MINISTRY OF EDUCATION AND TRAINING

VIETNAM ACADEMY OF SCIENCE AND TECHNOLOGY

GRADUATE UNIVERSITY SCIENCE AND TECHNOLOGY

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STUDY on ANTI-MITOTIC ACTIVITY against CANCER CELL LINES MCF-7 and JURKAT T of EXTRACTS and ACTIVE SUBSTANCES from CULTURED MYCELIA and FRUITING BODIES of *Cordyceps neovolkiana* DL0004 and *Isaria cicadae* F0004

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SUMMARY OF BIOTECHNOLOGY DOCTORAL THESIS

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Reviewer 2: …

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INTRODUCTION

1. The urgency of the thesis

Natural products, with their diverse compositions and chemical structures, have been extensively studied for their anti-cancer potential for more than half a century and are considered a rich source of bioactive compounds and therapeutic potential. For *Cordyceps* spp., more than 200 biologically active compounds including nucleosides, sterols, cyclic peptides, flavonoids, dihydrobenzofurans, polyketids, polysaccharides, alkaloids, ergosterols and polyphenols have been isolated and identified. Studies in Vietnam are mainly carried out at the level of extracts of fungal strains belonging to the genus *Cordyceps* and there are very few studies on the analysis and determination of the antimitotic activity of the obtained substances. Therefore, "Study on anti-mitotic activity against cancer cell lines MCF-7 and Jurkat T of extracts and active substances from cultured mycelia and fruiting bodies of *Cordyceps neovolkiana* dl0004 and *Isaria cicadae* f0004" ' is necessary, to proactively create a source of raw materials and a premise for research into the application of insect parasitic fungi in Vietnam.

2. Objecttive of the thesis

Evaluation of the antimitotic activity of *Cordyceps* extracts as a basis for the application of extracts and key compounds obtained from *Cordyceps* in Vietnam.

3. Research contents

Extracting mycelia and fruiting bodies of the cultivated mushrooms; screening extracts for potential cytotoxicity on two cancer cell lines MCF-7 and Jurkat T; Study on the antimitotic properties of the potential extracts; isolation and identification of compounds with antimitotic activity from potential extracts.

CHAPTER 1. OVERVIEW

1.1. Overview of *Cordyceps*

1.1.1. Introduction of Cordyceps

Cordyceps are classified in the family Clavicipitaceae based on the cylindrical sporangia, the thickness of the apex, and the sporangia. Their host spectrum is very wide, often belonging to the group of insects and arthropods.

1.1.2. Overview of C. neovolkiana and I. cicadae

According to the taxonomy of Kobayasi (1982), *C. neovolkiana* belongs to: Family (familia): Clavicipitaceae ; Genus: *Cordyceps*. According to Sung (2007), *Isaria cicadae* belongs to the family (familia): Cordycipitaceae; Genus: *Isaria*.

1.1.3. Economic value of Cordyceps mushrooms

Researching by Data Bridge Market Research predicts, the global market will reach 1,167.50 million USD by 2027, growing at a compound annual growth rate of 10.55% during the forecast period from 2020 to year 2027.

1.2. Study on cultivation of *Cordyceps*

Besides *O. sinensis* and *C. militaris*, many other potential *Cordyceps* and insect parasitic fungi are exploited and applied such as *C. takaomontana, I. tenuipes and C. nutans, C. cicadae,...*

1.3. Chemical Constituents and bioactivities of *Cordyceps*

1.3.1. Chemical Constituents of *Cordyceps*

a) Polysaccharid: In general, polysaccharides from *Cordyceps* are difficult to analyze and identify because of their complex structure, a triple right-handed helix conformation with different lengths and ratios of pentose and hexose side chains.

b) Protein and other nitrogen-containing compounds: Cordyceps contains proteins, peptides, polypeptides, polyamines, all essential amino acids, and several common and rare cyclic dipeptides. *Cordyceps* also contains small amounts of polyamines, such as 1,3-diamino propane, cadaverin, spermidin, spermin, and putrescin.

c) Sterol: Several sterols are found in *Cordyceps*, including the common sterol in many fungal cell walls, ergosterol.

d) Others: Các hợp chất phân cực của cao chiết *C. sinensis* gồm có alcohol và aldehyde. Một vài hợp chất ức chế miễn dịch cũng được tìm thấy trong *Cordyceps*.

Polar compounds of the *C. sinensis* extract include alcohols and aldehydes. Some immunosuppressive compounds are also found in *Cordyceps*.

1.3.2. Antimitotic activity of Cordyceps

a) Antimitotic activity of C. sinensis: There is much evidence that supports the efficacy of *C. sinensis* as an anticancer drug because of its role as an immune response activator.

b) Antimitotic activity of C. militaris: C. militaris mushroom has long been used in Asian countries as a health-promoting and supportive medicine for cancer patients..

c) Antimitotic activity of other Cordyceps: For *C. neovolkiana*, studies on phylogenetics, determination of anticancer activity as well as other biological activities are less studied.

CHAPTER 2. MATERIALS AND METHODOLOGY

The studies were carried out according to the scheme: creating extracts from cultivated mushroom mycelia and fruiting bodies → screening the extracts for potential cytotoxicity on 2 cell lines MCF-7, Jurkat T → identification the characterization Antimitotic (cell cycle arrest, apoptosis induction) of potential extracts → isolated and identified compounds with antimitotic activity from potential extracts.

2.1. Materials

Strain *C. neovolkiana* DL0004 was obtained from Langbiang, Lam Dong province and Strain *I. cicadae* F0004 was obtained from Dak Lak province, Vietnam.

2.2. Methodology

2.2.1. Methods for obtaining extracts and compounds

a) Methods of artificial culture of Cordyceps mushroom mycelia and fruiting bodies

The process of mycelium and fruiting bodies culture were carried out: creating primary seed from the original stock → creating secondary seed from primary seed culture different environments and conditions to create a mycelium system (surface liquid culture) or produce fruiting bodies (semi-solid-state fermentation).

b) Extraction method

Extraction and compounds were obtained according to the method of Nguyen Kim Phi Phung (2007).

