ASSESSMENT OF PHYTOCHEMICAL COMPOSITION, ANTIMICROBIAL, ANTIOXIDANT ACTIVITIES, AND CYTOTOXICITY OF THE ETHANOL EXTRACT FROM *CORDYCEPS MILITARIS*

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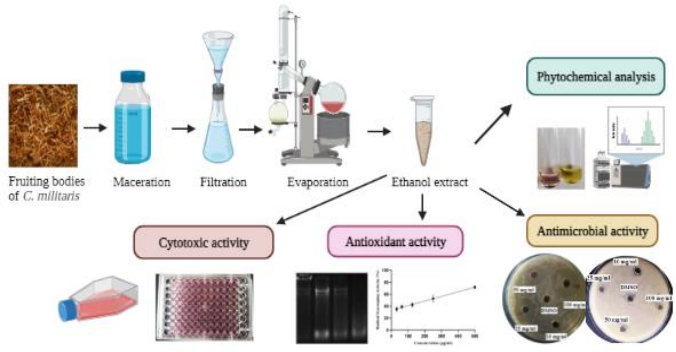
ABSTRACT

Cordyceps militaris (*C. militaris*) has been utilized as an herbal medicine with diverse therapeutic properties in Asian countries. It can be a prominent resource of secondary metabolites that possesses remarkable biological activities. Herein, this study investigated the phytochemical screening, antimicrobial, antioxidant, and cytotoxic activities of the fruiting bodies of *C. militaris* using ethanol solvent. Qualitative phytochemical screening of the ethanol extract revealed the presence of proteins, carbohydrates, phenolics, tannins, flavonoids, and saponins, especially cordycepin and adenosine. The agar well diffusion method demonstrated that the ethanol extract exhibited sensitive activity against *Staphylococcus aureus* (ZOI = 30.33 ± 2.08 mm) and moderate activities against at for *Salmonella spp.* (ZOI = 16.33 ± 1.52 mm), *Escherichia coli* (ZOI = 9.33 ± 1.15 mm) as well as *Aspergillus niger* (ZOI = 7.83 ± 0.76 mm) at 100 mg/mL concentration. The 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity with the half-maximal inhibitory concentration (IC_{50}) of 217.85 ± 33.16 µg/mL was conducted for the extract at 500 µg/mL. The protective effect against oxidative damage to DNA induced by hydrogen peroxide and UV radiation was observed with the treatment. Moreover, the results assessed *C. militaris* extract inhibited the growth of the MDA-MB-231 cell line compared to control. At the same time, these treatments affected the increase of the cell proliferation of normal human fibroblast cell line at 134.85 ± 35.18%. The data in this work indicated that *C. militaris* extract had significant biological properties and could be applied to herbal supplements and medicines in the pharmaceutical industry.

Keywords: *Cordyceps militaris*, medicinal mushrooms, antioxidant, antimicrobial, cytotoxicity, secondary metabolites.

Graphical abstract



1. INTRODUCTION

The genus *Cordyceps* is one of the largest genera in the family *Clavicipitaceae* and has been well known as the traditional medical mushrooms for the application of functional foods and medicines. There are at least 750 species identified in Asia, Europe, and North America [1]. The diversity of this genus is distributed in Korea, China, Nepal, and other areas due to the humid climate and tropical habitats and depends on the association between Cordyceps species and hosts [2]. They invade insects as well as insect larvae by harmonizing the life cycles of their host for their survival and growth. Consequently, there are many various secondary metabolites produced during the interaction between this genus and their host in order to respond to the host defense such as proteins, nucleic acid, nucleotides, sterols, peptides, polysaccharides, flavonoids, alkaloids, phenolics, and others [3]. Based upon these specific mechanisms, *Cordyceps* species have become promising sources of natural products.

