Screening for some biological activities of Cultured *cordyceps neovolkiana*

Chi-Dung Nguyen, Thu Huynh, Minh-Hiep Dinh

***Abstract*— *Cordyceps* has been demonstrated to possess a myriad of biological compounds and effects. There are various strains of *Cordyceps*. In this study, we evaluated some biological activities of cultured *Cordyceps neovolkiana* extracts. The result exhibited that all *C. neovolkiana* extracts almost showed no toxicity effect on HepG2 cells at the concentration of 100 µg/ml. Besides, all *C. neovolkiana* extracts also were not α-glucosidase inhibitory activity at the concentration from 1000 to 8000 µg/ml. However, some extracts had ABTS˙ free radical scavenging potential with IC_{50} values between 4129.92 ± 25.12 and 4926.25 ± 41.01 µg/ml. In addition, at 200 µg/ml, the EtOH extract exhibited 64.57 ± 6.30 % ($p<0.001$) of PBMC proliferation inhibition. In conclusion, these data revealed biological activities of cultured *C. neovolkiana*, suggesting that further studies would be necessary.**

***Index Terms*— Antioxidant, *Cordyceps neovolkiana*, cytotoxic, α-glucosidase, PBMC**

1 INTRODUCTION

Cordyceps and related species are a special group of fungi within Hypocreales (Ascomycota) that are parasites of insects, *Elaphomyces*, nematodes, and plants [7]. More than 400 *Cordyceps* species have been described worldwide with the highest diversity in East Asia and Southeast Asia. *C. neovolkiana* is a genus in *Cordyceps* group [2]. *Cordyceps* fungi are mostly regarded as bio-controls in agriculture and as precious traditional herbals in Vietnamese and Chinese traditional medicines [1]. Besides, *Cordyceps* extracts isolated from cultured *Cordyceps* had demonstrated to exhibited potential bioactivities, especially antioxidant, cytotoxic and immunomodulatory activities. Hence, this study concentrated on the antioxidant, cytotoxic and immunomodulatory effects of cultured mycelial *C. neovolkiana* extracts.

To our knowledge, no information is available on the fraction extracts of cultured *C. neovolkiana*. In Vietnam, the fungus has been isolated in Lam Dong province. Therefore, the aim of the study is to determine some biological activities of cultured *C. neovolkiana*.

2 MATERIALS AND METHODS

2.1 Materials

Cultured mycelia of *Cordyceps neovolkiana* was from Nguyen Long Joint Stock Company, Lam Dong Province. The HepG2 cell line was from Department of Genetics, Faculty of Biology and Biotechnology, University of Science, VNU-HCM. 2,2-azinobis-3-ehtylbenzothiazolin-6-sulfonic acid (ABTS), p-nitrophenyl-α-D-glucopyranoside (pNPG), fetal bovine serum (FBS), trypan blue (w/v), phytohemagglutinin – PHA, Dulbecco's Modified Eagle's Medium and RPMI 1640 medium (supplemented with 10% FBS, 10 mM HEPES, 2 mM L-glutamine, 50 µg/ml Gentamicin, 100 U/ml Penicillin, 1000 µg/ml Streptomycin, and 0.25 µg/ml Amphotericin B) were purchased from Sigma Aldrich, Inc., USA. Human peripheral blood mononuclear cells (PBMCs) were obtained from three healthy volunteers who did not use drug for 7 days. Sodium dodecyl sulfate - SDS ($C_{12}H_{25}NaO_4S$) and 3-(4, 5-dimethylthiazol-2- yl)-2, 5 diphenyl tetrazodium bromide (MTT) were purchased from Merck Inc. (Germany). Other chemicals and reagents were analytical grade.

Manuscript Received on March 15th, 2017, Manuscript Revised on November 01st, 2017.

We would like to thank Dr. Binh-Nguyen Truong for providing cultured Cordyceps spp. used in this study.

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2.2 Methods

2.2.1Preparation of C. neovolkiana extracts

The cultured *C. neovolkiana* biomass was dried and extracted in 96% ethanol (EtOH). Then, petroleum ether (PE), ethyl acetate (EtOAc), n-buthanol (BuOH) and water (W) fractions were obtained in ascending sequence of polarity by liquid-liquid extraction with EtOH extract. Polysaccharide (PS) from residues was extracted by hot water extraction [9].

