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FREE RADICAL SCAVENGING EFFECT OF EXTRACTS FROM *Isaria cicadae* F0004 IN VIETNAM

Khac Ky Lam[1,2,3], Thi Nguyet Huynh[1,2], Minh Hiep Dinh[4], Dai Hung Ngo[5,*]

[1]Faculty of Biology-Biotechnology, University of Science, Ho Chi Minh City, Vietnam
[2]Vietnam National University, Ho Chi Minh City, Vietnam
[3]Industrial University of Ho Chi Minh City, Vietnam
[4]Department of Agriculture and Rural Development of Ho Chi Minh City, Vietnam
[5]Thu Dau Mot University, Binh Duong province, Vietnam

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ABSTRACT

Isaria cicadae, currently identified as *Paecilomyces cicadae* (Miq.) Samson (Cordycipitaceae, Hypocreales, Ascomycetes), is a parasitic fungus on cicadas and is considered a folk medicine in traditional medicine. In this research, the antioxidant activity of some extracts from the biomass and fruit bodies of *Isaria cicadae* F0004 isolated in Vietnam was investigated. The results showed that the Ethyl acetate (EA) fraction extract of the biomass of *Isaria cicadae* F0004 exhibited the best antioxidant activity in vitro, with IC_{50} values of 159.73 ± 7.33 μg/mL (DPPH assay) and 379.25 ± 7.33 μg/mL (ABTS assay). Meanwhile, the EA fraction extract of the fruit bodies of *I. cicadae* F0004 showed antioxidant activity with IC_{50} values of 90.8 ± 2.36 μg/mL (xanthine oxidase inhibition), 68.631 ± 0.632 μg/mL (protein membrane protection), 19.042 ± 1.072 μg/mL (lipid membrane protection). Furthermore, the EA fraction extract of the fruit bodies of *I. cicadae* F0004 exhibited a strong capability for DNA damage protection at 100 μg/mL concentration and $\Delta OD_{4000\ \mu g/mL}$ = 0.422 ± 0.014 (reducing power). Therefore, the EA fraction extract of *I. cicadae* F0004 serves as an effective free radical scavenger and a potential source of natural antioxidants, and this study contributes more information on the biological activities of *I. cicadae* F0004 from local raw materials.

Keywords: *Isaria cicadae*, free radical scavenging activity, biomass, fruit bodies, RAW 264.7 cells.

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[*]*Corresponding author email*: hungnd@tdmu.edu.vn

INTRODUCTION

Free radicals including reactive oxygen species (ROS) and reactive nitrogen species (RNS), are generated intracellularly by cellular metabolism and exogenous agents that have been recognized as both beneficial and harmful. Overproduction of ROS (arising from the mitochondrial electron transport chain or overstimulating NADPH) leads to oxidative stress, a harmful process that can be an important mediator of cellular structural damage, including lipids and protein membranes and DNA (Valko et al., 2007). Thus, the neutralization of free radicals as well as the ability to inhibit enzymes that activate oxidation processes (such as xanthine oxidase - an important enzyme catalyzing the hydroxylation of hypoxanthine to xanthine and xanthine to uric acid which is excreted by kidneys) and the ability to protect against cellular damage is essential.

Genus *Isaria* is widely recognized as a medicinal mushroom to improve human health and well-being and has been emerging as a miracle cure for many common ailments in Eastern and Chinese medicine. Nowadays Vietnam is one of the countries with a lot of research to promote the discovery of bio-active sources for public health care. Among them, the species *Isaria cicadae*, which molecular record for the first authentication from Vietnam was the entomopathogenic fungus T011, collected in the coffee garden in Dak Lak province (Lao et al., 2021).

Among the *Isaria* strains exploited and cultured, *I. cicadae* can also be cultivated in controlled laboratories and may therefore have an important role in health care (Li et al., 2014). Multiple pharmacological activities have been reported for wild *Cordyceps cicadae*, cultured *C. cicadae*, and mycelia of *I. cicadae* because of their multiple bioactivities and no significant toxicity of their phytochemicals, such as cordycepin, cordycepic acid, adenosine, guanosine, uridine, inosine, thymidine, other nucleosides, ergosterol and other peroxides, D- mannitol,... found in the fruiting bodies, biomass, and mycelium (Zhang et al., 2019). To be able to actively isolate, cultivate, and obtain biomass and fruiting bodies with many biologically active substances from native *Cordyceps* sources, *Isaria cicadae* F0004 from the Dak Lak province in Vietnam was selected and cultured to obtain fruit bodies and biomass. The aim of this research is to investigate the free radical scavenging activity of extracts from the biomass and fruiting bodies of *I. cicadae* F0004 on RAW 264.7 cells.

