Studying on cytotoxic activity of ethyl acetate extracts and isolated substances from cultured *Isaria cicadae* F0004 in Vietnam against the MCF-7 cell lines and Jurkat cell lines

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Abstract

*Isaria cicadae* species of the *Isaria* genus have been isolated in many Asian countries, including China, Korea, Thailand, and Vietnam. Furthermore, *Isaria cicadae* content potential medicinal source in the prevention and treatment of cancer, strengthening the immune system. This study aims to investigate the cytotoxicity against MCF-7 and Jurkat T cell lines of the extract and isolated potential compounds from *Isaria cicadae* in Vietnam. The results showed that ethyl acetate (EA) extract from the fruit body of the *Isaria cicadae* F0004 strain had high cytotoxic activity against both MCF-7 and Jurkat cell lines at the concentration of 100 μg/mL with the IC$_{50}$ value was reached 17.15 ± 1.68 and 10.37 ± 0.61 μg/mL respectively. The constituents of the EA extract from the fungus *Isaria cicadae* F0004 were isolated by column chromatography and preparative chromatography. Then, the structures were determined by spectroscopy $^1$H–NMR, $^1$C–NMR. It was obtained 5 compounds including uracil, 1–O–ethyl–β–D–ribofuranose, ergosterol, p–hydroxybenzoic acid, protocatechuic acid. Protocatechuic acid isolated from *Isaria cicadae* F0004 that showed cytotoxic activity on MCF-7 and Jurkat cell lines with IC$_{50}$ values of 5.97 ± 0.36 and 3.15 ± 0.64 (mM) respectively. This study is the basis for further research on the cytotoxic activity of the fungus *Isaria cicadae* F0004 in vitro.

1. Introduction
Isaria cicadae (I. cicadae)- the anamorph of Cordyceps cicadae was originally described by Miquel in 1838, after which many scientific names such as Isaria basili, Sphaeria sinclairi and Paecilomyces cicadae were developed [1]. The chemical composition of I. cicadae mainly includes organic acids, amino acids, lipids and phospholipids, nucleosides, carbohydrates and their derivatives. Many other compounds such as adenosine, guanosine, uridine, inosine, thymidine and other nucleosides (ergosterol and ergosterol peroxide) and D–mannitol present in I. cicadae have many biological activities [2].

In Vietnam, I. cicadae were found in Dak Lak province and cultured successfully on artificial medium. Our previous study showed that the ethyl acetate (EA) extract of fruit-body I. cicadae F0004, that collected in Vietnam, has ability to inhibit the proliferation of MCF-7 and Jurkat cancer cell lines with IC50 value 17.15 ± 1.68 µg/mL and 10.37 ± 0.61 µg/mL respectively. To elucidate the major cytotoxic components of MCF-7 and Jurkat cancer cell lines, the main compound from the EA extract was isolated.

2. Materials and Methods

2.1 Cells and culture medium

Cell lines were provided by Department of Genetics, Faculty of Biology and Biotechnology, University of Science, HCM-VNU, were purchased from the American Type Culture Collection (Manassas, VA, USA) including breast cancer cells MCF-7 (HTB-22) and human Jurkat T (TIB-152). Cells were cultured at 37°C and 5% CO2 for 24 hours in Eagle’s Minimum Essential medium (E’MEM) 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) FBS (Sigma) and coverage of 70%– 80%.

2.2 Cell growth inhibition assay - Sulforhodamine B assay (SRB)

Cell viabilities were estimated by SRB assay based on the ability of SRB to bind to proteins in the cell, the amount of total protein, or the number of cells correlated with SRB dye. For SRB test, 100 µL of cancer cell lines (density 10^4 cell/100 well) were cultured in 96-well plates by E’MEM medium, incubated under 5% CO2 at 37°C for 24 hours then, mixed with 100 µL of extract and incubated. After 2 days, adding 50µL of cold trichloroacetic acid 50% (w/v) for 1 – 3 hours and washed 5 times with distilled water (200 µL/well) and dried at room temperature for 12-24 hours. 100 µL 0.2% (w/v) SRB was added for 20 min per each well and washing 4 times with 1% acetic acid and dried at room temperature. After that, added 200 µL of 10 mM Tris each well, shake by orbital shaker. The absorbance was 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 percent of cell inhibition was calculated as the following formula: %I =[(1-ODs/Odc).100] %, in which ODs = absorbance value of test sample and ODc = absorbance value of control [3].