Among these species, *Cordyceps militaris (C. militaris)* is one of the most popular members being artificially cultivated successfully because of the market demands and its values [2]. Traditionally, *C. militaris* has been utilized as an herbal medicine in Asian countries to improve the longevity and vitality of the human body [4]. The important constituents derived from the fruiting body as well as cultured mycelia of this fungus contain cordycepin, adenosine, polysaccharides, fatty acids, and amino acids [3]. These compositions have possessed beneficial activities including anticancer, antibacterial, antioxidant, and anti-inflammatory effects [5]. However, the various methods for the artificial culture of *C. militaris* not only lead to changes in pharmacological properties but also affect the production of secondary metabolites under laboratory conditions. In addition, the various polarity of solvents might affect the isolation of bioactive compounds between fruiting bodies and cultured mycelia of this mushroom. Therefore, the objectives of this study were to investigate the bioactive compositions, antimicrobial, antioxidant properties, and cytotoxicity of the ethanol extract of cultivated *C. militaris* from fruiting bodies under laboratory conditions.

2. MATERIALS AND METHODS

2.1. Material

C. militaris was supplied from the Laboratory of AT Group Company, Ho Chi Minh City, Vietnam. The mushrooms were stored at 4°C before utilizing for the next process. The following bacteria and fungi used were *Staphylococcus aureus*, *Escherichia coli*, *Salmonella spp.*, and *Aspergillus niger* which were obtained from the Faculty of Biology and Environment, Ho Chi Minh University of Food Industry, Vietnam. Two cell lines including MDA-MB-231 human breast adenocarcinoma (ATCC, HTB-26) and HF normal human fibroblast isolated from the skin tissue in this research were supplied from the Laboratory of Tissue Engineering and Biomedical Materials, Vietnam National University - University of Science, Ho Chi Minh, Vietnam.

2.2. Preparation of ethanol extract

After being harvested from a cultivated medium, the fresh fruiting bodies were chopped with a kitchen knife into small pieces from 2 to 4 mm. These pieces were dried at 40°C for 48 h in a hot air machine (UF55 Memmert, Germany) and then ground into the powder using a blender (HR2118/01 Philips, Netherlands). The powder (200 g) was macerated in 70% ethanol (2 liters) for 24 h at room temperature. Then, the solution was filtered and concentrated by the rotary evaporator (HS-2005S-N Hahnvapor, Korea) at 45°C. The solution was dried using a

freeze-dryer (MDF-C8V1 Series Panasonic, Japan) to obtain *C. militaris* ethanol extract. The dried powder was stored at 4°C in dark bottles until being used for subsequent assays.

2.3. Qualitative phytochemical analysis

The determination of bioactive constituents in the ethanol extract from fruiting bodies of *C. militaris* was conducted utilizing methods referred to previous documents including Biuret test for proteins [6], Molisch's tests for carbohydrates [7], ferric chloride and lead acetate tests for phenolics [8], lead acetate tests for tannins [8], alkaline reagent test for flavonoids [9], and foam test for saponins [9]. The presence of adenosine and cordycepin was also confirmed by liquid chromatography-mass spectrometry (LC/MS) analysis following the guidelines of the Center of Analytical Services and Experimentation (CASE), Ho Chi Minh City, Vietnam.

2.4. Antimicrobial activity determination

Antimicrobial activity was carried out utilizing the agar well diffusion method [10]. For bacterial pathogens, one Gram-positive bacteria (*Staphylococcus aureus*), as well as two Gram-negative strains (*Escherichia coli, Salmonella spp.*) were tested in this study. Meanwhile, *Aspergillus niger* was also studied as a fungal pathogen. The microbial reference cultures were incubated at 37°C and performed on standard nutrient media containing Mueller Hinton agar (*Staphylococcus aureus, Escherichia coli, Salmonella spp*.) and potato dextrose agar (*Aspergillus niger*). Densities of bacterial and fungal cultures were determined by the spectrophotometer at wavelength 600 nm with $1x10^8$ CFU/mL and $1x10^6$ CFU/mL, respectively. The *C. militaris* extract was dissolved in dimethyl sulfoxide (DMSO) solvent and tested at 10, 25, 50, and 100 mg/mL concentrations. DMSO (99.9%, v/v) was used as the negative control. The inoculum of each strain was prepared in sterile sodium chloride 0.9% and swabbed on the top of the solidified media. The wells were prepared with a sterile blue tip and 50 µl of the concentrations of extract were loaded into the wells. The plates were incubated at 37°C for 24 h to monitor the antimicrobial effect. These experiments were repeated in at least triplicate. The diameters of the circular inhibition zones (ZOI) were measured in millimeters (mm) with the metric ruler for scale.

