IN VITRO CYTOTOXICITY OF *CORDYCEPS MILITARIS* EXTRACTS ON DIFFERENT HUMAN CANCER CELL LINES

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Abstract:

This study was conducted to evaluate the *in vitro* cytotoxicity of *C. militaris* extracts against cancer cells. The cytotoxic activity was tested using Resazurin reduction microplate assay (REMA). The *in vitro* assay for the *C. militaris* extracts was measured using three human cancer cells: Human hepatocellular carcinoma (HepG2), Human breast adenocarcinoma (MCF-7), and Cervical adenocarcinoma (HeLa). The REMA assay indicated that Ethyl alcohol (EtOH) and Ethyl acetate (EtOAc) extract of *C. militaris* exhibited significant cytotoxicity on MCF-7, HepG-2 and HeLa cancer cells in the dose-dependent manner. *C. militaris* extracted with EtOAc exhibited stronger cytotoxicity on MCF-7 than HepG2 and HeLa. The results obtained indicated that the probable use of the *C. militaris* extract in preparing recipes for cancer-related ailments. Further studies will identify bioactive compounds and elucidate more detailed molecular mechanisms of cell death.

Keywords: *Codyceps militaris*; Cordycepin; Adenosine; Cytotoxicity

Introduction

C. militaris is a medicinal mushroom that has been widely used in Asia for the treatment of various diseases. This fungus is an herbal drug known for its use as a source of therapeutic bioactive compounds (Wasser & Weis, 1999; Smith *et al.*, 2002). It has been widely used as a folk tonic food in Asia extensively (Ying *et al.*, 1987). Cordyceps is relatively considered to be a non-toxic medicinal mushroom, besides a few negative published data (Shrestha *et al.*, 1987). The main bioactive components that possess medicinal property include cordycepin (3'-deoxyadenosine) and adenosine (Cunningham *et al.*, 1950). These compounds have pharmacological actions, including anti-cancer (De Silva *et al.*, 2012), anti-tumor, antioxidant (Ramesh *et al.*, 2012), anti-hyperlipidemia (Guo *et al.*, 2010) and anti-fungus (Kim *et al.*, 2003). However, in *C. militaris* more nucleosides than 10 types which are related to the function of the central nervous system (Das *et al.*, 2010).

Apart from being used in usual surgical methods, radiotherapy, and chemotherapy, herbal medicine can also be used as the primary complementary and alternative medicines for treating various cancers, including colorectal cancer (Zhai *et al.*, 2013; Ling *et al.*, 2014; Lee *et al.*, 2015). Cordycepin is one of the cytotoxic analogs of nucleoside, first tested as a chemotherapy agent (Jeong *et al.*, 2011; Tuli *et al.*, 2013; Yoon *et al.*, 2018). In the present study, we investigated the cytotoxic effect of *C. militaris* extract with EtOH and EtOAc on Human breast adenocarcinoma MCF-7, Human hepatocellular carcinoma HepG2 and Cervix adenocarcinoma HeLa cells death.

Materials and Methods

Chemicals and materials

Resazurin sodium salt was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Ethyl alcohol (EtOH) (AR grade) and Ethyl acetate (EtOAc) (AR grade) were purchased from BDH (Poole, UK). Dulbecco's minimum essential medium, Fetal bovine serum (FBS), 100X Penicillin/Streptomycin solution, 0.25 % Trypsin-EDTA and the other cell culture reagents were purchased from GIBCO®, Invitrogen Corporation (Carlsbad, CA, USA).