2.2.2Cytotoxic assay

The cytotoxic screening was measured by the Sulforhodamine B method of the US National Cancer Institute. The HepG2 cells were cultivated on a 96-well plate at an initial density of 10^4 cells/well and pre-incubated for 24 hours before treating with a 100 µg/ml of samples (using Dimethyl sulfoxide (DMSO) 5% to dissolve samples) and continuously incubating for 48 hours. After that, 10% Trichloroacetic acid (TCA) was added before keeping at 40^0C for 1-3 hours, removing broth and drying at room temperature. Following that, the 0.2% Sulforhodamine B was added to dye cells at room temperature for 10-15 min. The Sulforhodamine B was removed by 1% acetic acid. The results were screened by adding 200 µl 10 mM Tris-base and measuring at 492 nm and 620 nm. The positive control was camptothecin at concentration of 0.07 µg/ml. The cytotoxic activity was calculated by $(1- A_c/A_s)$ x 100% with: $A_{(c/s)} = A_{492} - A_{620}$, 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; A_c and A_s are the absorbance values of the control (DMSO 0.25%) and tested samples, respectively.

2.2.3Determination of the α-glucosidase inhibitory

α-glucosidase breaks down starch and disaccharides to glucose related to diabetes. Assay for α-glucosidase inhibition was performed by slight modification of a previously published method (Ma *et al*, 2011). Briefly, solutions of α-glucosidase and its substrate (p-nitrophenyl α-D-glucopyranoside pNPG) were prepared in phosphate buffer (100 mM, pH 6.9). 5% DMSO was used as a preferred solvent for preparation of inhibitor solutions. The inhibition assays were conducted by adding inhibitor solution (50 µl) to 40 µl of enzyme solution (0.2 unit/ml) in 100 mM phosphate buffer (pH 6.8) followed by room temperature for 20 minutes. After pre-incubation, 40 µl of 3 mM substrate (pNPG) prepared in phosphate buffer was added to the mixture to initiate enzymatic reaction. The reaction mixture was incubated at room temperature for 30 minutes, and the reaction was stopped by addition of 130 µl of 0.2 M Na_2CO_3. Acarbose was used as a positive control. The α-glucosidase activity was determined by measuring the p-nitrophenol released from pNPG at 405 nm using an Elx 800 Micro plate reader. The % inhibition was calculated using the following equation: Inhibition (%) = $[1 – (A_{sample} – A'_{sample})/(A_{control} – A'_{control})]$ x 100%, where A_{sample} and $A_{control}$ are the absorbance values of the tested and control (DMSO 5%) samples, respectively; A'_{sample} and $A'_{control}$ are the absorbance values of the tested and control (DMSO 5%) samples without α-glucosidase. IC_{50} values of potent inhibitors were determined by testing 5 serial dilutions of inhibitors and were calculated by using the program Microsoft Excel.

2.2.4$ABTS^{\cdot+}$ radical scavenging assay

The measurement of $ABTS^{\cdot+}$ radical scavenging activity was used with some modifications [3]. 7 mM $ABTS^{\cdot+}$ was mixed with a 2.45 mM potassium persulphate solution. The reaction mixture was left to settle at room temperature for 12-16 h in the dark before using. $ABTS^{\cdot+}$ solution was diluted with phosphate buffer to adjust its absorbance to within 0.70 ± 0.02 at 734 nm. Then, 3 ml of $ABTS^{\cdot+}$ solution was mixed with 100 µl of various concentrations of samples. Vitamin C was considered as a positive control. Finally, the absorbance was measured at 734 nm after reaction at room temperature for 30 minutes.

The $ABTS^{\cdot+}$ free radical scavenging activity was calculated by the following equation: $(1 − A/A_0)$ x 100%, where A and A_0 were the absorbance values in the presence and absence of the test samples, respectively. Each experiment was carried out in triplicates and consequences were exhibited as mean % antioxidant activity ± SD. The concentration of 50% inhibition (IC_{50} value) based on the percentage of $ABTS^{\cdot+}$ radicals scavenged was calculated from the plotted graph of the means at the concentrations of the samples.

2.2.5Preparation of PBMC and lymphoproliferation test

PBMC were isolated from heparinized human peripheral blood of healthy donors. Blood cells were obtained by centrifuging (20^0C, 900 rpm, 30 min) the mixture of blood and normal saline (v/v:1/1) on Ficoll-Paque (v/v:4/3) gradients as

described by manufacturer's protocol (Sigma-Aldrich). The PBMC layers were collected and washed with normal saline solution to remove red blood cells, then centrifuged (20^0C, 800 rpm, 10 min). The PBMC were dissolved with erythrocytolytic solution from 3-5 minutes at room temperature, and centrifuged (20^0C, 2000 rpm, 5 min). The cells were re-suspended in RPMI-1640 medium supplemented with 10% FBS.