MATERIALS AND METHODS

Materials

I. cicadae F0004 strain was collected in the Eakar district, Dak Lak province Vietnam in the year 2017. Both biomass and fruit bodies were cultured and collected by static liquid culture method and semi-solid culture method.

Cultural conditions to obtain the biomass and fruit body

Strains were activated to restore fungal-like activity after seed keeping. Transplantation of strains was conducted from agar to a more nutrient-rich liquid medium to rapidly increase the number of *I. cicadae* F0004 mycelia before inoculating the culture medium. After that, the medium was inoculated into an Erlenmeyer flask containing 200 mL of autoclaved potato glucose medium and incubated at 20–25 ^{o}C for 10–12 days.

Biomass was cultured on a liquid-static medium in polypropylene plastic containers. The composition of the medium was as follows: 200 mL of nutrient solution with 20% potato juice, 0.05% sucrose, 0.006% peptone, 0.004% high yeast, 0.0005% KH_2PO_4, and 0.0002% $MgSO_4.7H_2O$. The liquid seed 4% was cultured statically for 5 days at 23 ^{o}C. Biomass was collected after 40 days of inoculation. It was dried at 60 ^{o}C to a constant weight and stored in a dry place at 20 ^{o}C.

After secondary propagation, the mycelium was inoculated into a semisolid culture medium consisting of brown rice and millet with a weight of 40 g/box in different ratios, together with 70 mL of distilled

water/box. This culture medium box was sterilized at 121 ºC for 30 min. Then, 5mL of mycelium was added into the culture medium (5 mL/box), which was incubated at 22 ± 2 ºC in the dark for 20 days. After that, the culture medium box was brought to the light to stimulate fruiting. The fruit bodies were collected on day 60 and then dried at 60 ºC to a constant weight and stored in a dry place at 20 ºC.

Extracts preparation

The extraction method described by Nguyen (2007) was followed, with adjustments. Samples were completely extracted with ethanol 96% (EtOH) in 48 hours and then evaporated at 50 ºC to obtain EtOH extracts. To reduce the active ingredients to fractions of the total extract, solvents of increasing polarity, including petroleum ether (PE), ethyl acetate (EA), n-butanol (BuOH), and distilled water (H_2O), were used and then evaporated at 50 ºC. The biomass culture was precipitated with ethanol 96% (4 ºC) to collect exo-polysaccharides (EPSs). The biomass residues were dried and boiled in distilled water at a ratio of 1:10 (w/v). The biomass was divided into three parts, and each part was boiled for 60 min. Then, the biomass was filtered and centrifuged to remove residues and evaporated to obtain polysaccharide extract (IPS). All extracts were stored at 4 ºC in dark conditions.

DPPH radical scavenging assay

This experiment was followed by Zheng et al. (2008) procedure with adjustments. Add 0.5 mL of sample to 0.75 mL DPPH (1,1-diphenyl-2-picrylhydrazyl) solution, vortex and incubate for 30 minutes at room temperature. All samples were measured photo-metrically at 517 nm. This experiment was repeated 3 times. The DPPH radical–scavenging activity (RSA) (%) was calculated and the results were expressed in IC_{50} values.

$$RSA_{DPPH} = \left[OD_{DPPH} - \left(OD_{sample} - OD_{methanolic\ sample} \right) \right] / OD_{DPPH} * 100$$

Where: OD is the optical density.

ABTS radical scavenging assay

ABTS (2,2'-azino-bis (3-ethylbenzothia-zoline-6-sulfonic acid)) stock solution was prepared by dissolving ABTS (7 mM) to K2S2O8 (2.45mM) at the ratio 1:1 (v/v), then incubating for 16 hours in the dark. ABTS ready-to-use solution was diluted by PBS buffer (pH 7.4) until OD values at 734 nm were 0.7 ± 0.02. The reaction mixture contained 3 mL ABTS ready-to-use solution and 100 µL of the sample. Incubating this mixture for 30 min in the dark and measuring at 734 nm (Nenadis et al., 2004). Repeating 3 times. The ABTS RSA (%) was calculated and the results were expressed in IC_{50} values.

$$RSA_{ABTS} = \left[OD_{ABTS\ solution} - OD_{sample} \right] / OD_{ABTS\ solution} * 100\%$$

Where: OD is the optical density.