2.3 Isolation of components

Biomass and fruit bodies of artificial I. cicadae were dried and extracted with ethanol each for 24 hours at room temperature. The ratio of raw materials and ethanol is 1:2, after 24 hours of collection, add more ethanol and repeat until all substances are obtained. The biomass moisture content was 8.5% and the fruit body moisture content was 6.9%. The extracts were evaporated at 40–50°C under reduced pressure and dissolve in water. The solution was extracted by the liquid-liquid method with ethyl acetate in a ratio of 1:1 (v/v), then, evaporating to obtain EA extract. The EA extract (7 g from 460 g original material) was fractionated by normal phase chromatography column using solvent of n-hexane, chloroform, ethyl acetate and methanol as eluent and obtain four fractions H (2.28 g), C (3.39 g), E (0.49 g), and M (0.80 g). Fraction H after evaporation of the solvent, was recrystallized to yield colorless crystals of compound N1 (70 mg). Fraction C after evaporation of the solvent partially soluble in chloroform is separated into the C1 (0.26 g) and the remaining is C2 fraction. The C1 was rechromatographed on a silica gel column eluted with mobile phase of chloroform:acetone (95:5) give amorphous compound N1 (4.3 mg) and N2 (3 mg). The C2 fraction was also rechromatographed the same to obtain 8 fractions C1.1-C1.8. Fraction C1.3 (763.2 mg) was rechromatographed with solvent
chloroform:acetone (acetone ratio increases gradually from 0%, 5%, 10%... to 100% to collect compounds N_4 (8.7 mg) and N_5 (8 mg). Fraction C_{1.6} (590.2 mg) was applied to Sephadex column, eluted with methanol 100% to get six fraction C_{2.6(1-6)}. Purifying fraction C_{2.6.5} by modulation chromatography to obtain compound N_6 (26.3 mg). The structural formulas of these compounds were determined by nuclear magnetic resonance (NMR) method at the Central Analysis Department of University of Science, Vietnam National University Ho Chi Minh City, 227 Nguyen Van Cu Street, District 5, Ho Chi Minh City.

2.4 Statistical analysis

The in vitro experiments were performed in triplicate and expressed as mean ± standard deviation (SD) of the sample examined. Statistical analysis were performed using One-way ANOVA. P-value < 0.05 were considered statistically significant.

3. Results and discussion

3.1 Isolation of components

The EA extract will be divided into smaller fractions using solvents of increasing polarity hexane (H), chloroform (C), ethyl acetate (EA), methanol (MeOH). When using the solvent to dissolve the respective fractions, the 3 segments H, EA and MeOH did not have an insoluble precipitate. The C fraction, which had an insoluble part in C, was segregated into the C_{1} fraction (the precipitate was soluble in MeOH), and the remainder was soluble in C as the C_{2} fraction. Six compounds of five fractions were determined by ^1^H-NMR, ^13^C-NMR spectroscopy methods.

N_1 has the molecular formula C_4H_4N_2O_2, white amorphous powder, ^1^H-NMR (500 MHz, DMSO-d_6): δ_H 5.42 (1H, d, 7.6 Hz, H–5) and 7.38 (1H, d, 7.6 Hz, H–6). ^13^C-NMR (125 MHz, DMSO–d_6): δ_C 151.7 (C–2), δ_C 164.8 (C–4), δ_C 100.1 (C–5), δ_C 142.6 (C–6). These data were consistent with reported data on uracil [4].