2.2.2. Method to screen cytotoxic activity and apoptosis induction of extracts and compounds

The method of investigating cytotoxic activity by SRB. Method to investigate the ability to induce apoptosis: cancer cells were analyzed for cell cycle by flow cytometry.

2.3. Statistical analysis method

Data are expressed as mean standard deviation; experiments were repeated 3 times. The data were statistically processed using GraphPad Prism 9.0.0.121 software. Values with $p < 0.05$ are accepted as having a statistically significant difference.

CHAPTER 3. RESULTS AND DISCUSSIONS

3.1. Production of mycelium extracts and fruiting bodies of *C. neovolkiana* DL0004 and *I. cicadae* F0004.

The average dried mycelium per box of two strains of *C. neovolkiana* DL0004 and *I. cicadae F0004* were 1.76 grams/box and 2.63 grams/box, respectively, after 35 days of surface culture. The average dried fruit bodies per box of two strains of *C. neovolkiana* DL0004 and *I. cicadae* F0004 were 0.52 grams/box and 1.37 grams/box, respectively, after 45 days of semi-solid-state fermentation.

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

3.2.1. Cytotoxic activity of C. neovolkiana extracts DL0004 *against MCF-7 and Jurkat T cell lines*

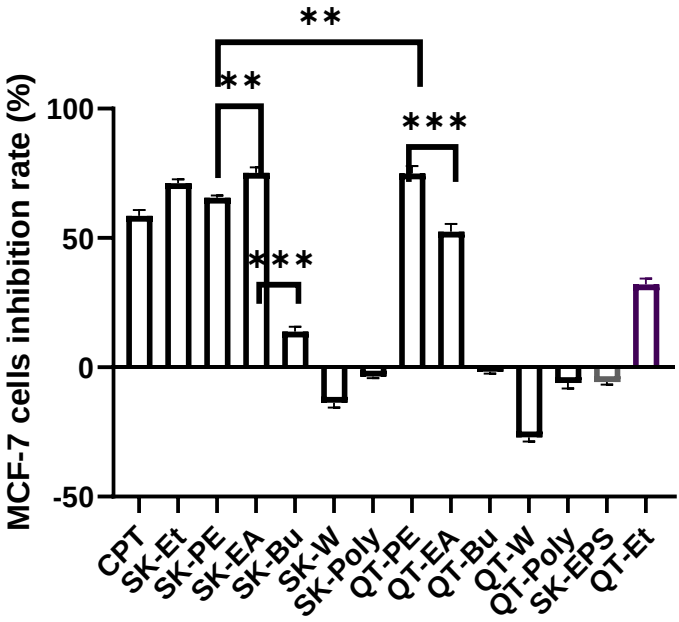


Fig 3.2. MCF-7 cells inhibition rate of *C. neovolkiana* DL0004 mycelium extract and fruiting body at a concentration of 100 µg/mL

(Notes: *: $p < 0,05$; **: $p < 0,01$; ***: $p < 0,001$)

The cytotoxic activity of MCF-7 and Jurkat T cancer cell lines were very different between the extracts, and the low polarity EA and PE extracts were capable of cytotoxicity of MCF- and MCF- cancer cell lines. 7 and Jurkat T were higher than other extracts.

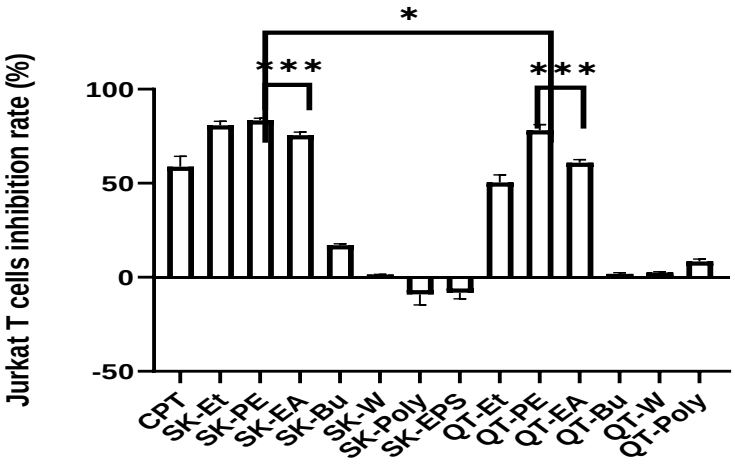


Fig 3.3. Jurkat T cells inhibition rate of *C. neovolkiana* DL0004 mycelium extract and fruiting body at a concentration of 100 µg/mL

3.2.2. Hoạt tính gây độc của các cao chiết I. cicadae F0004 đối với các dòng tế bào MCF-7 và Jurkat T

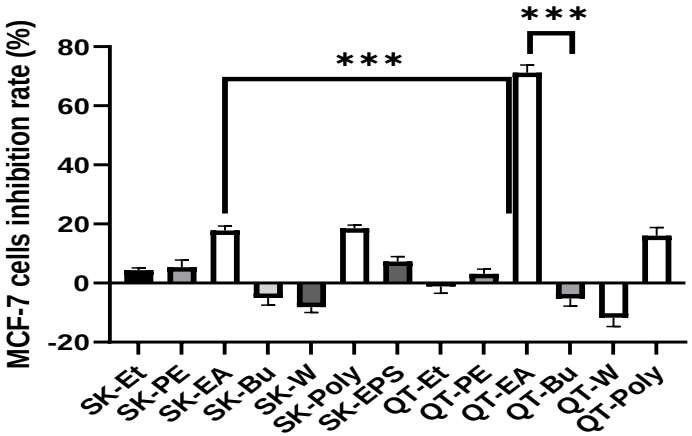


Fig 3.4. MCF-7 cells inhibition rate of *I. cicadae* F0004 mycelium extract and fruiting body at a concentration of 100 µg/mL