Reducing power assay

The samples were mixed with 0.5 mL phosphate buffer (0.2 M, pH 6.0) and 0.5mL $K_3[Fe(CN)]_6$ (1%). Then incubated the mixture for 20 min at 50 ºC. Add 0.5 mL TCA (10%) and the reaction mixture was centrifuged for 10 min, at 5,000 rpm. After that, add 0.8 mL supernatant solution to 2 mL distilled water and 0.4 mL $FeCl_3$ (1%) with adjustments (Ferreira et al., 2007). Finally, the mixtures were measured at 700 nm. Repeating 3 times, the results were expressed in OD values.

Xanthine oxidase inhibition

The stock sample solutions were prepared by dissolving sample extracts in DMSO 5%, then diluting the stock sample

solutions in phosphate buffer. Then mixed with 5 µL XO enzyme (5U) and incubated for 15 min, at 25 °C. Added 80 µL Xanthine (150 µM) to the reaction mixture, mixed and incubated for 30 min, at 25 °C (Noro et al., 1983). Finally, the reaction mixture was added 25 µL HCl (1 M) and measured at 290 nm. Repeating 3 times and the results were expressed in IC_{50} values.

Cell viability assay

Added 20 µL of sample in every concentration per well to RAW 264.7 cells (180 µL/10^4 CFU) that had been incubated in 96-wells, then incubated in 48 hours (Taciak et al., 2018). After that, the cells were washed with 100 µL Phosphate buffered salinemedia (PBS) and added 50 µL MTT 1X (MTT/DMEM), continue incubating for 4 hours. Finally, the mixtures were added 100 µL DMSO to dissolve formazan and incubated overnight. The reaction mixtures were measured at 562 nm by Microplate Reader (Perkin Elmer). The cytotoxicity or the cell survival rate (S) was calculated and compared to control cells.

$$\text{Cell viability}(\%) = \text{OD}_{\text{sample}} / \text{OD}_{\text{control}} * 100\%$$

Where: OD is the optical density.

Cell membrane proteins oxidation assay

This experiment was followed by Levine et al. (1990) procedure with adjustments. RAW 264.7 cells (5×10^3 CFU) were lysates in a buffer medium (Tris-HCl 25 mM pH 7.8, EDTA 2 mM, NaCl 180 mM, Triton X-100 1%). Then incubated it with 5 µL of the sample at 37 °C for 30 min. The cells were mixed with $FeSO_4$ (10 µL, 400 µM), H_2O_2 (20 µL, 0–1 mM), distilled water (5 µL), respectively, and continued incubating at 37 °C for 1 hour. After that, add 400 µL TCA (20%) and centrifuged the mixture. The solid was suspended with 150 µL of 2,4-dinitrophenyl hydrazine which was mixed in HCl (2 M), then incubated the mixtures at room temperature for 40 min. The cell membrane protein was precipitated with TCA (20%) and washed with EtOH:EA (1:1) solution, then suspended in guanidine hydrochloride (500 µL, 6M). The reaction mixtures were continuously incubated at 37 °C for 15 min, then centrifuged at 6000 rpm for 5 min. The supernatant was measured at 370 nm. Simultaneously, the blank sample was prepared similarly to the extracts sample, using HCl (2 M) instead of 2,4-dinitrophenyl hydrazine.

Cell membrane lipid oxidation assay

This study was followed by Kikuzaki & Nakatani (1993) procedure with adjustments. RAW 264.7 cells (5×10^3 CFU) after washing 3 times by phosphate buffered saline (PBS) media, were mixed with 5 µL PBS and 5 µL of sample solution at the different concentrations. The mixtures were added $FeSO_4$ (10 µL, 400 µM) and H_2O_2 (20 µL), respectively, and incubated at 37 °C for 1 hour. Then added cold TCA solution (10%) was at the ratio 1:2 (v/v) and centrifuged to obtain supernatant. After that, the reaction mixtures were added TBA (1%) (1:1, v/v) and incubated at 90 °C for 30 min (Kikuzaki & Nakatani, 1993). Cooled and centrifuged and measured at 528 nm.