2.5. Antioxidant activity assay

Antioxidant activity was measured by the scavenging power of the diphenyl picryl hydrazine (DPPH) assay [11]. The stock solution of the ethanol extracts of *C. militaris* was prepared at 10 mg/mL and diluted in ethanol at experimental concentrations from 31.25 to 500 µg/mL. The stock solution of DPPH (Sigma-aldrich, Germany) was dissolved in methanol at 0.1 mM concentration. As a standard, ascorbic acid (Sigma-aldrich, Germany) was used as a control. The dilutions of ascorbic acid ranging from 2 to 10 µg/mL

were also prepared. The ratio between the concentrations of the extract and DPPH was set at 1:1 in a 96-well plate. After incubation for 30 min in dark, the absorbance was calculated at 540 nm by the spectrophotometer. These experiments were repeated in triplicate. The percentage of radical scavenging activity was calculated by the following formula: RSA (%) = [(ODcontrol - ODconcentration)/ ODcontrol] x 100%. The IC_{50} value is determined as the concentration of antioxidant constituents to reduce the initial DPPH concentration by 50%.

2.6. DNA damage inhibition assay

Inhibition of DNA damage was evaluated in the presence of hydrogen peroxide H_2O_2 and UV radiation [12]. A volume of 24 µl including 10 µl of pBL21 plasmid DNA, 10 µl of different concentrations of the extract (2 and 8 mg/mL), and 4 µl of 30% H_2O_2 was prepared for the experiment. Negative control was only untreated UV-irradiated pBL21 plasmid DNA and positive control was only treated UV-irradiated pBL21 plasmid DNA without the extract. All samples were placed directly on the surface of a UV transilluminator for 30 min. After irradiation, the Gel Doc system visualized DNA damage inhibition on agarose gel electrophoresis at 1.2%.

2.7. Cell culture maintenance

Cell lines including MDA-MB-231 cells and HF cells were cultured in DMEM medium (Gibco, USA) supplemented with 10% (v/v) fetal bovine serum (Sigma-aldrich, Germany). Antibiotic-antimycotic (Corning, USA) solution was added to the medium. The cells were cultured in humidified 5% CO_2 atmosphere at 37°C in the incubator. MDA-MB-231 and HF cell lines were passaged every 2-3 days.

2.8. Cytotoxicity assay

Cytotoxicity assay was based on the measured cell metabolism using MTT method [13]. The cell density of 5 x 10^3 cells/well was placed into each well of a 96-well plate and left to attach overnight. The medium was then discarded and replaced with a 95 µl new medium. Subsequently, 5 µl of serial concentrations of the ethanol extract were added to each well. The dried powder was weighed and dissolved in 99.9% (v/v) DMSO to prepare stock solutions. The stock solution was diluted in a cell culture medium to reach experimental concentrations, then filtered by sterile 0.22 µm membranes. The *C. militaris* extract in the well ranged from 6.25 to 100 µg/mL. Control untreated wells were treated with 0.1% (v/v) DMSO. After 48 h of treatment, 5 µl of 5 mg/mL MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was added and incubated for 4 h. Then 100 µl of 99.9% (v/v) DMSO was added to each well to lyse cells and solubilize formazan crystals. The absorbance was measured at a wavelength of 540 nm. The following formula calculated the percentage viability of cells: Cell viability (%) = (ODconcentration – ODblank)/ (ODcontrol – ODblank') x 100%.

2.9. Statistical Analysis

Data were analyzed by Graphpad Prism 9 software (USA). Results were presented as Mean ± Standard Deviation (SD). All data were analyzed by two-tailed unpaired Student's t-tests and one-way analysis of variance (ANOVA) tests. The statistically significant differences were determined at $P \leq 0.05$ (*), $P \leq 0.01$ (**), $P \leq 0.001$ (***), $P \leq 0.0001$ (****).

3. RESULTS AND DISCUSSION

3.1. Analysis of phytochemical constituents

Results of the qualitative phytochemical analysis showed that secondary metabolites containing proteins, carbohydrates, phenolics, tannins, flavonoids, and saponins were present in the ethanol extract of *C. militaris* (Table 1). In particular, the ethanol extract possessed the adenosine and cordycepin contents at 49.5 mg/kg and 2589 mg/kg, respectively (Table 2). These compounds were responsible for bioactive properties in this medical mushroom such as anticancer, antioxidant, antimicrobial, antiviral, and anti-inflammatory activities [14].