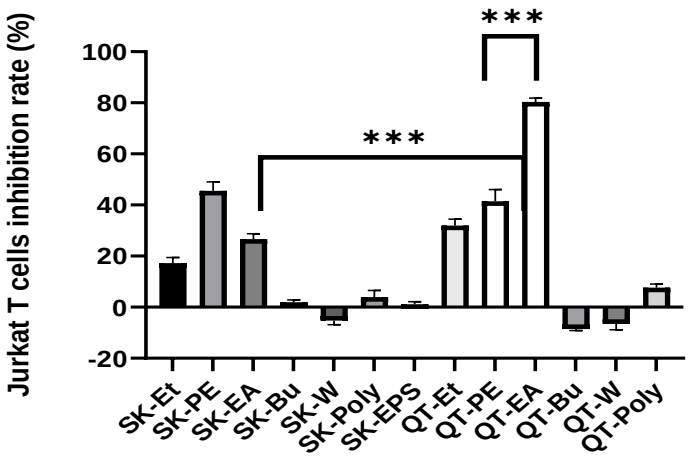


Fig 3.5. Jurkat T cells inhibition rate of *I. cicadae* F0004 mycelium extract and fruiting body at a concentration of 100 µg/mL

To proceed to the next steps, an overview was needed based on the following criteria: the extracts were considered to have high cytotoxic activity against cells in vitro with an $IC_{50} \leq 20$ µg/mL (the extracts were high active and have potential as raw materials for creating anti-cancer drugs); IC_{50} value: 21 - 200 µg/mL (moderate activity), IC_{50} value: 201 - 500 µg/mL (weak activity) and $IC_{50} > 501$ µg/mL (inactive) according to NCI criteria.

3.2.3. Identification of extracts with high cytotoxicity

PE mycelium extract from *C. neovolkiana* DL0004 had IC_{50} values with MCF-7 and Jurkat T of 26.94 ± 1.62 and 15.5 ± 0.19 (µg/mL), respectively and EA from fruit body extract from *I. cicadae* F0004 had IC_{50} values with MCF-7 and Jurkat T of 17.15 ± 1.68 and

10.37 ± 0.61 (µg/mL), respectively, which were extracts with potential cytotoxic *in vitro* activity against MCF-7 and Jurkat T cancer cells.

3.3. Study on MCF-7 and Jurkat T cells apoptosis induction with potential extracts

3.3.1. Study on PE extract of C. neovolkiana DL0004 mycelium induced apoptosis of MCF-7 and Jurkat T cancer cells

a) MCF-7 and Jurkat cells morphology after staining with AO/EB

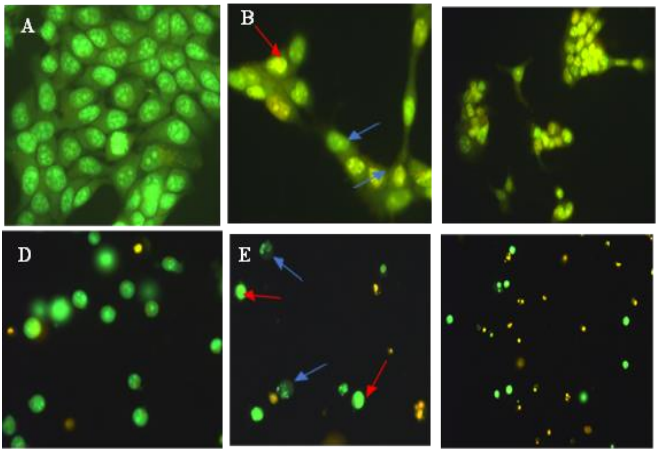


Fig 3.6. Morphology of MCF-7 and Jurkat T cells induced by *C. neovolkiana* DL0004 mycelium PE extract after AO/EB staining

: Fragmented nuclear cells : Cells with condensed nucleus

A: MCF-7 cells control **B**: PE extract after 48 h **C:** CPT after 48 h

D: Jurkat T Cells control **E:** PE extract after 48 h **F:** CPT after 48 h

PE extract strongly induces apoptosis of Jurkat T cells.

b) Cell cycle analysis by flow cytometry

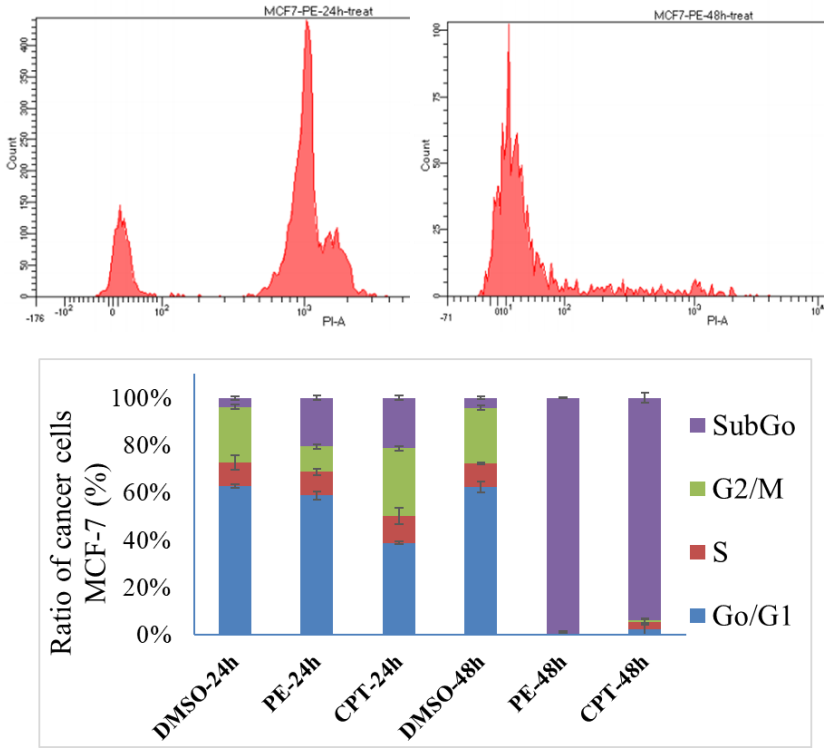
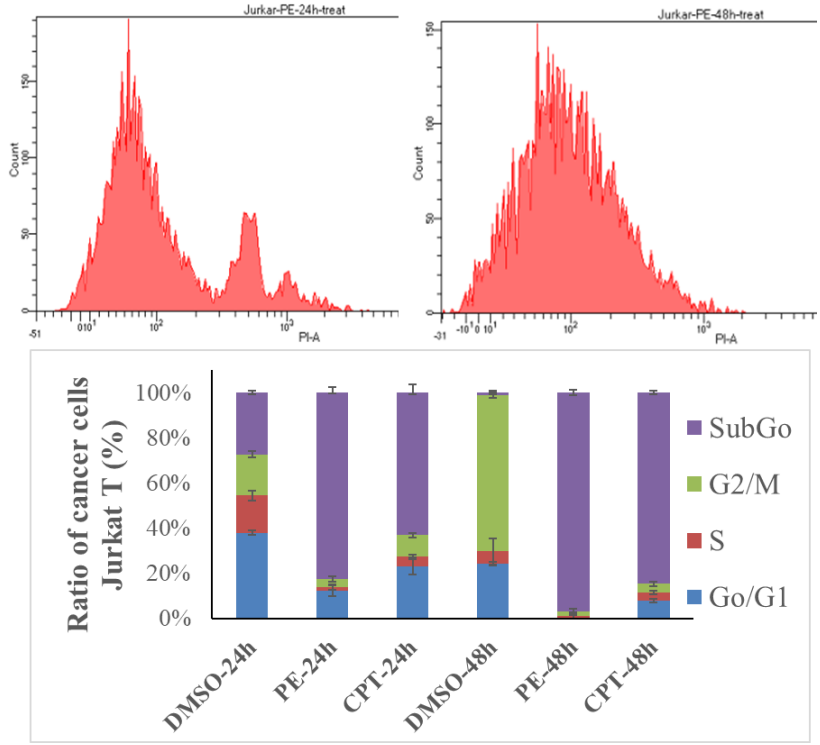