Determination of free radical mediated DNA damage

This method was described by Milne et al. (1993) with adjustments. RAW 264.7 cells after washing 2 times by PBS, were added 5 mL of Trypsin and incubated for 15 min. The cell was mixed with 1 mL PBS has 5 mM EDTA and centrifuged at 1000 rpm for 10 min, at 25 °C, then collected the cell supernatant (repeated 2 times). The cell supernatant was added RNAase (25 µL, 0.5 mg/mL), proteinase K (10 µL, 10 mg/mL), SDS (25 µL, 10%), CH_3COONa (350 µL, 0.2 M). Then incubate the mixture at 37 °C for 30 min and at 55 °C for 55 min, respectively.

The mixture was added phenol: chloroform: isoamyl alcohol (25:24:1) solution at the ratio 1:1 (v/v), and centrifuged

at 12,000 rpm for 5 min, 4 ºC (repeated 2 times) to collect the supernatant. Added cold ethanol 70% (1:1, v/v) to the supernatant and centrifuged at 12000 rpm for 5 min. Then the solid was dissolved with TE buffer (50 µL, pH 8.0) to obtain DNA molecules. The sample reaction mixture included 4 µL of DNA, 1 µL distilled water, 1 µL $FeSO_4$ and 1 µL H_2O_2, respectively. Then incubated it at room temperature for 10 min and mixed with EDTA (1 mL, 0.5M). Finally, electrophoresis of the sample reaction mixture on agarose gel (1%) for 20 min with 100V. The results of agarose gel electrophoresis were projected under a UV projector with VisionCapt software.

Statistical analysis

The results were shown as MEAN ± SD. All experiments of this study were replicated in triplicate and all data were performed using Graphpad Prism (8.4.3 ver). ANOVA one–way and ANOVA two-way multiple range tests provided mean comparisons with the level of statistical significance set as $P < 0.05$ (Kallel et al., 2014).

RESULTS

DPPH radical scavenging

The results of the DPPH radical scavenging of extracts from the biomass and fruit bodies of *I. cicadae* F0004 are expressed as IC_{50} values. These results are shown in Table 1.

Table 1. IC_{50} of DPPH radical scavenging activity of the biomass and fruit bodies extracts of *Isaria cicadae* F0004

IC_{50} (µg/mL)	BM	FB
Vitamin C	2.45 ± 0.07^{a}	
EtOH	752.08 ± 27.7^{de}	692.12 ± 22.33^{f}
PE	772.88 ± 21.76^{e}	$1,267.12 \pm 32.58^{g}$
EA	159.73 ± 7.33^{b}	198.34 ± 6.72^{h}
BuOH	689.72 ± 35.55^{d}	662.52 ± 8.14^{i}
H_2O	380.27 ± 15.83^{c}	419.45 ± 21.35^{j}
IPS	-	-
EPS	-	-

Notes: BM (Biomass); FB (Fruit body); EtOH (ethanol); PE (Petroleum Ether); EA (Ethyl acetate); BuOH (Buthanol); H_2O (water); IPS (Polysaccharide); EPS (Exo-polysaccharide); Vitamin C as a positive control; The letters a, b, c, d in the same column represent the difference between the IC_{50} values of the extracts at $p < 0.05$ level; (-) Unidentified IC_{50}.

Table 1 showed that the EA extracts of both biomass and fruit body of *I. cicadae* F0004 had IC_{50} values lower than those of other medicinal fungi, and the EA extracts of the biomass of *I. cicadae* F0004 had the lowest IC_{50} value (159.73 ± 7.33 µg/mL). Like adenosine, cordycepin which is a derivative of adenosine, isolated from *I. cicadae* has been shown to have antioxidant properties. Another adenosine derivative, N-(2-hydroxyethyl) adenosine was isolated and proven to exhibit antioxidant activity by measuring its radical scavenging effect on 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals (Xiaofeng Zhang et al., 2019). Therefore, EA fraction extracts is a source of potential secondary metabolites that show DPPH RSA. On the other hand, the radical scavenging activity of *I. cicadae* are higher than some medicinal fungi such as *Cantharellus cibarius* (IC_{50} = 929.8 ±36 µg/mL) and *Pleurotus porrigens* (IC_{50} = 1,025 ± 37 µg/mL) (Ebrahimzadeh et al., 2010), *Cordyceps militaris* fruiting body extract (IC_{50} = 1,640 µg/mL) and mycelium extract (IC_{50} = 1,200 µg/mL) (Prommaban et al., 2022), respectively. When compared with some fungi in the genus *Isaria*, *I. cicadae* showed better DPPH radical scavenging activity than *Isaria tenuipes* mycelium extract (IC_{50} = 5,180 µg/mL), the activity is not as good as that of the fruiting body

extract of *I. tenuipes* (IC_{50} = 50 μg/mL) (Prommaban et al., 2022).

