A COMPARISON OF THE CYTOTOXIC ACTIVITY OF EXTRACTS FROM FRUITING BODIES AND MYCELIAL BIOMASS OF *CORDYCEPS NEOVOLKIANA* (DL004) FUNGUS

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ABSTRACT

Cordyceps neovolkiana is an insect-parasitic fungus, naturally distributed in the Langbiang mountain, Tam Dao and Ba Vi national parks of Vietnam. This study assessed and compared the *in vitro* cytotoxic activity of crude extracts from fruit bodies and biomass of *C. neovolkiana* fungus against human Jurkat (acute T cell leukemia) and breast carcinoma (MCF-7) cells using the sulforhodamine B (SRB) assay. We obtained the extracts via the ethanol extraction and liquid-liquid extraction with four different solvents:ethanol (EtOH) petroleum ether (60 – 80 ^{o}C) (PE), ethyl acetate (EA), butanol (BU) and water (W), successively. The result shows that the cytotoxic potential of biomass and fruit-body extracts were determined by the IC_{50} value at 0 – 100 µg/mL concentration. The PE-fruit-body extract displayed the highest cytotoxic activity against MCF-7 and Jurkat cell lines with IC_{50} values of 76.30 ± 1.20 µg/ml and 37.18 ± 1.39 µg/ml, respectively. Besides, the PE- biomass extracts showed the highest cytotoxic activity against CF-7 and Jurkat cell lines with IC_{50} values of 26.94 ± 1.62 µg/mL and 15.50 ± 0.19 µg/ml, respectively. Obviously, we need to study further about the cytotoxic mechanism PE biomass extracts on MCF-7 and Jurkat cells and chemical composition analysis of extracts to possibly use as a promising anticancer drug material.

Keywords: Cordyceps neovolkiana, biomass, fruit body, fungus, carcinoma.

1. INTRODUCTION

Cordyceps is a group of the insect-parasitic fungi with high pharmacological values that has been used in traditional medicine for treating some diseases such as cancer, asthma, bronchitis, impotence, immune regulation and anti-aging [1]. At present, over 400 types of *Cordyceps* have been found, described and distributed mainly in the wet tropics and temperate regions in East

Asia and Southeast Asia [2].In Vietnam, more than 10 strains of *Cordyceps* have been discovered and studied such as DL004 (*C. neovolkiana),* DL0038A (*C.takaomontana*), DL006, DL0015,etc.[3]. However, in the world, only two *Cordyceps* species are widely researched and applied including *Ophiocordyceps sinensis* and *Cordyceps militaris*- mostly found in China. Thus, further research on these strains is needed to develop local medicinal mushrooms in nature which could replace the rare and endangered yet wildly-collected *Cordyceps.*

Cordyceps neovolkiana is a species in *Cordyceps* group, recorded by Kobayasi*et al.* in 1941 and has been found in the LangbiangMountain. Its mycelial biomass and fruit bodies have been researched and successfully cultured in Vietnam. Some initial studies suggest that *C. neovolkiana* biomass extracts contain bioactive compounds such as adenosine, cordycepin, polysaccharide, phytosterol, polyuronic and display potential bioactivities suchas antioxidant, cytotoxic and immunomodulatory effects. Specifically, the $CHCl_3$biomass extract had cytotoxic ability to three cancer cell lines: HeLa, NCI-H460 and MCF-7 from 74.98 % to 82.07 %; EAand PE also had cytotoxic activity, though low, against NCI-H460 and MCF-7 [4]. *C. neovolkiana* biomass extracts also had been demonstratedtohave ABTS• free radical scavenging potential (IC_{50} values between 4129.92 ± 25.12 μg/ml and 4926.25 ± 41.01 μg/ml) and at 200 μg/ml, the EtOH extract exhibited peripheral blood mononuclear cell (PBMC) proliferation inhibition [5].

In this study, we evaluate and compare the *in vitro* cytotoxic activity of crude extracts from fruit bodies and biomass of *C. neovolkiana* DL004 fungus against human Jurkat T (acute T cell leukemia) and breast carcinoma (MCF-7) cell lines.