The lymphoproliferation test was modified from MTT colorimetric assay by Mosmann [5]. 100 µl of cell suspension adjusted to 1×10^5 cells was applied into each well of a 96-well flat-bottomed plate with 100 µl fresh medium that containing different concentrations (0, 50, 100, 200 µg/ml) of extracts. The plates were incubated in 5% CO_2-air humidified atmosphere at 37 ^{0}C for 48 hours. Subsequently, 20 µl MTT (5.5 µg/ml) was added into each well. The plates were continually incubated in 5 % CO_2-air humidified atmosphere at 37 ^{0}C for 4 hours in dark. Then, 50 µl 20% SDS in 0.02 M HCl was added into each well, and then incubated for 16 hours. Absorbance was measured with an ELISA plate reader at 570 nm and 620 nm. All experiments were conducted at least three times [5].

The activity of each extract on PBMC proliferation was calculated by the following formula:

$$G(\%) = (OD_{(570\text{-}620)\ samples}/OD_{(570\text{-}620)\ control} - 1) \times 100\%$$

3 RESULTS AND DISCUSSION

3.1 Cytotoxicity effect

The resuts indicated that the EtOH and fraction extracts of cultured mycelia *C. neovolkiana* displayed weak cytotoxic activity on HepG2 cells (Fig. 1). In this assay, camptothecin was used as a positive control, which inhibited about 58.65 ± 3.57 % of the HepG2 growth at a concentration of 0.07 µg/ml. At 100 µg/ml, the cytotoxic activity of EtOH extract on HepG2 was the highest (about 30.19%). Wang *et. al* [11] investigated the inhibitory activity of HepG2 proliferation of EtOH extracts and fraction extracts of cultured mycelia *C. sinensis* that were contrast to our present study.

3.2 ABTS$^{\cdot+}$radical-scavenging activity

The *in vitro* antioxidant activity of the extracts was determined by using the ABTS$^{\cdot}$ radical scavenging assay. As shown in Fig. 2, the IC_{50} values of EtOH and fraction extracts in ABTS free radicals scavenging assays ranged from 4129.92 ± 25.12 µg/ml to 4926.25 ± 41.01 µg/ml, and > 5000 µg/ml, whereas the IC_{50} value of vitamin C was about 34 ± 1 µg/ml. Compared to antioxidant activities of polysaccharide extract of *C. neovolkiana* [6], the IC_{50} values of ABTS free radical scavenging activity of polysaccharide extract of *C. neovolkiana* was 2952 ± 26.56 µg/ml. The results in this study showed that the antioxidant activity *in vitro* of polysaccharide extracts of *C. neovolkiana* was higher than solvent fraction extracts of *C. neovolkiana*. Besides, the IC_{50} values of ABTS free radical scavenging activity of IPS and EPS from *Cordyceps sinensis* were 1885.90 ± 2.91 to 4417.96 ± 91.16 µg/ml, respectively [10]. The comparison exposed higher antioxidant activity of polysaccharide *C. sinensis* extracts than fraction *C. neovolkiana* extracts. In addition, Wu *et al* [13] studied the antioxidant activity of polysaccharide fractions purified from *Cordyceps militaris*. The result attributed to their electron transfer or hydrogen donating ability. It has been suggested the existence of hydroxyl group in polysaccharides could donate electrons to reduce the radicals to a more stable form or reacts with the free radicals to terminate the radical chain reaction. There was a direct correlation between antioxidant activities and reducing power. Furthermore, the presence of reductant associated with the reducing power. Reductant has been shown to exert antioxidant action by breaking the free radical chain by donating a hydrogen atom.

3.3 The α-glucosidase inhibitory activity

The *in vitro* α-glucosidase inhibitory activity of extracts of *C. neovolkiana* were tested at different concentrations of 125, 250, 500, 1000, 2000, 4000 and 8000 µg/ml. In this study, acarbose was used as a positive control with IC_{50} = 3579.12 µg/ml. Results indicated that all extracts were not α-glucosidase inhibitors. At 8000 µg/ml, α-glucosidase inhibitory activity of EtOAc extract was the highest with a inhibitory rate of 32.17%. So, it can be concluded that extracts of *C. neovolkiana* were not efficient for α-glucosidase inhibiting drug.