Similarly, these results corresponded to several previous studies that have reported similar constituents from the fruiting bodies of *C. militaris* by various solvents [15]. It also indicated that ethanol was the effective solvent for isolating secondary metabolites because of its high polarity. Therefore, the presence of bioactive compounds in *C. militaris* extract may result from different nutritious elements and environmental factors related to the cultivation process.

Table 1. Phytochemical analysis from the ethanol extract of fruiting bodies of *C. militaris*

No.	Phytochemicals	Result
1	Proteins	+
2	Carbohydrates	+
3	Phenolics	+
4	Tannins	+
5	Flavonoids	+
6	Saponins	+

"+": Present, "-": Absent

Table 2. Adenosine and cordycepin contents from the ethanol extract of fruiting bodies of *C. militaris*

No.	Phytochemicals	Result
1	Adenosine	49.5 mg/kg
2	Cordycepin	2589 mg/kg

3.2. Antimicrobial effects of *C. militaris* extract

In the case of the antimicrobial assay, *C. militaris* extract was active against different strains (Figure 1). These results demonstrated the highest zone of inhibition against *Staphylococcus aureus*, 30.33 ± 2.08 mm, compared with other strains at 100 mg/mL concentration (Figure 1C). In addition, the other two bacterial strains also showed moderate activity toward the extract at 9.33 ± 1.15 mm for *Escherichia coli* and 16.33 ± 1.52 mm for *Salmonella spp.* (Figure 1A-1B). On the other hand, the extract exhibited *Aspergillus niger* with the least responsive at 7.83 ± 0.76 mm (Figure 1D). Furthermore, the antimicrobial properties were increased following the increasing doses tested. All microbial strains were resistant to DMSO (99.9% v/v) used as the negative control. In line with our findings, other previous studies have demonstrated antimicrobial effects against broad bacteria and fungi by extracting secondary metabolites of *C. militaris* from fruiting bodies [16]. In the current study, antimicrobial effects of secondary metabolites from the extract were more active against Gram-positive bacteria than Gram-negative strains and fungi. Owing to differences in the structure of their cell surfaces, the outer membrane of Gram-positive bacteria which possesses teichoic and lipoteichoic acids in the peptidoglycan layer allows hydrophobic compounds throughout the diffusion pathway [17]. This may explain the sensitivity of *Staphylococcus aureus* towards the ethanol extract from *C. militaris*.