2. MATERIALS AND METHODS

2.1. Materials

2.1.1. Fungal strain and seed culture

Cultured mycelia of *Cordyceps neovolkiana* was supplied by Nguyen Long Joint Stock Company in Lam Dong Province [6]. It was maintained on potato dextrose agar (PDA) (including 200 g/l potato extract, 20 g/L glucose, 20 g/l agar) at 4^{o}C. The strain was activated on the same medium at 23 ^{o}C for 10 days in a petri dish. 200ml of a seed cultured with 200 g/L potato extract and 50 g/l sucrose was inoculated with two seed agar discs (Φ = 8 mm) for 10 days at 23 ^{o}C without shaking.

2.1.2. Cell line and culture medium

Cancer cell lines: breast cancer cells MCF-7 and Jurkat cells were from the Department of Genetics, Faculty of Biology and Biotechnology at University of Science, HCM-VNU. Cells were cultured in the E'MEM medium (supplemented with 2 mM L-glutamine, 20 mM HEPES, 0.025 μg/ml amphotericin B, 100 UI/ml penicillin G, 100 μg/ml streptomycin, 10 % (v/v) and Fetal Bovine Serum (FBS)) were purchased from Sigma Aldrich, Inc., USA, in an atmosphere of 5 % CO_2, at 37 ^{o}C for 24 hours and coverage of 70-80 %.

Solvents used were:ethanol (EtOH), petroleum ether (PE), butanol (BuOH), ethyl acetate (EtOAc) and other chemicals and reagents.

2.2. Methods

2.2.1. Preparation of mycelial biomass and fruit bodies

Mycelial biomass: The fungus was cultured in a liquid medium (consists of 20% potato extract, 0.05 % sucrose, 0.006 % peptone, 0.004 % yeast extract, 0.0005 % KH_2PO_4 and 0.0002 $MgSO_4.7H_2O$) with a ratio of 4 % seed at 23 ^oC without shaking. The mycelial biomass was harvested and washed with water after 40-day cultivation.

Fruit bodies: The fungus was cultured in a semi-solid medium (consists of 27.1 % brown rice, 27.1 % millet, 5 % silkworm pupae powder and 70 mL of nutrient broth containing 0.04 % glucose, 0.005 % peptone, 0.0015 % $MgSO_4.7H_2O$ and 0.0015 % K_2HPO_4) with a ratio of 12.5 % seed, incubated 15 days in dark at 23^oC. Then, it was brought to light room for the development of fruit and harvested after 45-day cultivation.

2.2.2. Preparation of extracts

After dried at 60 ^oC for about 3 days, the mycelial biomass and fruit bodies were grounded into powder. The dried powder was extracted with ethanol 96 % with ratio 1:10 (1g material: 10 ml ethanol) for two days at room temperature. The extract was collected by drip method and repeated until the color of extracts was out. Ethanolic extract (EtOH) were obtained after removing solvent by evaporation. The residue of ethanol extraction were extracted with distilled water at 65 ^oC for one hour to obtain polysaccharide extracts (PS) by precipitating it with ethanol 96 % (1:4, v/v) [6]. The EtOH were extracted with four various solvents (including petroleum ether 60–80 ^oC (PE), ethyl acetate (EA), butanol (BuOH) and water (W), successively) by using the liquid-liquid extraction method. All the extracts were obtained by evaporation and then dissolved in 5% dimethyl sulfoxide (DMSO) to reach appropriate concentrations.

2.2.3. Sulforhodamine B (SRB) assay

SRB is widely used for screening in cytotoxic experiments based on the binding ability of the SRB dye to the protein complex of the cell. The test was conducted according to the method of the Department of Genetics, Faculty of Biology and Biotechnology at University of Science, HCM-VNU. Cancer cell lines were seeded by E'MEM medium in 96-well plates (at 70–80 % confluency) and incubated under 5 % CO_2 at 37 ^oC for 24 hours. Then, they were treated with various concentrations of extracts for 48 hours. The cells were fixed with 50 µl of 50 % (w/v) cold trichloroacetic acid for 1–3 hours, washed 5 times with distilled water and dried at room temperature for 12-24 hours.