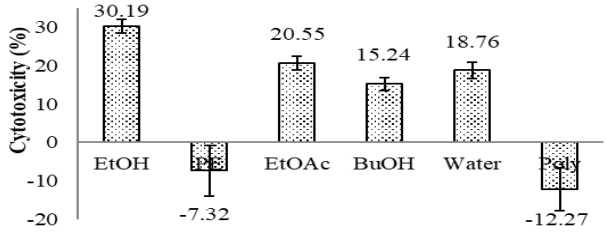

Fig. 1 Cytotoxic activity on HepG2 cells of extracts at 100 µg/ml

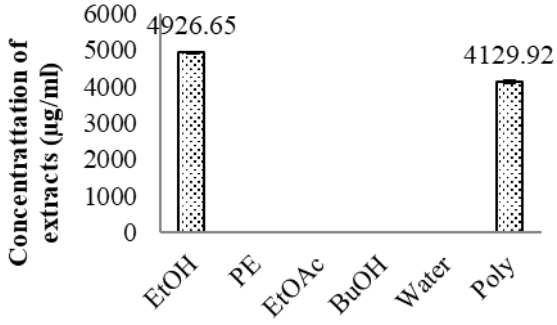

Fig. 2 IC_{50} values (µg/ml) of ABTS radical scavenging activity

TABLE 1
THE COMPONENTS OF COMPLETE BLOOD COUNT OF DONORS

Samples	Leukocyte (cell/mm^3)	Neutrophil (%)	Lymphocyte (%)	Monocyte (%)	Eosinophil (%)
Normal	4000-11000	40-76	24-44	3-10	1-3
N1	7000	63.15	31.58	5.27	0
N2	5300	63.26	26.53	10.21	0
N4	6300	64.28	30.95	4.47	0