Fig 3.7. Ratio of MCF-7 cells at different cell cycle phases after induction of *C. neovolkiana* DL0004 mycelium PE extract

These results show that PE extract has the ability to stop the MCF-7 cell cycle at S phase and put cells into Sub G0 phase. After 24 h, MCF-7 cells were cell cycle stopped at S phase and Jurkat cells were stopped at G1 phase. However, when the induction time was increased to 48 h, both cell lines were completely inhibited by the cell cycle, all cells entering the resting Sub/G0 phase or undergoing apoptosis..



Hình 3.8. Ratio of Jurkat T cells at different cell cycle phases after induction of *C. neovolkiana* DL0004 mycelium PE extract

c) Quantification of apoptotic cells by Annexin V/PI staining

After 48 h of induction, the percentage of MCF-7 cells with late apoptosis increased but not significantly.

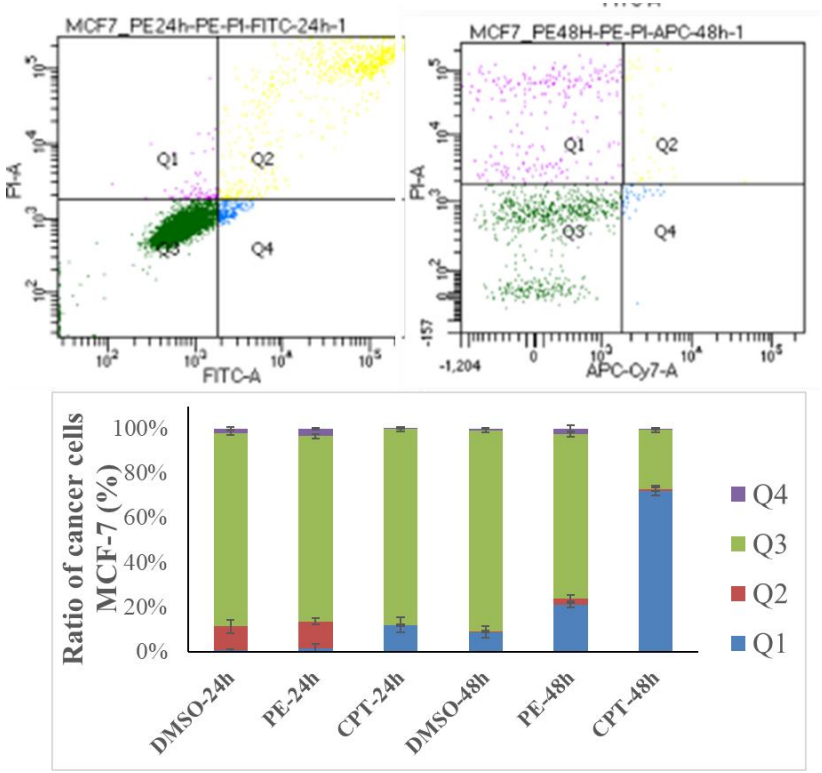


Fig 3.9. Ratio of MCF-7 cells after induction with *C. neovolkiana* DL0004 mycelium PE extract and Annexin V/PI staining

PE extract induced apoptosis of Jurkat T cells better than MCF-7 cells. Simultaneously, the mycelium PE extract *C. neovolkiana* DL0004 also caused cells necrosis.

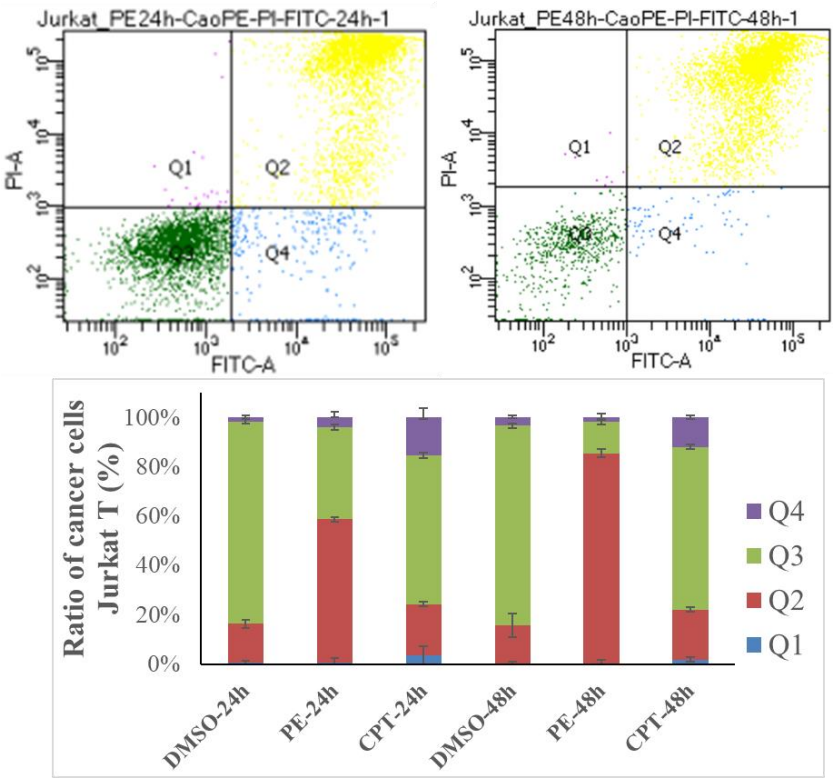


Fig 3.10. Ratio of Jurkat T cells after induction with *C. neovolkiana* DL0004 mycelium PE extract and Annexin V/PI staining