ABTS radical scavenging

Similarly, ABTS is used to survey the compounds in extracts to be able to donate H^+ and electrons to free radicals to create structurally stable products. These results are shown in Table 2.

The results (Table 2) showed that EA fraction extracts from both biomass and fruit body of *I. cicadae* F0004 have the lowest IC_{50} values, 379.25 ± 7.33 μg/mL (biomass) lower than 417.11 ± 11.36 μg/mL (fruit bodies). This proves that the EA extract contains many potential secondary metabolites capable of neutralizing ABTS, especially the EA extract from the biomass of *I. cicadae* showed that the EA fraction is a solvent with potential secondary metabolites that have a higher ABTS radical scavenging activity than hexane, chloroform, and distilled water. However, the EA extract of the biomass of *I. cicadae* was not shown to have a better ABTS radical scavenging activity than *Garnoderma lucidum* (IC_{50} = 1.06 ± 0.05 μg/mL) (Quy & Xuan, 2019), *C. militaris* mycelium (IC_{50} = 460 μg/mL) (Prommaban et al., 2022). But its DPPH scavenging activity is not as good as that of the fruiting bodies of *C. militaris* (IC_{50} = 240 μg/mL) and *I. tenuipes* (IC_{50} = 220 μg/mL) (Prommaban et al., 2022) which showed that to determine the antioxidant capacity of an active ingredient, besides determining the free radical scavenging capacity, we must also do more tests.

Table 2. IC_{50} of ABTS radical–scavenging activity of the biomass and fruit bodies extracts of *Isaria cicadae* F0004

IC_{50} (μg/mL)	BM	FB
Vitamin C	59.59 ± 0.81[a]	
EtOH	1,718.83 ± 22.67[f]	895.81 ± 13.22[i]
PE	> 4,000	> 4,000
EA	379.25 ± 7.33[b]	417.11 ± 11.36[g]
BuOH	482.25 ± 5.34[c]	671.41 ± 5.35[h]
H_2O	1,234.21 ± 15.83[d]	1,211.19 ± 25.78[k]
IPS	410.73 ± 15.48[b]	957.1 ± 22.72[j]
EPS	1,613.183 ± 8.46[e]	-

Notes: BM (Biomass); FB (Fruit body); EtOH (ethanol); PE (Petroleum Ether); EA (Ethyl acetate); BuOH (Buthanol); H_2O (water); IPS (Polysaccharide); EPS (exo-polysaccharide); Vitamin C as a positive control; The letters a, b, c, d in the same column represent the difference between the IC_{50} values of the extracts at $p < 0.05$ level; (-) Unidentified IC_{50}.

Reducing power

Reducing power is a method to evaluate the presence of reducing agents in samples (Lam Khac Ky et al., 2021). These results are presented in Table 3.

Table 3 showed that the ΔOD values of EA extracts from biomass and fruit bodies of *I. cicadae* F0004 are higher significantly than the others at the same concentrations. In addition, almost of biomass extracts have ΔOD values higher significantly than the fruit body extracts, except BuOH extract and H_2O extract, and EA of fruit body extract is the highest ΔOD values among all survey extracts. Combined with the results from DPPH and ABTS radical scavenging, the EAs of both the biomass and fruiting bodies containing many compounds of medium polarity have antioxidant activity *in vitro*. Therefore, in order to select the most potential compounds, it is necessary to further investigate the ability to inhibit enzymes and protect cells from oxidizing agents.