N_2 has white amorphous powder, the molecular formula C_{7}H_{16}N_{2}O_{5}, ^1^H-NMR (500 MHz, DMSO–d_6): δ_H 4.83 (1H, d, 4.7), 4.13 (2H, dd, 6.3 - 5.0), 3.88 (2H, d, 4.7), 3.91 (1H, td, 5.75 - 3.92), 3.66 (1H, dd, 11.54 - 3.95), 3.54 (1H, dd, 11.58 - 5.42), 3.42 (2H, dd, 9.6 - 7.1), 3.73 (1H, m), 1.12 (3H, t, 7.1). ^13^C-NMR (125 MHz, DMSO – d_6): δ_C 108.7 (C–1), 85.7 (C–2), 72.7 (C–3), 76.7 (C–4), 64.7 (C–5), 64.0 (C–6), 15.9 (C–7). These data were consistent with reported data on 1–O–Ethyl–β–D–ribofuranose [5].

N_3 has white amorphous powder, spectral data of ^1^H-NMR (500 MHz, CDCl_3) and ^13^C-NMR (125 MHz, CDCl_3) agreed with published of Li et al. (2015) about ergosterol (C_{28}H_{44}O) [6].
3.2 Cell cytotoxicity ability

Table 1. Cytotoxic activity of fractions of EA extract at 100 µg/mL, 48h. The in vitro experiments were performed in triplicate and expressed as mean ± standard deviation (SD) of the sample examined.

| Fraction | Cytotoxic activity (%) | Compound obtained |
|----------|------------------------|-------------------|

Figure 1. NMR spectroscopy results and structures of compounds extracted from I. cicadae F0004

A. $^1$H-NMR (500 MHz, DMSO–$d_6$) of N$_1$
B. $^{13}$C-NMR (125 MHz, DMSO–$d_6$) of N$_1$
C. $^1$H-NMR (500 MHz, DMSO – $d_6$) of N$_2$
D. $^{13}$C-NMR (125 MHz, DMSO – $d_6$) of N$_2$
E. $^1$H-NMR (500 MHz, CDCl$_3$) of N$_3$
F. $^{13}$C-NMR (125 MHz, CDCl$_3$) of N$_3$
G. $^1$H-NMR (500 MHz, Acetone–$d_6$) of N$_4$
H. $^{13}$C-NMR (125 MHz, Acetone–$d_6$) of N$_4$
I. $^1$H-NMR (500 MHz, Acetone–$d_6$) of N$_5$
K. $^{13}$C-NMR (125 MHz, Acetone–$d_6$) of N$_5$

N$_4$ has white amorphous powder. $^1$H-NMR (500 MHz, Acetone–$d_6$): $\delta$ 6.92 (2H, d, 8.8 Hz, H–2,6), and 7.91 (2H, d, 8.8 Hz, H–3,5). $^{13}$C-NMR (125 MHz, Acetone–$d_6$): $\delta$C 161.7 (C–4), 121.8 (C–1), 115.1 (C–2, 6), 131.5 (C–3, 5), 166.7 (C–7). These data were consistent with reported data on p-hydroxybenzoic acid (C$_7$H$_6$O$_3$) [7].

N$_5$ was consistent with report of Erukainure et al. (2017) about protocatechuic acid [8]. N$_5$ has the molecular formula C$_7$H$_8$O$_4$, white amorphous powder. $^1$H-NMR (500 MHz, Acetone–$d_6$): $\delta$H 6.89 (1H, d, 8.3 Hz, H–5). $\delta$H 7.47 (1H, dd, 8.3 and 2.1 Hz, H–6) and $\delta$H 7.53 (1H, d, 2.0 Hz, H–2); $^{13}$C-NMR (125 MHz, Acetone–$d_6$): $\delta$C 123.1 (C–1), $\delta$C 117.4 (C–2), $\delta$C 145.5 (C–3), $\delta$C 150.6 (C–4), $\delta$C 115.6 (C–5), and $\delta$C 123.5 (C–6).