3.3.2. Results of evaluating the ability to induce apoptosis of EA extract from fruit body of I. cicadae F0004

a) Morphology of MCF-7 and Jurkat cancer cells after AO/EB staining

EA extract from fruit body of *I. cicadae* F0004 showed signs of inducing apoptosis of MCF-7 cancer cells after 48 hours of induction, but the sign was not clear.

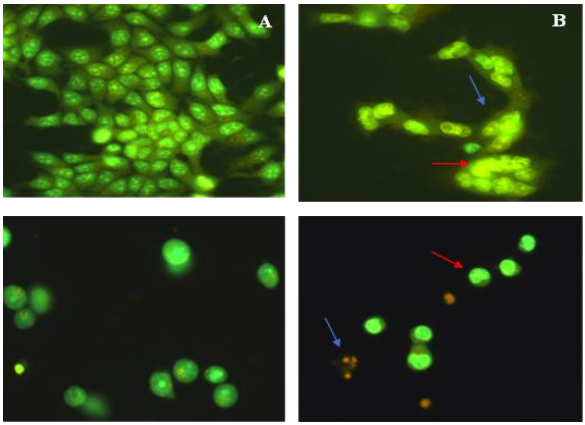


Fig 3.11. Morphology of MCF-7 and Jurkat T cells induced by *I. cicadae* F0004 mycelium PE extract after AO/EB staining

b) Cell cycle analysis by flow cytometry

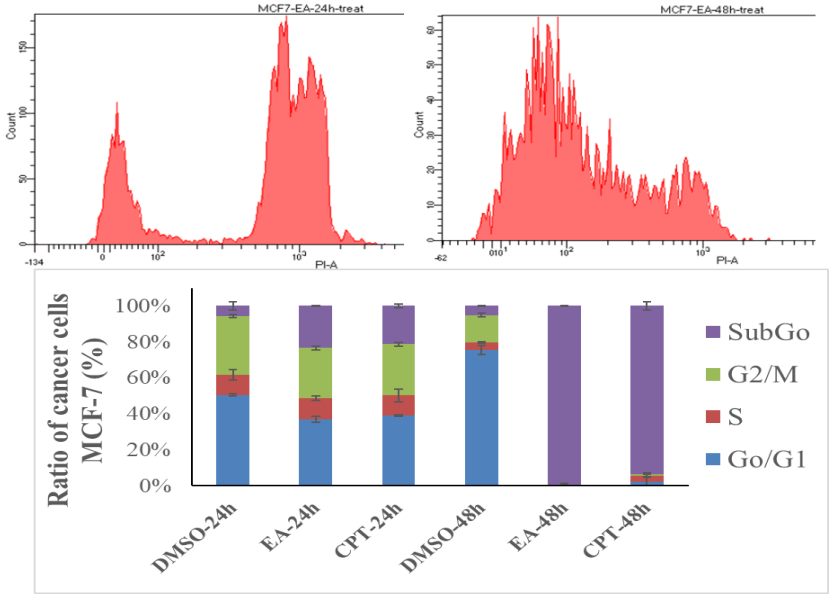


Fig 3.12. Ratio of MCF-7 cells at different cell cycle phases after induction of *I. cicadae* F0004 mycelium PE extract

The ratio of 48-hour-induced MCF-7 entering SubG0 increased sharply compared with 24-hour induction. Results after 24 hours and 48 hours of induction with EA extract of fruiting body *I. cicadae* F0004 showed that MCF-7 cells stopped the cell cycle at G1 phase. MCF-7 cells after 48 h were completely inhibited at 17,15 µg/mL, the cell entered the subG0 phase.

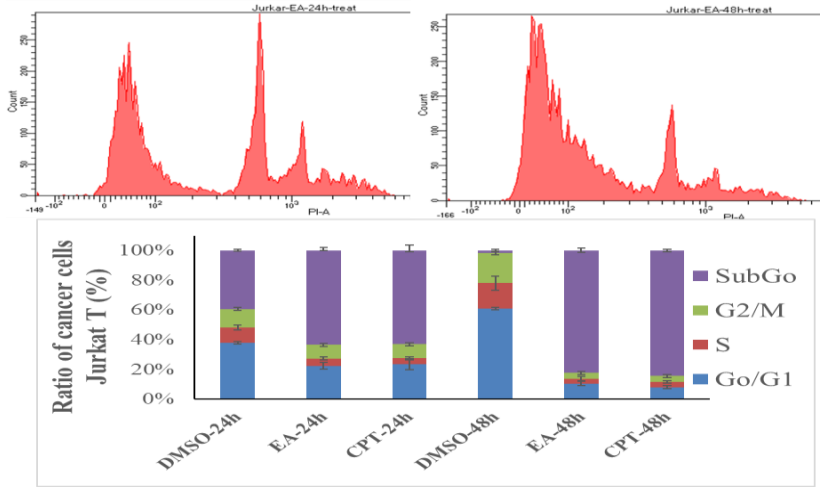


Fig 3.13. Ratio of Jurkat T cells at different cell cycle phases after induction of *I. cicadae* F0004 mycelium PE extract

The results of flow cytometry analysis showed that the Jurkat T cancer cell population when induced with EA extract tended to increase the ratio of cells in sub-G0 in real time.

c) Quantitative results of apoptotic cells by annexin V/PI staining

After 24 and 48 hours of induction, it was shown that EA extract of fruiting body of *I. cicadae* F0004 could strongly inhibit the mitotic process of MCF-7 cancer cells in real time by the mechanism of necrosis.