After that, added with 100 µl 0.2 % (w/v) SRB (Sigma) into each well for 20 min and washed 4 times with 1 % acetic acid, the cell plates were shaken with 10 mM Tris base on an orbital shaker to solubilize the protein-bound dye (approximately 10 min). The absorbance was then determined by ELISA reader at 492 nm and 620 nm wavelength. Camptothecin at concentration of 0.05 µg/ml was used as a positive control.

The rate of cell inhibition was calculated according to the following formula:

Inh % = (1-[As/Ac]×100)%, where As = absorbance value of test sample; Ac = absorbance value of control. $A(c/s) = A_{492} - A_{620}$, where A_{492} and A_{620} are the absorbance values at 492 nm and 620 nm, respectively; $A_{(492/620)} = A_{cells} - A_{blank}$, where A_{cells} and A_{blank} are the absorbance values in the presence and absence of cells, respectively.

3. RESULTS AND DISCUSSION

3.1. Extraction yield

Ethanol was an effective solvent for extraction of phytochemicals in fungi and plants as it has the ability to dissolve most the polar and non-polar compounds. Based on the different degree of polarization, PE, EA, BuOH and W solvents were examined as potential separating agents for liquid-liquid extraction. As a result, 6 extracts from biomass and 6 extracts from fruit bodies of *C. neovolkiana* fungus were obtained. In general, the extraction yields of the biomass were higher than those of the fruit bodies, except for the PS. Among those examined, the rate of EtOH-biomass extract was 41.07 % and was much higher than the proportion of EtOH-fruit-body extract (16.69 %). This indicates that there are many soluble substances in EtOH in biomass.

Similarly, the results for liquid-liquid extracts of biomass were also higher than those of fruit bodies. The most obvious differences in W and PE extracts were 18.57 %, 10.50 % in biomass and 8.99 %, 3.53 % in fruit bodies, respectively. Noticeably, EA extract occupied low percentages both in biomass (1.12 %) and fruit body (0.78 %) (Fig. 1).

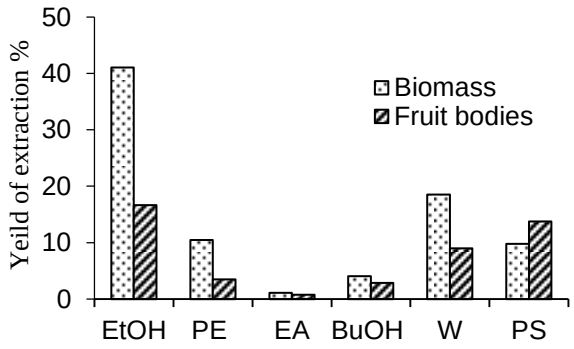

Figure 1. The comparison of the extraction yield between biomass and fruit bodies of *Cordyceps neovolkiana* (DL004) fungus.

In conclusion, both the biomass and fruit bodies of artificial *C. neovolkiana* DL004 contained non-polar and polar organic compounds. In particular, polarized and very polar compounds occupy most by the high BuOH, W and PS fractional efficiency. However, their chemical composition and characteristics have still not been researched clearly.

3.2. Cytotoxic activity of extracts against MCF-7 and Jurkat T cell lines

There are many extracts from other types of *Cordyceps* as well as C. *neovolkiana* that show potential anti-cancer activities such as *C. sinensis, C. militaris* and *C. takaomontana* [7, 8]. Our research was conducted to check and compare the cytotoxic activity of these extracts against MCF-7 (characteristic of adherent cell line) and Jurkat T (characteristic of suspension cell line) cell lines at a concentration of 100 µg/mL by using SRB assay.

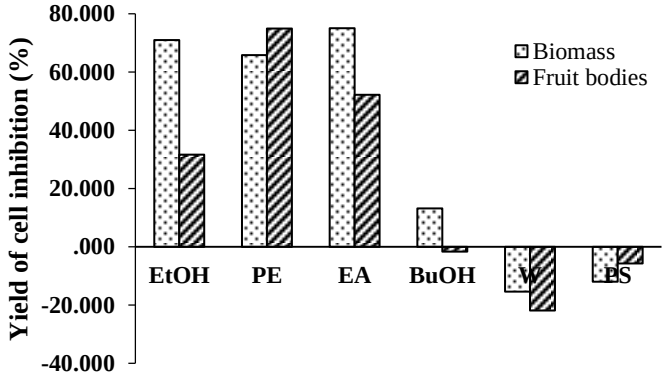

Figure 2. The percentage of cell inhibition of extracts from biomass and fruit bodies of *Cordyceps neovolkiana* (DL004) fungus against MCF-7 cell line at 100 µg/mL concentration.