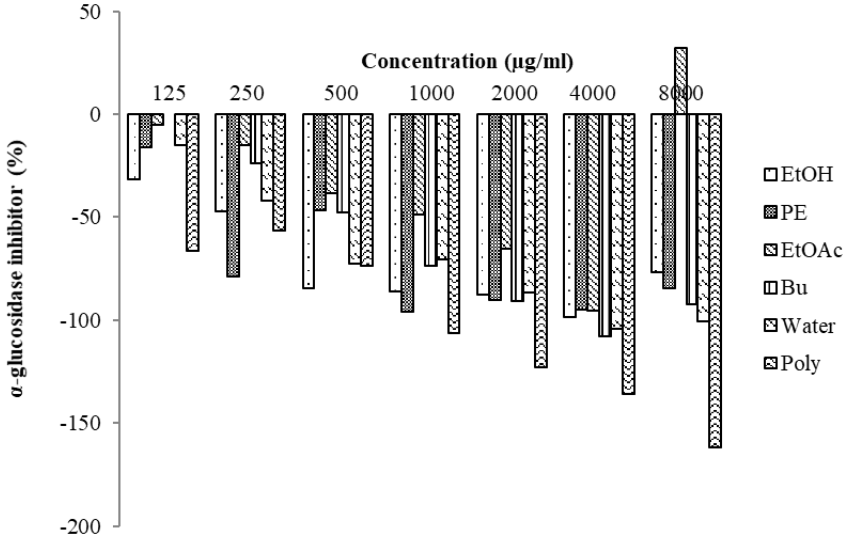

Fig. 3 The rate of α-glucosidase inhibitory of C. neovolkiana extracts

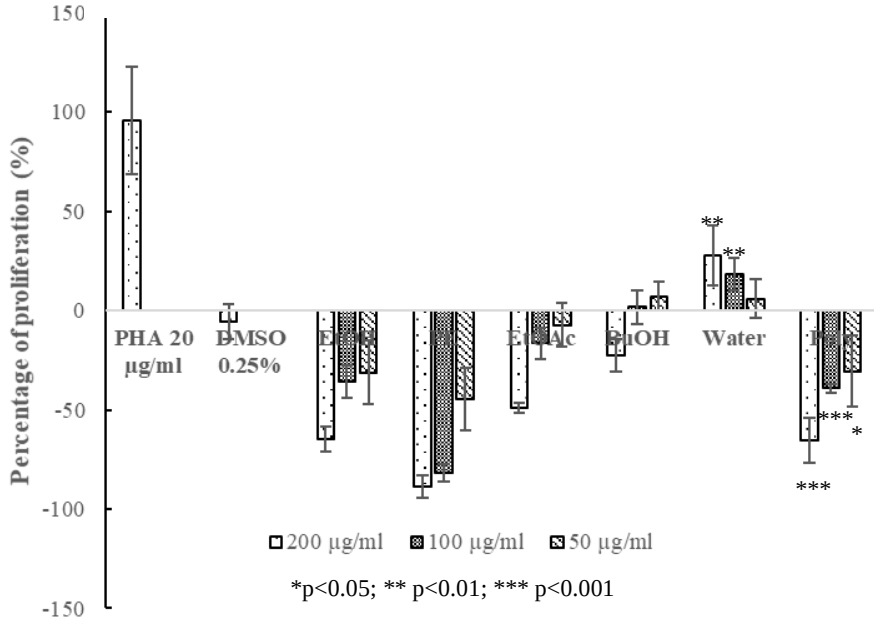

Fig. 4 Effects of C. neovolkiana extracts in PBMC proliferation

3.4 The immunomodulatory effect

Generally, the results showed both enhancing and inhibitory effects of *C. neovolkiana* extracts in human PBMC. The positive control is PHA, a mitogen for T lymphocytes can bind to N-acetylgalactosamine glycoproteins expressed on the surface of T cells then activate the cells to produce cytokines and proliferate, which stimulated the proliferation of PBMC to 95.80 ± 27.20%. Besides, both of water and DMSO control showed slight changes of -6.38 ± 4.18% and -5.65 ± 8.97%, respectively.

The EtOH extract is crude extract containing all of components in solvent fractions of different polarities. At 200 µg/ml, the EtOH extract exhibited a strong inhibition of PBMC proliferation of -64.57 ± 6.30% ($p<0.001$). In addition, the results of Wang *et al* [11] indicated that EtOH extract displayed strong cytotoxic activity on human hepatocellular (HepG2 and Hep3B) and colorectal (HT-29 and HCT 116) carcinoma cells, with an average of low IC_{50} value (27 µg/ml). The PE and EtOAc extracts obtain components from weak to moderate polarity. All concentration of both were active in inhibiting the proliferation of PBMC, significantly (Fig. 4, $p<0.05$).

In contrast, others containing polar compounds demonstrated a trend of high immunostimulating activity. The water extract at 100 µg/ml enhanced steadily the proliferation of PBMC with a stimulation index equal to 27.87 ± 15.19%, significantly ($p<0.01$). Following that, the *C. neovolkiana* extract possesses approximately the enhancement of water extract.

Wei *et al* [12] concluded that hot-water extract of *C. sinensis* influences to the maturation of human monocyte-derived dendritic cells. Moreover, CS-primed mature dendritic cells displayed increased production of IL-12 and IFN-γ when co-cultured with allogeneic T cells. It indicates that the *C. sinensis* extract may help T_h cells to differentiate into Th1 cells and suggests that *C. sinensis* can be applied as a promising adjuvant in immunotherapy. In addition, natural compounds have traditionally been used and already been proven to be nontoxic. Several studies were reported the *in vivo* used of *C. sinensis*. Nevertheless, no significant toxic effects were observed in models of Wang *et. al* indicating that *C. sinensis* is relatively safe for therapeutic purposes [11].

4 CONCLUSSION

C. neovolkiana extracts showed weak toxicity effect on HepG2 cells and were not got in α-glucosidase inhibitory activity. In contrast to that, *C. neovolkiana* extracts are able to stimulate the proliferation of PBMC, especially the water extract. In additional, *C. neovolkiana* extracts were potential antioxidant materials. These data suggest that further studies on *in vivo* models would be necessary.

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Khảo sát một số hoạt tính sinh học của nấm *Cordyceps neovolkiana* nuôi cấy

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***Tóm tắt*—** Nhóm Cordyceps đã được biết đến với nhiều hợp chất và hoạt tính sinh học. Nhóm Cordyceps rất đa dạng. Trong nghiên cứu này, chúng tôi đánh giá một số hoạt tính sinh học từ cao chiết sinh khối của Cordyceps neovolkiana. Kết quả thí nghiệm đã chỉ ra rằng cao chiết sinh khối của Cordyceps neovolkiana hầu như không có khả năng gây độc tế bào tại nồng độ 100 µg/ml. Bên cạnh đó, tất cả cao chiết sinh khối của Cordyceps neovolkiana không có khả năng ức chế α-glucosidase ở nồng độ từ 1000 đến 8000 µg/ml. Tuy nhiên, Cao chiết phân cồn và cao phân đoạn có khả năng bắt gốc tự do ABTS từ 4129,2 ± 25,12 tới 4926,25 ± 41,01 and > 5000 µg/ml. Thêm vào đó, tại nồng độ 200 µg/ml, cao cồn ức chế tăng sinh PBMC 64,57 ± 6,30 % ($p<0,001$). Điều này chứng tỏ việc nghiên cứu về hoạt tính sinh học của các cao chiết và hoạt chất từ cao chiết Cordyceps neovolkiana là cần thiết.

***Từ khóa*—** Cordyceps neovolkiana, sinh khối, cao chiết, α-glucosidase, hoạt tính sinh học