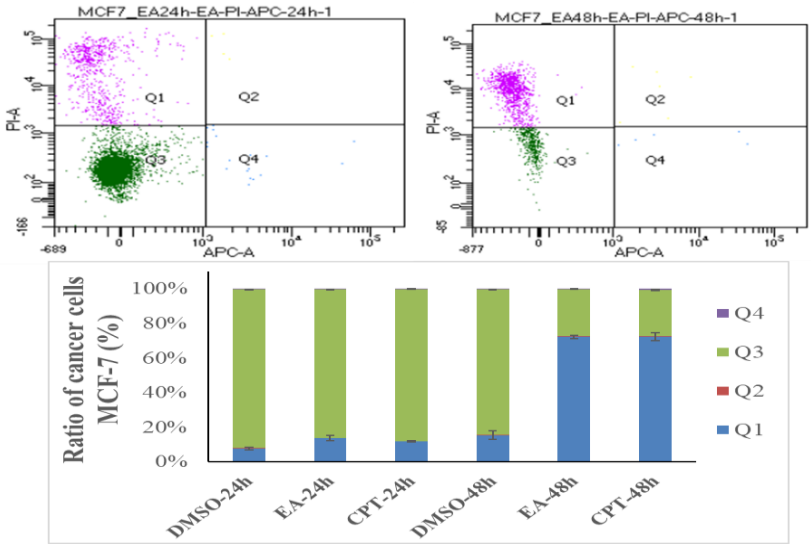


Fig 3.14. Ratio of MCF-7 cancer cells after induction of EA extract of fruiting body *I. cicadae* F0004 and Annexin V/PI staining

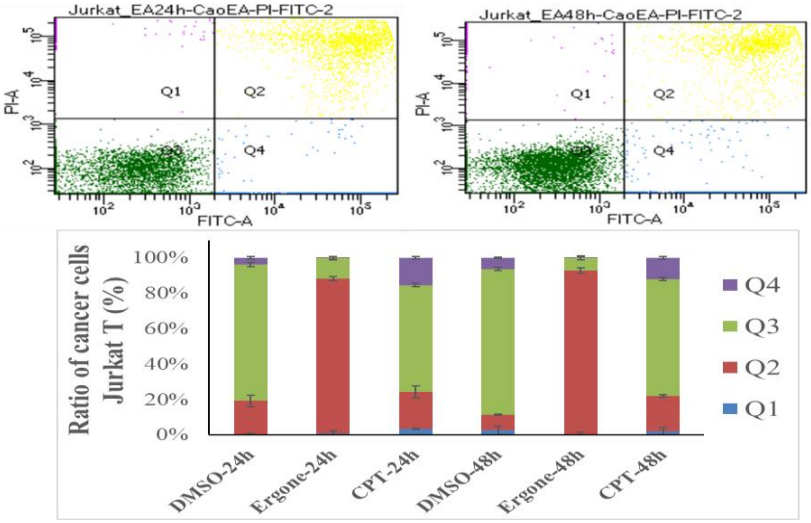


Fig 3.15. Ratio of Jurkat T cancer cells after induction of EA extract of fruiting body *I. cicadae* F0004 and Annexin V/PI staining

EA extract of fruit body *I. cicadae* F0004 was able to induce apoptosis of Jurkat T cells. When increasing the extraction induction time to 48 h, the percentage of cells entering apoptosis did not differ from 24 h.

3.4. Isolation, purification and identification of compounds of extracts with antimitotic potential

The PE extract of the mycelium *C. neovolkiana* DL0004 and the EA extract of the fruiting body *I. cicadae* F0004 had strong cytotoxicity and the ability to induce apoptosis with two cancer cell lines, MCF-7 and Jurkat T, selected for isolated, identified some compounds as well as continue to investigate the cytotoxic activity and induce apoptosis of MCF-7 and Jurkat T cancer cells with potential compounds.

3.4.1. Isolation of substances from the PE extract of C. neovolkiana mycelium DL0004

The PE extract (7.2 g) was fractionated by normal-phase-column chromatography using the solvent system n-hexane–EtOAc–acetone (10:1:1–4:1:1) as the eluent obtained 08 segments: PE1, PE2, PE3, PE4, PE5, PE6, PE7, PE8. *C. neovolkiana* DL0004 mycelium PE extract was isolated and identified 05 substances: (CN1) ergone, (CN2) ergosterol peroxide, (CN3) cerevisterol, (CN4) melithasterol B, (CN5) ergosterol.

3.4.2. Isolation of substances from EA extract of fruit body Isaria cicadae F0004

The EA extract of fruit body *I. cicadae* F0004 (7g) were fractionated by normal-phase-column chromatography with organic solvents including hexane, chloroform, ethyl acetate and methanol. EA extract of fruit body *I. Cicadae* F0004 were isolated and identified

06 substances: (IC1) uracil, (IC2) 1–O–Ethyl–β–D–ribofuranose, (IC3) ergosterol, (IC4) p–hydroxybenzoic acid, (IC5) protocatechuic acid, (IC6) nicotinic acid.

3.5. Results of evaluating the cytotoxic activity of MCF-7 and Jurkat T on cancer cells

Ergone with IC_{50} value that was toxic to MCF-7 and Jurkat T cell lines at 17.24 ± 1.31 and 39.30 ± 1.53 g/mL, respectively, was selected to further investigate the characterization and induction apoptosis on two cancer cell lines MCF-7 and Jurkat T.

3.6. Results of ergone induction of MCF-7 and Jurkat T cancer cell apoptosis

3.6.1. Morphology of MCF-7 and Jurkat T cells after induction

The results showed that in the batch of ergone-induced Jurkat T cancer cells, there was early apoptosis and late apoptosis.