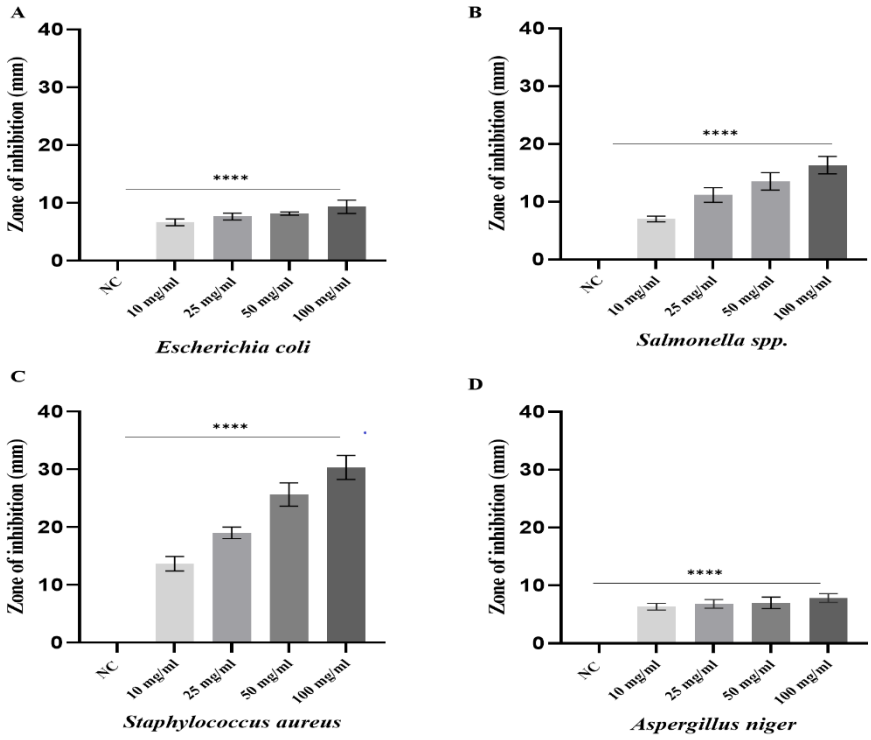


Figure 1. Antimicrobial effects of *C. militaris* extract. (A-D): Zones of inhibition of experimental concentrations against microbial. (A). *Escherichia coli*. (B). *Salmonnella spp*. (C). *Staphylococcus aureus*. (D). *Aspergillus niger* (D). NC: negative control (DMSO, 99.9% v/v). Each value represents as Mean ± SD of three independent experiments. The statistical difference was analyzed by one-way ANOVA tests (**** $P < 0.0001$).

3.3. Antioxidant effects of *C. militaris* extract

The antioxidant activity of *C. militaris* had been investigated by DPPH procedure utilizing ascorbic acid as a reference standard. The results described the inhibitory concentration IC_{50} of the extract was 217.85 ± 33.16 µg/mL whereas the reference drug was 5.31 ± 0.79 µg/mL (Figure 2A-2B). The radical scavenging effects of the extract reached 71.7 ± 2.05 % at the reaction concentration of 500 µg/mL. In addition, the percentage of the scavenging activity was concentration-dependent manner. Other research reported that the IC_{50} value from fruiting bodies of *C. militaris* was determined at 4.16 mg/mL by DPPH assay [5]. Another study described the antioxidant ability of this mushroom to scavenge toward free radicals with IC_{50} at 167.13 ± 2.22 µg/mL [18]. The antioxidant property could be different because of various elements including the bioactive compounds, synergistic effects among them, or extraction methods. The antioxidant effect of this extract might be due to the presence of bioactive compounds like cordycepin, adenosine, flavonoids, and phenolics which act as free radical scavengers. Hence, these results suggested that secondary metabolites of the *C. militaris* extract could be responsible for the antioxidant activity in the current study.

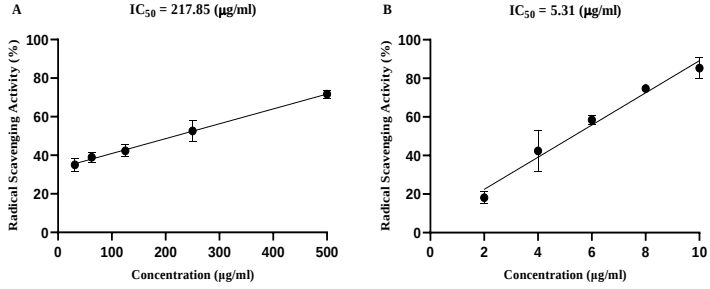


Figure 2. Antioxidant effects of *C. militaris* extract. (A). Radical scavenging capacity of the extract from 31.25 to 500 (µg/mL). (B). Antioxidant activity of ascorbic acid as positive control. Data were expressed as Mean ± SD of three independent experiments.

3.4. Protection of DNA against oxidative damage of *C. militaris* extract

To evaluate the ability of *C. militaris* extract to protect DNA by photolyzing hydrogen peroxide and UV radiation, the pBL21 plasmid DNA was treated with 2 and 8 mg/mL concentrations of the extract. Figure 3 illustrates the electrophoretic pattern of plasmid pBL21 in the presence or absence of the treatment. In detail, DNA derived from pBL21 plasmid expressed two bands on agarose gel electrophoresis without the treatment in lane 1 (L1). Meanwhile, the UV irradiation in the presence of hydrogen peroxide destroyed plasmid DNA in lane 4 (L4). On the other hand, the results showed that treatments with 2 and 8 mg/mL doses were unchanged in the DNA conformation in lane 2 and lane 3 (L2-L3). Several studies have reported secondary metabolites from the plant extracts may protect the process of DNA damage owing to the presence of flavonoids and phenolic constituents [19]. Hence, these observations suggested that *C. militaris* extract possessed the protective effects of DNA damage following its antioxidant potential.