It was obtained 3 new compounds compared to the strain Isaria cicadae isolated from China including 1-O-ethyl-β-D-ribofuranose, p-hydroxybenzoic acid and protocatechuic acid. According to the study results of Nxumalo et al (2020), the nucleotides extracted from Cordyceps cicadae include uracil, uridine, 2'-deoxyuridine, inosine, guanosine, thymidine, adenine, adenosine, 2'-deoxyadenosine, cordycepin (3'-deoxyadenosine) and N6-(2-hydroxyethyl)-adenosine (HEA) [9]. Besides, ergosterol was also found in this fungal strain. He et al. (2018) isolated ergosterol peroxide in the culture of Paecilomyces cicadae [10], Sun et al. (2007) studied the chemical composition of I. cicadae conidia powder (Zhejiang, China) by super-performance liquid chromatography coupled to mass spectrometry (UPLC-ESI-Q-TOF-MS). The results obtained 8 substances, including: myriocin, beauvericin, cordycepic acid, N6-(2-hydroxyethyl)-adenosine, uridine, guanosine, cordycepin and adenosine [11].
Table 1 results show that the C2 fraction has cytotoxic activity, therefore continue to investigate the cytotoxic potential of MCF-7 and Jurkat cancer cell lines for compounds p-hydroxybenzoic acid and protocatechuic acid.

Table 2. Cytotoxic activity of p-hydroxybenzoic acid and protocatechuic acid against MCF-7 and Jurkat cancer cell lines after 48h of treatment. The in vitro experiments were performed in triplicate and expressed as mean ± standard deviation (SD) of the sample examined.

| Compound | IC50 (mM) | MCF-7 | Jurkat |
|----------|-----------|-------|--------|
| N4       | -         | -     | -      |
| N5       | 5.97 ± 0.36 | 3.15 ± 0.64 |

Uracil, 1-O-ethyl-β-D-ribofuranose, ergosterol were collected from H and C1 fractions that exhibits low cytotoxicity against MCF-7 and jurkat cancer cell lines. That means these compounds have weak cytotoxic activity on these cancer cells. However, compounds have been shown to have important biological activities such as are anti-oxidant, immune-supportive, etc... In particular, ergosterol is a sterol with important cell membrane roles that has been found in many fungi and is a provitamin D2 in humans[12]. Besides, p-hydroxybenzoic acid can combine with protocatechuic acid to potentially support enhanced cytotoxic activity similar to those studied by Wang et al. (2018) demonstrated that p-hydroxybenzoic acid had a low rate of inhibition of MCF-7 cells, but when combined with adriamycin, increased cytotoxicity and apoptosis on MCF-7 [13]. Yin et al. (2009) demonstrated that protocatechuic acid has antitumor activity through increasing apoptosis or preventing invasion and metastasis of human breast cancer cells MCF-7, lung cancer A549, HepG2 cells, HeLa cells, cervical cancer cells and LNCaP prostate cancer cells [14]. According to the results of Steng et al. (2000), the protocatechuic acid extracted from Hibiscus induced apoptosis in human leukemia cells through decreased phosphorylation of retinoblastoma (RB) tumorigenesis and the expression of human leukemia cells present Bcl-2. The rate of cell death increased with the concentration and induction time (70%, 2 mM, at 48 h), protocatechuic acid exerts anti-proliferative effects through phosphorylation and degradation of RB; inhibiting Bcl-2 protein in cancer cells [15]. In general, EA extract and protocatechuic acid extracted from I. cicadae isolated in Vietnam have the ability to induce cytotoxicity of MCF-7 and Jurkat cancer cell lines. This study is the premise for further studies on this potential fungus.

4. Conclusion
The cytotoxic ability of the fungus Isaria cicadae in the world has been demonstrated. Compared with other fungal strains of other countries, EA extract from fruit body of Isaria cicadae F0004 isolated in Vietnam has strong cytotoxic activity on MCF-7 and Jurkat cancer cell lines. Five compounds from EA extract of Isaria cicadae F0004 fruit body were isolated, including uracil, 1-O-ethyl-β-D-ribofuranose, ergosterol, p-hydroxybenzoic acid, protocatechuic acid. In this study, protocatechuic acid exhibited cytotoxic activity against cancer cell lines MCF-7 and Jurkat with IC50 values of 5.97 ± 0.36 and 3.15 ± 0.64 (mM), respectively. Further studies are needed to determine the mechanism of the extracts and compounds isolated from this potential fungal strain.

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