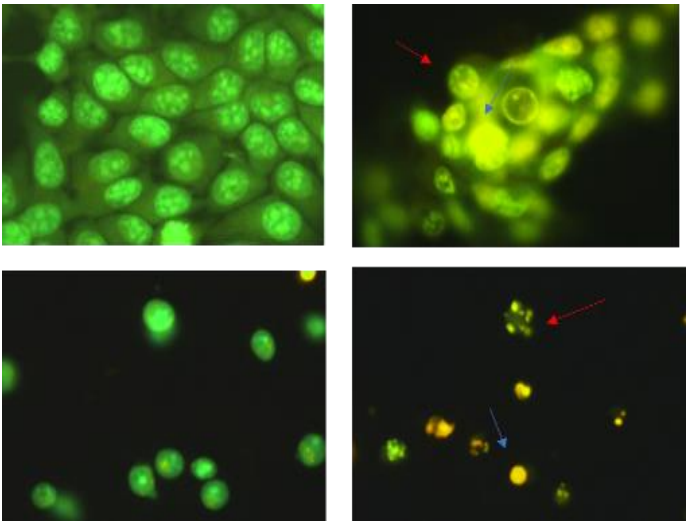


Fig 3.16. MCF-7 and Jurkat T cells after induction with ergone

3.6.2. Cell cycle analysis by flow cytometry

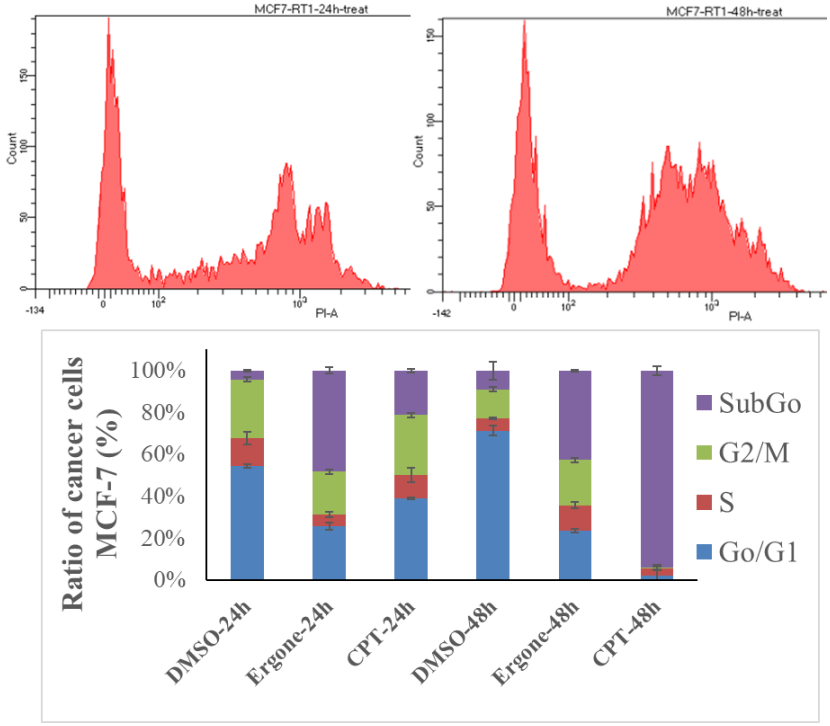


Fig 3.17. Ratio of MCF-7 cells at different cell cycle phases after induction of ergone from the PE extract of *C. neovolkiana* DL0004

Ergone induced MCF-7 cell apoptosis at 24 h and 48 h, and the results were not significantly changed at 24 h and 48 h. Ergone induces mitotic resistance in the Jurkat T cancer cell line by stop the cell cycle in the G1 phase, at the G0/G1 point and consequent induction of apoptosis.

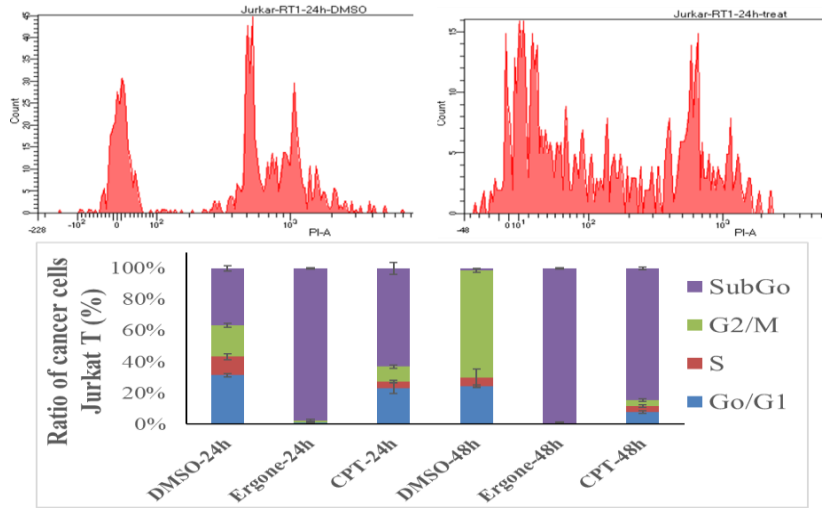


Fig 3.18. Ratio of Jurkat T cells at different phases of the cell cycle

3.6.3. Quantification of apoptosis-induced cells by Annexin V/PI staining

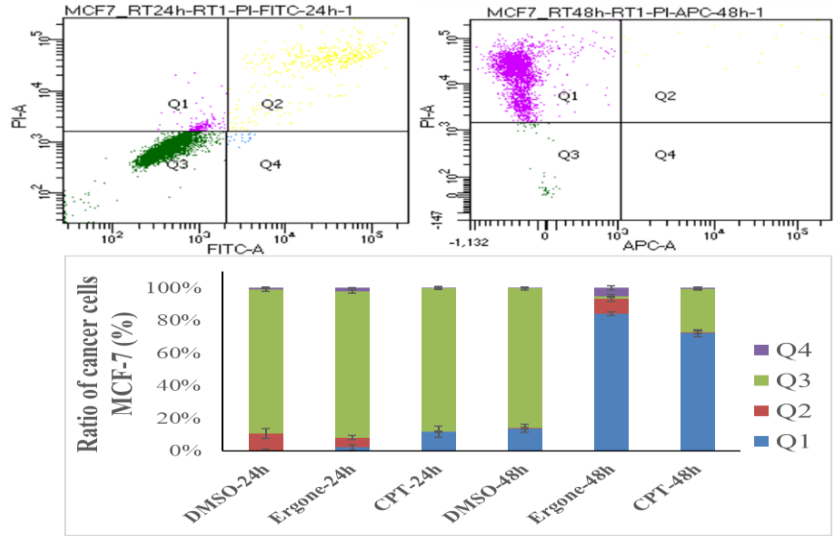


Fig 3.19. Ratio of MCF-7 cells induced by ergone

The above results showed that ergone has the ability to induce apoptosis in the MCF-7 cancer cell line.