The results show that EtOH, PE and EA extracts had high cytotoxic capacity (more than 50 %) but capacity is different between extracts of the mycelial biomass and fruit bodies. Specifically, EtOH and EA extracts of the mycelial biomasses had stronger cytotoxic activity (71.05 %, 75.14 %) than those of fruit bodies (31.61%, 52.19 %), respectively. Meanwhile, cytotoxic capacity of fruit bodies was higher than biomass in PE extract, 74.97 % and 65.85 %, respectively (Fig. 2). The BuOH extract had rates of cell inhibition inconsiderably-less than 20 %, which means over 80 % of the MCF-7 cells was still alive when treated by BuOH extract. Notably, W and PS extracts were capable of stimulating cell proliferation by both of biomass and fruit bodies.

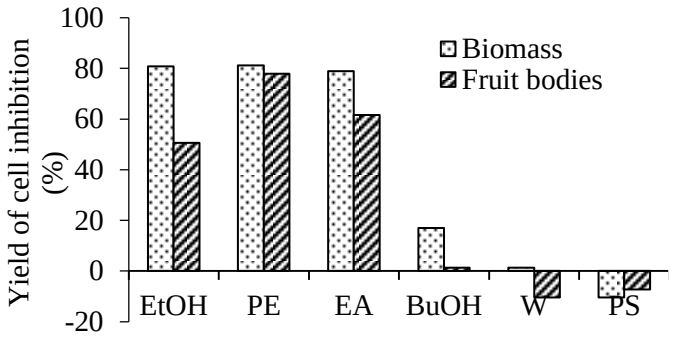

Figure 3. The percentage of cell inhibition of extracts from biomass and fruit bodies of *Cordyceps neovolkiana* (DL004) fungus against Jukat cell line at 100 µg/mL concentration.

Similar to MCF-7 cells, three extracts of EtOH, PE and EA had high cytotoxic activity against Jurkat T cell line, however cytotoxic activity of mycelial biomass extracts was stronger approximately 80 % and was the highest at PE extract with 81.2 %, followed by EtOH extract with 80.78 % and EA extract with 78.90 %. This activity of three extracts from fruit bodies were 77.87 %, 61.54 % and 50.53 %, correspondingly. Besides, BuOH, W and PS extracts did not considerably affect or stimulate the development of the cells (Fig. 3). Based on the result of the cytotoxic activity of extracts, we only selected the EtOH, PE, BuOH extracts of biomass and PE, EA extracts of fruit bodies to determine their IC_{50} values against MCF-7 and Jurkat T cell lines (Table 1).

Table 1. Determination of IC_{50} value of EtOH, PE and EA extracts from mycelial biomass and fruit bodies on MCF-7 and Jurkat T cell line (µg/mL).

Extract	MCF-7		Jukat	
	Biomass (µg/mL)	**Fruit bodies (µg/mL)**	**Biomass (µg/mL)**	**Fruit bodies (µg/mL)**
EtOH	37.60 ± 2.27	-	17.88 ± 0.78	-
PE	26.94 ± 1.62	-	15.50 ± 0.19	37.18 ± 1.39
EA	78.13 ± 3.27	76.30 ± 1.20	35.68 ± 0.29	64.36 ± 5.99

"-" extracts did not defined the IC_{50} value.