Table 3. ΔOD at 4,000 μg/mL of reducing power of the biomass and fruit bodies extracts of *Isaria cicadae* F0004

	BM	FB
Vitamin C	> 4,000[a]	
EtOH	0.099 ± 0.00[e]	0.194 ± 0.004[i]
PE	0.096 ± 0.001[e]	0.214 ± 0.017[h]
EA	0.339 ± 0.006[b]	0.422 ± 0.014[f]
BuOH	0.145 ± 0.003[d]	0.137 ± 0.005[j]
H_2O	0.198 ± 0.004[c]	0.120 ± 0.001[j]
IPS	0.193 ± 0.003[c]	0.271 ± 0.003[g]
EPS	0.096 ± 0.007[e]	-

Notes: BM (Biomass); FB (Fruit body); EtOH (ethanol); PE (Petroleum Ether); EA (Ethyl acetate); BuOH (Buthanol); H_2O (water); IPS (Polysaccharide); EPS (exo-polysaccharide); Vitamin C as a positive control; The letters a, b, c, d, e, f, g, h, i and j in the same column represent the difference between the IC_{50} values of the extracts at $p < 0.05$ level; (-) Unidentified ΔOD.

Xanthine oxidase inhibition

This study is based on the inhibition of uric acid formation with xanthine. The extract has XO inhibitory activity which amounts of uric acid are less, and the OD (Optical density) was decreased. The results of this study are shown in Table 4.

Generally, almost of extracts show XO inhibitory activity, except EtOH extract of biomass, H_2O extract and EPS extract of fruit bodies (Table 4). Especially, the $IC_{50} = 90.8 \pm 2.36$ μg/mL of EA extract from fruit bodies is close to allopurinol (positive control). Thus, EA extracts in both *I. cicadae* biomass and fruit bodies are two potential extracts that have XO inhibitory activity.

Based on the results of the DPPH assay, ABTS assay and XO inhibition assay, EA extract of both biomass and fruit bodies was chosen to determine the ability to protect cell components under oxidizing agents in RAW 264.7 cells.

Table 4. IC_{50} of XO inhibition of the biomass and fruit bodies extracts of *Isaria cicadae* F0004

IC_{50} (μg/mL)	BM	FB
Allopurinol	82.5 ± 1.82[a]	
EtOH	-	278.51 ± 10.89[f]
PE	> 1,000	> 1,000
EA	104.56 ± 7.09[b]	90.8 ± 2.36[e]
BuOH	185.73 ± 13.51[c]	235.55 ± 68.24[f]
H_2O	179.98 ± 7.69[c]	-
IPS	176.64 ± 24.02[c]	214.43 ± 13.31[f]
EPS	610.57 ± 48.53[d]	-

Notes: BM (Biomass); FB (Fruit body); EtOH (ethanol); PE (Petroleum Ether); EA (Ethyl acetate); BuOH (Buthanol); H_2O (water); IPS (Polysaccharide); EPS (exo-polysaccharide); Allopurinol as a positive control; The letters a, b, c, d, e and f in the same column represent the difference between the IC_{50} values of the extracts at $p < 0.05$ level; (-) Unidentified IC_{50}.

Cell viability

The results of RAW 264.7 cells survival rate (%) after treatment with EA extracts and EtOH extracts (control sample) from the biomass and fruit bodies of *I. cicadae* F0004 are shown in Table 5.

Table 5 showed that RAW 264.7 cells have a survival rate of over 70% in all survey

extracts from the biomass and fruit bodies of *I. cicadae* F0004 at 100 μg/mL. Therefore, concentrations of extracts from the biomass and fruit bodies of *I. cicadae* F0004 between 0 and 100 μg/mL to treat RAW 264.7 cells were selected for the next experiments.

Table 5. Percentage survival of RAW 264.7 cells after treatment with EA extracts and EtOH extracts from the biomass and fruit bodies of *Isaria cicadae* F0004

		Percentage survival of RAW 264.7 cells				
μg/mL		0	25	50	100	200
BM	EtOH	100	82.82 ± 1.97^{a}	80.61 ± 2.58^{a}	72.13 ± 1.64^{b}	60.96 ± 1.25^{c}
	EA	100	87.72 ± 3.27^{a}	77.72 ± 1.21^{b}	72.39 ± 2.87^{c}	67.41 ± 3.58^{d}
FB	EtOH	100	89.54 ± 5.92^{a}	84.74 ± 9.26^{ab}	75.82 ± 7.68^{b}	64.25 ± 6.9^{c}
	EA	100	106.66 ± 7.58^{a}	85.46 ± 22.48^{b}	79.85 ± 3.91^{c}	56.15 ± 13.47^{d}

Notes: BM (Biomass); FB (Fruit body); EtOH (ethanol); EA (Ethyl acetate); The letters a, b, c and d in the same column represent the difference between the IC_{50} values of the extracts at $p < 0.05$ level.

