discussion

In this study, we successfully extracted and characterized a protein-rich fraction from *C. militaris* fruiting bodies and evaluated its anticancer potential. The extraction of protein from *C. militaris* using an alkaline-isoelectric precipitation method (pH 12.0 to 7.0) is a highly effective strategy for generating a stable, bioactive-rich isolate. While the extreme alkalinity at pH 12.0 is essential for disrupting the complex chitinous cell walls and maximizing protein solubility through electrostatic repulsion, it is acknowledged that such conditions may induce partial protein denaturation or structural unfolding [10]. However, the process yielded a significant amount of protein-rich isolate (72.61%) with a protein content of 41.90%, confirming the efficiency of the alkaline extraction method followed by isoelectric precipitation. The resulting isolate exhibited low water activity (a_w = 0.22), indicating excellent stability and resistance to microbial spoilage, which are essential attributes for the development of functional food ingredients or pharmaceutical preparations.

A pivotal finding of this research is the substantial enrichment of cordycepin within the protein-rich isolate. The concentration of cordycepin increased approximately tenfold, from 103 mg/100 g in the raw mushroom powder to 1048 mg/100 g in the protein-rich isolate. Cordycepin is a well-established anticancer agent known to interfere with RNA synthesis and induce apoptosis [11]. The enrichment observed suggests that the protein extraction process simultaneously concentrates this bioactive nucleoside, possibly due to non-covalent binding with fungal proteins or co-precipitation during the pH adjustment. Conversely, adenosine content decreased,

implying that adenosine may be more soluble in the discarded supernatant or less stable under the alkaline conditions employed.

The biological evaluation demonstrated that the protein-rich fraction or total isolate (a combination of bioactive proteins and enriched nucleosides) possesses potent and selective anticancer activity. The isolate was particularly effective against MCF-7 human breast adenocarcinoma cells, with an IC_{50} of 8.68 μg/mL. This high potency suggests that the breast cancer cell line is highly susceptible to the bioactive constituents of the fraction. The protein-rich isolate was found to be significantly enriched with Cordycepin, a bioactive nucleoside documented to induce apoptosis in malignant cells by concurrently activating the extrinsic (death receptor) and intrinsic (mitochondrial) pathways. This programmed cell death was mediated through the activation of key initiator and executioner enzymes, specifically caspase-3, -8, and -9 through p38/JNK signaling pathway or ERK/Slug signaling pathway [7,12,13]. The isolate also showed moderate activity against HT-29 colorectal and HepG2 liver cancer cells. The lower sensitivity of Caco-2 cells (IC_{50} 159.01 μg/mL) compared to HT-29 cells highlights the heterogeneity of cancer cell responses, even within the same tissue type (colorectal), and underscores the need for targeted therapeutic approaches.

Perhaps the most significant outcome of this study is promising selectivity of the protein-rich fraction. Current cancer chemotherapies are often limited by their toxicity to healthy tissues [1]. In our study, the protein-rich isolate exhibited very low cytotoxicity toward normal human dermal fibroblasts (HDFn), with an IC_{50} value (475.56 μg/mL) that was nearly 55 times higher than that for MCF-7 cells. This wide therapeutic window indicates that the protein-rich isolate can effectively inhibit cancer cell proliferation at concentrations that are safe for normal somatic cells. This selectivity profile supports the potential of *C. militaris* protein-rich isolate as a candidate for further development as a safe adjuvant therapy or nutraceutical product for cancer management.

Future investigations should prioritize the identification and characterization of the molecular constituents within the isolated fraction, employing SDS-PAGE or proteomic analysis to pinpoint the specific peptides or proteins responsible for the observed biological activity. Furthermore, the underlying molecular mechanisms governing this selectivity have to be elucidated.

5. Conclusions

This study demonstrates that the alkaline extraction and isoelectric precipitation of *C. militaris* fruiting bodies effectively yield a stable, protein-rich isolate with significant therapeutic potential. A major highlight is the tenfold enrichment of cordycepin (increasing to 1048 mg/100 g), a potent bioactive nucleoside known for inducing apoptosis in malignant cells. Biological assays revealed that this isolate possesses promising selectivity in this model, particularly against MCF-7 breast cancer cells (IC_{50} = 8.68 μg/mL), while remaining nearly 55 times less toxic to healthy human fibroblasts (IC_{50} = 475.56 μg/mL). These findings suggest that the *C. militaris* protein-rich isolate offers a wide therapeutic window, positioning it as a promising, safe candidate for functional foods or adjuvant cancer therapies, though further molecular identification and isolation, in vivo validation and mechanistic studies are required to fully realize its clinical utility.

Author Contributions

S.R.: Data curation, data analysis, writing—original draft & preparation and final editing. S.S.: Preparation and identification of mushroom materials. T.S. & N.H.: preparation of protein-rich isolate, Physicochemical characterization analysis and Cytotoxicity test. Y.H. & W.P.: Validation method and phytochemical Analysis. P.S.: Writing—reviewing and editing. All authors have read and agreed to the published version of the manuscript.

Funding

This research received no external funding; however, it was supported by internal funds from the Department of Medical Sciences.

Institutional Review Board Statement

Not applicable.

Informed Consent Statement

Not applicable.

Data Availability Statement

Not applicable.

Acknowledgments

The authors are grateful to Siriporn Butseekhot from the Expert Center of Innovative Health Food, Thailand Institute of Scientific and Technological Research (TISTR), Thailand for her facilitating the protein isolation methods and equipment. Sarayut Radapong gratefully acknowledges the support of the Department of Medical Sciences, Ministry of Public Health.

Conflicts of Interest

The authors declare no conflict of interest. Given the role as Editorial Board Member, Sarayut Radapong had no involvement in the peer review of this paper and had no access to information regarding its peer-review process. Full responsibility for the editorial process of this paper was delegated to another editor of the journal.

Use of AI and AI-Assisted Technologies

During the preparation of this work, the authors used Gemini 1.5 Pro to generate the graphical abstract. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the content of the published article.

Abbreviation

IFOAM	The International Federation of Organic Agriculture Movements
a_w	Water activity
UHPLC	Ultra High-Performance Liquid Chromatography
LOD	Limit of Detection
LOQ	Limit of Quantification
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
IC_{50}	The half-maximal inhibitory concentration

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