Culture condition of C. militaris

The healthy fruiting body of *C. militaris* was obtained from the Lungyood farm Saraburi Province, Thailand was the culture at 20˚C in dark for 7 days on potato dextrose agar (PDA). Mycelium was cultured continuously in liquid medium at 20˚C for 21 days, so as to obtain mycelium pellets. The basic medium for the fruiting body of C. *militaris* was used by modification from Lungyood Chaemprasert farm and sterilized by autoclaving at 121 °C for 30 min. The seed starter culture with 5 mL into bottle culture and incubated in the dark at 22˚C for mycelium stage, after 14 days, controlled with a 14 h light/10 h dark cycle at 18 °C for stimulation stage. Fruiting body stage controlled with a 12 h light cycle at 22 °C. Sixty-day-old were harvested and dried at 50 °C for the next analysis

Preparation of the extract

The powder of *C. militaris* were extracted using 1000 mL of solvent (EtOH and EtOAc) in a flask, incubated at 37°C for 48 h using at 200 rpm. After that, separating the clear parts using a centrifuge at 10,000 rpm for 30 min and re-extraction. Collect all clear supernatant and a centrifuge at 10,000 rpm for 30 min. Next step, filtered using Filter Paper, Whatman filter No. 4 and then evaporated the solvent by evaporator at 42 °C. Freeze-dried and store the crude extract at -20 °C for experimentation.

Human Cancer Cell Lines and Culture

Human breast adenocarcinoma MCF-7 (ATCC® HTB-22™), Human hepatocellular carcinoma HepG2 (ATCC® HB-8065™) and Cervical adenocarcinoma HeLa (ATCC® CCL-2™) cell line was obtained from the American Type Culture Collection (ATCC, Manassasa, VA, USA). Cancer cells were maintained in DMEM medium contemning with 4 mM L-glutamine, 10% FBS and 1% Penicillin/Streptomycin solution incubated at 37˚C in a humidified incubator with 5% CO_2 atmosphere and the new medium culture was replaced every 3 days. Trypsinization with 0.25% trypsin-EDTA and the fresh DMEM was renewed every 3 day.

Cell viability

Cell viability for cancer cells was detected using REMA is carried out according to the Kuete *et al*. (2013). Cancer cells (5×10^3 cells/well) were added to 96-well plates for 24 h at 37˚C in an incubator. The medium was discarded, and cancer cells were exposed to a crude extract of different concentrations (final concentration 0-500 µg/mL) dissolved in the DMEM media at 37˚C for 24 h. Resazurin reagent final concentration 0.001% w/v added to replace media and incubated for 1 h. Product of resazurin reduction measured at excitation wavelength of 530 nm

and an emission wavelength of 590 nm by a microplate reader (Synergy 4HT Microplate Reader). Each bar represents the mean ± SEM from three experiments. The viability was evaluated based when compared to the control that not treated with the crude extract. Maximal cancer cell killing effect (*E*max) and half-maximal inhibitory concentration (IC_{50}) values were calculated using GraphPad Prism version 8 (GraphPad Software, San Diego, CA, USA) according to the National Cancer Institute guideline (Boyd, 1997).

Statistical analysis

Repeat all 3 tests to find mean and standard deviation using One-way ANOVA and Duncan's Multiple Range Test with SPSS version 19.0 (IBM, Armonk, NY, US), considering the differences. Significantly when $p < 0.05$