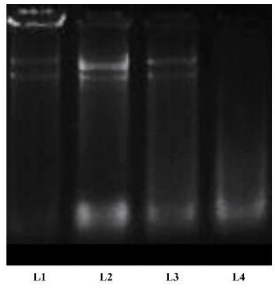


Figure 3. Effect of DNA damage inhibition of *C. militaris* extract. (L1). Untreated UV-irradiated pBL21 plasmid DNA (negative control). (L2). pBL21 plasmid DNA with 2 mg/mL of the extract. (L3). pBL21 plasmid DNA with 8 mg/mL of the extract. (L4). Treated UV-irradiated pBL21 plasmid DNA (positive control).

3.5. Cytotoxicity of *C. militaris* extract on different cell lines

To investigate the cytotoxic activity on MDA-MB-231 breast adenocarcinoma and HF fibroblast cell lines, various dilutions from 6.25 to 100 µg/mL concentration of the extract were evaluated by MTT assay. Figure 4 illustrated that *C. militaris* extract exhibited the negligible inhibition of the MDA-MB-231 cell line at doses tested compared with negative control. The cell viability of MDA-MB-231 cells was determined at 75.43 ± 4.27 % with a 100 µg/mL concentration of the extract (Figure 4A). Moreover, treated cells turned round, shrunk, and reduced in size, whereas untreated samples appeared phenotypically as spindle-

shaped cells (Figure 4B). These results indicated that the highest concentration of *C. militaris* extract decreased the cell proliferation of MDA-MB-231 cells at 100 µg/mL even though the cytotoxic effect was not significant. On the contrary, these treatments were not cytotoxic to regular human fibroblast HF cell lines at the tested doses (Figure 5). All dose levels tested showed a positive effect on the growth of normal cells. It was evident that the cell proliferation of HF cells increased by 134.85 ± 35.18 % compared to cancer cells (Figure 5A). Notably, there were no noticeable morphological differences in HF cells between treated and untreated samples (Figure 5B). It was observed that the *C. militaris* extract less toxicity to HF normal cells than cancer cells. In agreement with our findings, the MDA-MB-231 cell viability was reduced in previous research by secondary metabolites derived from fruiting bodies of *C. militaris* [20]. Meanwhile, cordycepin being the major bioactive component of *C. militaris* has expressed many therapeutic potentials and biological functions for the drug development in pharmacy [14]. It was reported that cordycepin inhibits the MDA-MB-231 cell viability and induces cell death [21]. Thus, these effects could be explained by the presence of phytoconstituents of the ethanol extract, particularly cordycepin, which might account for the cytotoxicity in this study. Also, it has been demonstrated that this ethanol extract might develop as a functional food and herbal tea to enhance human health.

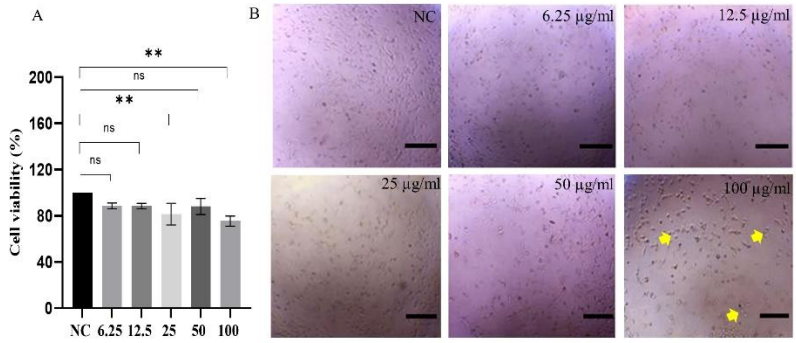


Figure 4. Cytotoxicity of *C. militaris* extract against MDA-MB-231 human breast adenocarcinoma cell line. (A). The percentage of cell viability of MDA-MB-231 cell line treated with the different concentrations of the extract from 6.25 to 100 (µg/mL) at 48 h. (B). Morphological changes of MDA-MB-231 cell line (yellow arrows). NC: negative control (DMSO, 0.1% v/v). Images captured at 40x magnification. Data were presented as Mean ± SD of three independent experiments. Statistical differences were analyzed by one-way ANOVA (** $P < 0.01$, ns > 0.05).