Cells were treated with these extracts at various concentrations of 0–100 µg/mL. The results showed that the PE extract of mycelial biomass had the highest cytotoxic activity on MCF-7 and Jurkat T cell lines with the IC_{50} of 26.94 ± 1.62 µg/mL and 15.50 ± 0.19 µg/mL respectively. Also, EtOH-mycelial-biomass extracts had high cytotoxic activity on Jukat with IC_{50} 17.88 ± 0.78 µg/ml. The IC_{50} value of other extracts were also determined at concentrations of 0–100 µg/mL, specifically on MCF-7: 37.60 ± 2.27 µg/mL with EtOH-mycelial-biomass extract; 78.13 ± 3.27 µg/mL with EA-mycelial-biomass; 76.30 ± 1.20 µg/mL with PE-fruit-body extracts, and on Jukat cell lines: 35.68 ± 0.29 µg/mL with EA-mycelial-biomass extract; 37.18 ± 1.39 µg/mL with PE-fruit-body extracts and 64.36 ± 5.99 µg/mL with EA-fruit-body extracts.

Cordyceps neovlkiana (DL004) was discovered early in 1941and in the Langbiang mountain, Vietnam. However, research on the medicinal values of this fungus have still been restricted. The results of SRB testing showed that EtOH, PE and EA extracts from DL004 mycelial biomass and fruit bodies had cytotoxic acivity. Both of mycelial biomass and fruit bodies can inhibit MCF-7 and Jurkat T carcinoma cells. However, there were differences between mycelial biomass and fruit bodies due to the difference in nutrient composition and culture conditions. This demonstrates that cytotoxic compounds are nonpolar or less polar and soluble in PE and EA solvents. All three extracts from biomass were higher than fruit bodies with two cell lines (It is possible that in the process of development, fruit bodies released a part of bioactive substances into semi-solid media). The results are similar to the research of Liu in 2014 on *Cordyceps militaris:* mycelia was exhibited antitumor activity against six tested human cancer cell lines better than fruiting bodies, with the range of IC_{50} (µg ml^{-1}) from 25.03 ± 1.37 to 39.81 ± 0.54. This also demonstrated that the fermented mycelia contained significantly higher amount of protein, crude fat, polysaccharides and minerals. PE extract had the highest activity with the IC_{50} of 26.94 ± 1.62 µg/ml against MCF-7, 15.50 ± 0.19 µg/ml against Jukat T and EtOH-biomass extract also had strong activity on Jukat T with IC_{50} value about 17.88 ± 0.78 µg/ml. Also, the results showed that extracts of *C. neovlkiana* had higher cytotoxic activity against Jukat T than MCF-7.

In particular, our results are consistent in cytotoxic activitywith some previous studies on *C. neovlkiana* and other *Cordyceps* [9 - 12]. EtOH, PE. CHCl3 and EA extracts have cytotoxic activity against the NCI-H460 and MCF-7 cell lines. Notably, phytosterol, cordycepin và adenosin had been detected in those extracts- esgrosterol were found in PE, adenosin and cordycepin were found in EtOH and EA extracts. Those are the major bioactive components making *Cordycepsin vitro* cancer resistant [13, 14]. PE biomass extract of *C. neovlkiana* has much higher cytotoxic activity (IC_{50} at 26.94 µg/ml) than Thao's research in 2012. The culture medium of *C. neovlkiana* of this study and Thao's are relatively different. The culture media could be affected by biomass of *C. neovlkiana* and biological activity of *C. neovlkiana* extract.

EA extract has lower cytotoxic activity (IC_{50} at 78.13 µg/ml) than EA extract from Wu's research (IC_{50} at 45 µg/ml) in 2007.

In short, results of our study demonstrated that the cytotoxic potential of the mycelial biomass extract was better than that of the fruit body. The differences regarding cultured conditions and medium constituents lead to the chemical composition, bioactive compounds and pharmacological efficiency of a cultured *Cordyceps* species sharing no similarity between the mycelial biomass and the fruit bodies.

4. CONCLUSION

Our research showed that the mycelial biomass extracts of *Cordyceps neovolkiana* fungus have tronger cytotoxic activity against MCF-7 and Jurkat T cells than the fruit bodies in three extracts: EtOH, PE and EA. This result was helpful and useful to the development of *C.neovolkiana* cultivation process for efficient production of functional foods or drugs. In order to understand better causes and mechanisms, there should be more in-depth research which analyze and determine the chemical composition, structure characteristics and bioactive compounds of those extracts as well as mechanism of cytotoxic activity. Hopefully, *C. neovolkiana* extracts would be used as a promising anticancer material in the future.

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