Results and Discussion

Effect of Cordyceps militaris crude extract on the survival of cancer cells

The fruiting body of *C. militaris* was grown for 60 daysharvested and dried at 50 ˚C in an incubator readilyfor extraction with different solvents. Evaporated and freeze-dried crude extract, followed dissolved crude extract at 10 mg/mL in the DMEM media to determine the *C. militaris* crude extract on the survival of MCF-7, HepG2 and HeLa cancer cells using Resazurin reduction assay. The results clearly demonstrated that *C. militaris* extract from EtOH and EtOAc showed the highest inhibitory effect on the survival of cancer cells effectively after treated with differenced solvent extract of *C. militaris* in a dose-dependent manner, except the extract with EtOH on HeLa cell, shown as the *E*max and IC_{50}. The *C. militaris* extract with EtOAc show the highest inhibited the survival effect on MCF-7 after incubate for 48 h (Figure 1A-B) (IC_{50} = 33.64±0.78 µg/mL) (Table 1) followed extract with EtOAc for 24 h (IC_{50} = 112.95±6.01 µg/mL) and extract with EtOH incubate for 48 h (IC_{50} = 112.35±3.04 µg/mL), respectively. In liver HepG2 (Figure 1C-D) and cervical HeLa cancer cell (Figure 1E-F), found that *C. militaris* form differenced extract affect the cell viability that significantly in a dose-dependent manner. *C. militaris* extract with EtOAc show the highest on Cytotoxicity HepG2 (IC_{50} = 63.99±13.01 µg/mL) (Table 2) and HeLa (IC_{50} = 92.24±0.58 µg/mL) (Table 3) after incubate for 48 h, respectively. *C. militaris* extract with EtOAc inhibited the survival of cancer cells than EtOH extract. However, MCF-7 cells are more sensitive to inhibited the survival of *C. militaris* extract with EtOAc than HepG2 and HeLa. From previous researchreports Asatiani *et al*. (2018) *C. militaris* extract with EtOAc and chloroform extract appeared to be the most active showing the most profound decrease in cell viability on HPAF-II, HCT116, PC3 and T47D, which is in line with our researchthat EtOAc has the best activity to inhibit cancercells. *C. militaris* extract affects the cytotoxicity on cancer cells, including lung carcinoma, B16 melanoma, lymphocytic, prostate (PC3), breast (MCF-7), hepatocellular (HepG2, Hep3B) and colorectal (HT-29 and HCT116) cells (Nakamura *et al*., 2015); (Huo *et al*., 2017). *C. militaris* extract with 50% ethanol exhibited significant MCF-7 cell inhibitory effects on human breast MCF-7 cancer cells (Lee *et al*., 2019).

Table 1: IC_{50} of *C. militaris* extract on MCF-7

Treatment	Incubation time	MCF-7 cancer cell	
		***E*max (%)**	**IC_{50} (µg/mL)**
EtOH	24 h	88.42±0.38^{c}	236.10±6.79^{c}
	48 h	87.73±0.60^{c}	112.35±3.04^{b}
EtOAc	24 h	91.21±0.71^{b}	112.95±6.01^{b}
	48 h	93.00±0.26^{a}	33.64±0.78^{a}

Each value is the mean ± SEM of three experiments. Small letters indicate significant differences in the column ($p < 0.05$).

Table 2: IC_{50} of *C. militaris* extract on HepG2

Treatment	Incubation time	HepG2 cancer cell	
		***E*max (%)**	**IC_{50} (µg/mL)**
EtOH	24 h	88.50±0.59^{c}	174.80±4.10^{c}
	48 h	87.43±0.11^{d}	135.60±3.54^{b}
EtOAc	24 h	92.48±0.13^{b}	134.20±2.69^{b}
	48 h	95.05±0.07^{a}	63.99±13.01^{a}

Each value is the mean ± SEM of three experiments. Small letters indicate significant differences in the column ($p < 0.05$).

Table 3: IC_{50} of *C. militaris* extract on HeLa

Treatment	Incubation time	HeLa cancer cell	
		***E*max (%)**	**IC_{50} (µg/mL)**
EtOH	24 h	21.31±1.15^{c}	>500
	48 h	39.59±0.83^{b}	>500
EtOAc	24 h	90.83±1.18^{a}	237.25±9.69^{a}
	48 h	92.24±0.58^{a}	161.90±3.96^{a}

Each value is the mean ± SEM of three experiments. Small letters indicate significant differences in the column ($p < 0.05$).