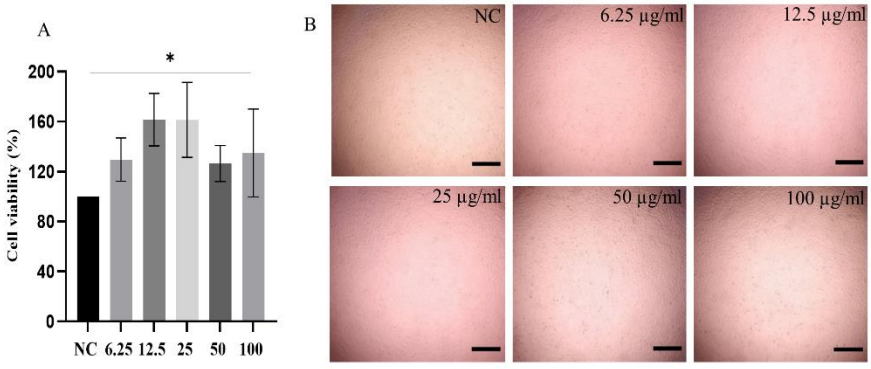


Figure 5. Cytotoxicity of *C. militaris* extract against HF human fibroblast cell line.

(A). The percentage of cell viability of HF cell line treated with the different concentrations of the extract from 6.25 to 100 (µg/mL) at 48 h. (B). Morphological changes of HF cell line. NC: negative control (DMSO, 0.1% v/v). Images captured at 40x magnification. Data were presented

as Mean ± SD of three independent experiments. Statistical differences were analyzed by one-way ANOVA (*P < 0.05).

4. CONCLUSION

This study revealed that *C. militaris* extract contained various bioactive groups such as proteins, carbohydrates, phenolics, tannins, flavonoids, and saponins, especially cordycepin and adenosine. The ethanol extract possessed the highest activity against *Staphylococcus aureus* compared with other bacteria and fungi. Furthermore, this extract was shown antioxidant potentials and was proportional to the protective ability by preventing oxidative DNA damage. It possessed slight cytotoxicity against MDA-MB-231 cell lines while there was no toxicity towards human fibroblast cells. Overall, this study may represent the *C. militaris* extract from fruiting bodies as a good choice for herbal supplements and medicines in pharmacy applications.

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TÓM TẮT

ĐÁNH GIÁ HOẠT TÍNH KHÁNG VI SINH, OXY HÓA, ĐỘC TÍNH VÀ HỢP CHẤT SINH HỌC THU NHẬN TỪ DỊCH CHIẾT ĐÔNG TRÙNG HẠ THẢO *Cordyceps militaris*

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Đông trùng hạ thảo *Cordyceps militaris* được dùng như thảo dược truyền thống tại nhiều quốc gia Châu Á và cung cấp các hợp chất sinh học quan trọng. Nghiên cứu này đánh giá định tính và định lượng các hợp chất sinh học, hoạt tính kháng vi sinh vật, kháng oxy hóa và độc tính từ cao chiết ethanol của quả thể nấm dược liệu được nuôi trồng này. Các nhóm hợp chất xuất hiện bao gồm protein, đường, phenolic, tannins, flavonoid, saponin, cordycepin và adenosine. Hoạt tính kháng vi sinh vật được xác định bằng đường kính vòng kháng khuẩn trên các vi khuẩn bao gồm *Staphylococcus aureus* (30,33 ± 2,08 mm), *Salmonella spp.* (16,33 ± 1,52 mm), *Escherichia coli* (9,33 ± 1,15 mm) và nấm *Aspergillus niger* (7,83 ± 0,76 mm). Hoạt tính kháng oxy hóa bằng khả năng bắt gốc tự do DPPH có phần trăm ức chế IC_{50} là 217,85 ± 33,16 µg/mL và có khả năng bảo vệ sự phân mãnh DNA. Cao chiết có khả năng gây độc tế bào ung thư vú MDA-MB-231 không đáng kể nhưng kích thích tăng sinh tế bào bình thường ở người là 134,85 ± 35,18 % tại 100 µg/mL. Như vậy, cao chiết có chứa các hợp chất sinh học có giá trị và có tiềm năng ứng dụng trong thực phẩm chức năng và phát triển thuốc.

Từ khóa: Cordyceps militaris, nấm dược liệu, kháng oxy hóa, kháng vi sinh vật, độc tính, hợp chất thứ cấp.