After 24 h of cell induction with ergone, the percentage of cells in the late stage of apoptosis increased by 68.62 ± 3.87% compared with the control. From the above results, it was found that ergone has a strong ability to induce apoptosis in the Jurkat T cancer cell line.

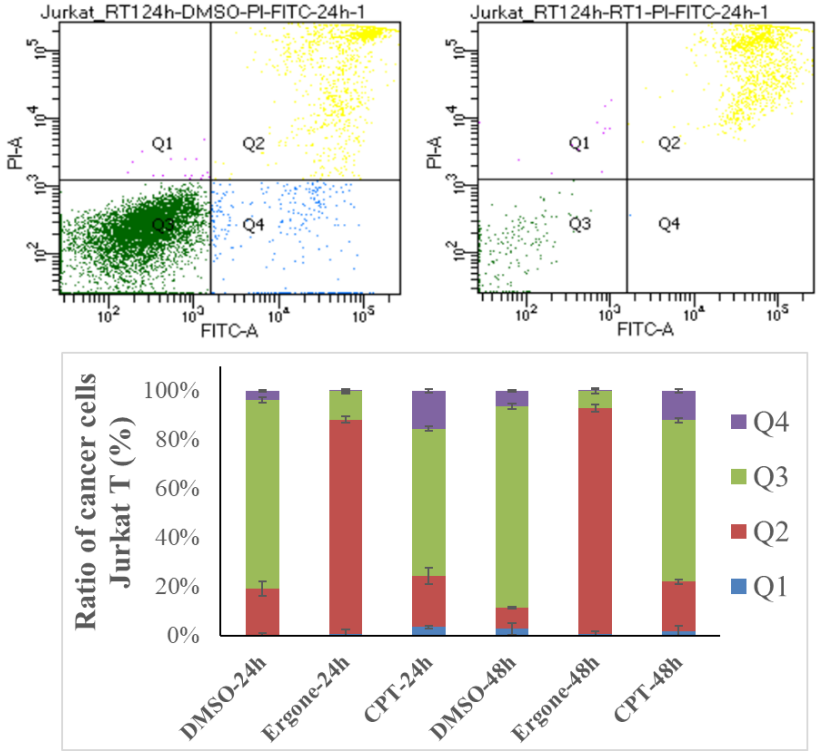


Fig 3.20. Ratio of Jurkat T cells apoptosis induction by ergone from the PE extract of *C. neovolkiana* DL0004

CONCLUSIONS AND RECOMMENDATIONS

Conclusions

It showed that 03 extracts with high cytotoxic activity were identified, including: EA extract of *C. neovolkiana* DL0004 mycelium inhibiting MCF-7 and Jurkat T cells, IC_{50} values were 78.13 ± 3.27 and 35.68 ± 0.29 µg/mL, respectively; PE extract of *C. neovolkiana* DL0004 mycelium inhibited MCF-7 and Jurkat T cells, IC_{50} values were 26.94 ± 1.62 and 15.50 ± 0.19 µg/mL, respectively; EA extract of fruit body *I. cicadae* F0004 inhibited MCF-7 and Jurkat T cells with IC_{50} values of 17.15 ± 1.68 µg/mL and 10.37 ± 0.61 µg/mL, respectively.

The mycelium extract of *C. neovolkiana* DL0004 and the extract of the body *I. Cicadae* F0004 were able to induce apoptosis in the cancer cell lines MCF-7 and Jurkat T.

It was Isolated and identified 11 compounds from 02 plants with high antimitotic activity, including: 05 compounds from PE extract from mycelium of *C. neovolkiana* DL0004 (ergone, ergosterol peroxide, cerevisterol, melithasterol B and ergosterol) and 06 compounds from the fruiting body EA extract of *I. cicadae* F0004 (uracil; 1–O–Ethyl–β–D–ribofuranose; ergosterol; p–hydroxybenzoic acid; protocatechuic acid and nicotinic acid).

Ergone from *C. neovolkiana* DL0004 mycelium PE extract has high antimitotic potential and induces apoptosis of MCF-7 and Jurkat T cancer cell lines.

Recommendations

Screening on anti-mitotic activity of extracts from mycelium and fruiting bodies of 02 fungal strains on other cancer cell lines.

More isolation and identification of compounds from the fractions.

Studying to determine the mechanism of anti-mitotic and anti-metastasis on cells, genes and protein expression of extracts and compounds exhibiting potential anti-mitotic activity on cancer cell lines.

Testing on the ability of extracts and compounds to inhibit cancer in animal models.

NEW CONTRIBUTIONS OF THE THESIS

The results of the thesis proved that the mycelium and fruiting bodies of 02 strains of *C. neovoliana* DL0004 and *I. cicadae* F0004 isolated and cultured in Vietnam have anti-mitotic activity and induce apoptosis in 02 MCF-7 cancer cell lines and Jurkat T.

It has Isolated and identified 11 compounds from the energizing PE extract of *C. neovolkiana* DL0004 and the fruiting body of EA of *I. cicadae* F0004. In which, there are three substances: 1–O–Ethyl–β–D–ribofuranose; p–hydroxybenzoic acid and protocatechuic acid were first reported on *I. cicadae*.

The results determined that ergone from PE extract of the *C. neovolkiana* DL0004 mycelium has potential mitotic resistance and apoptosis induction as a basis for practical application studies in the fields of biotechnology and biochemistry.

LIST OF PUBLICATIONS

The articles were published in national scientific journals

1. Doan Minh Quan, **Nguyen Chi Dung**, Dinh Minh Hiep (2015), *Extraction of ergosterol and characterization of anti-*

mitotic activity of petroleum-ether extracts from mycelium of Cordyceps spp. isolated in Vietnam, Tạp chí Khoa học và Công nghệ (ISSN 0866-708X), Viện Hàn lâm Khoa học và Công nghệ Việt Nam, tập 53, số 6B, trang 97-104

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