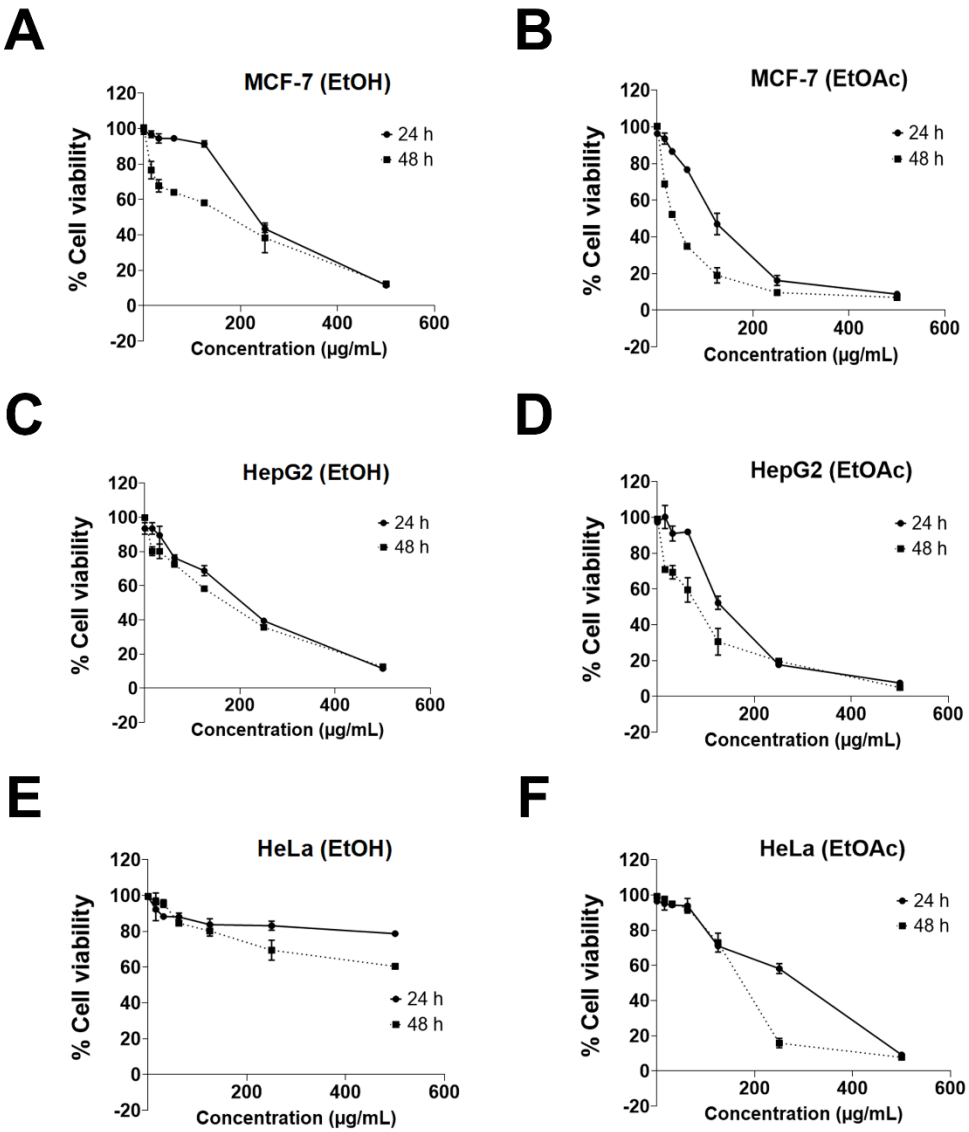

Figure 1: Effect of *C. militaris* from different solvent extract on survival of cancer cell, 1A-B; MCF-7 cancer cell, 1C-D; HepG2 cancer cell and 1E-F; HeLa cancer cell, each value is the mean ± SEM of three experiments. $p < 0.05$, compared to control.

Conclusion

In this study, the *C. militaris* extract with EtOAc affected the highest inhibition of the survival of MCF-7, HepG2, and HeLa cancer cells. In further research will be studied on the mechanisms of cytotoxicity of the other cancer cells. Data of study can be used for developing cancer drugs.

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