Cell membrane protein oxidation

The results of protecting RAW 264.7 protein membrane oxidation after treatment with extracts from the biomass and fruit bodies of *I. cicadae* F0004 are presented in the Table 6.

In Table 6, both EtOH and EA extracts from the biomass of *I. cicadae* F0004 have no difference among them, 89.93 ± 2.93 μg/mL (EtOH extract) and 82.65 ± 4.019 μg/mL (EA extract). According to that, phytochemical compounds in the EtOH extract from the biomass of *Isaria cicadae* F0004 have protein membrane protective activity similar to EA extract from the biomass of *I. cicadae* F0004. Meanwhile, EA extract from the fruit body of *I. cicadae* F0004 has IC_{50} (68.631 ± 0.632 μg/mL) lower significantly than EtOH extract from the fruit body of *I. cicadae* F0004 (102.385 ± 4.812 μg/mL). EtOH extract contains some compounds that interacted and affected the ability to link protein membranes. In addition, (Yadav et al., 2014) showed that EA extract in fungus containing flavonoids and polyphenols have antioxidant activity. Therefore, EA extract from the fruit bodies of *I. cicadae* F0004 is a potential extract that can protect the RAW 264.7 protein membrane.

Table 6. IC_{50} of RAW 264.7 protein membrane protection after treatment with extracts from the biomass and fruit bodies of *Isaria cicadae* F0004

IC_{50} (μg/mL)	EtOH	EA	BHA
BM	89.93 ± 2.93^{c}	82.65 ± 4.019^{c}	9.511 ± 0.574^{a}
FB	102.385 ± 4.812^{d}	68.631 ± 0.632^{b}	

Notes: BM (Biomass); FB (Fruit body); EtOH (ethanol); EA (Ethyl acetate); BHA (β-hydroxy acid) as the positive control; The letters a, b, c and d in the same column represent the difference between the IC50 values of the extracts at $p < 0.05$ level.

Cell membrane lipid oxidation

The results of inhibiting RAW 264.7 lipid membrane peroxidation after treatment with extracts from the biomass and fruit bodies of *I. cicadae* F0004 are shown in Table 7.

The IC_{50} values of EA extracts from the biomass and fruit bodies of *I. cicadae* F0004 are lower significantly than EtOH extracts from the biomass and fruit bodies of *I. cicadae* F0004. In addition, EA extract from the fruit bodies of *I. cicadae* F0004 has the lowest IC_{50}

value (19.042 ± 1.072 μg/mL). Therefore, EA extract from the fruit bodies of *I. cicadae* F0004 is a potential extract that can inhibit RAW 264.7 lipid membrane peroxidation. Moreover, EA extracts from biomass and fruit body of *I. cicadae* inhibited lipid peroxidation better than *C. militaris* fruiting body extract (380 μg/mL) and *C. militaris* mycelium extract (5530 μg/mL), *I. tenuipes* fruiting body extract (400 μg/mL) and *I. tenuipes* mycelium extract (490 μg/mL), respectively (Prommaban et al., 2022).

Table 7. IC_{50} of RAW 264.7 lipid membrane protection after treatment with extracts from the biomass and fruit bodies of Isaria cicadae F0004

IC_{50} (μg/mL)	EtOH	EA	BHA
BM	143.361 ± 14.232^{d}	61.62 ± 6.525^{c}	11.59 ± 0.17^{a}
FB	> 200	19.042 ± 1.072^{b}	

Notes: BM (Biomass); FB (Fruit body); EtOH (ethanol); EA (Ethyl acetate); BHA (β-hydroxy acid) as the positive control; The letters a, b, c and d in the same column represent the difference between the IC50 values of the extracts at $p < 0.05$ level.

Determination of free radical mediated DNA damage

The results of protecting RAW 264.7 DNA after treatment with extracts from *I. cicadae* biomass and fruit bodies are shown in Table 8 and Figure 1.

The DNA of RAW 264.7 was treated with *I. cicadae* biomass and fruit body extracts, having clear bands when the concentration of samples was increased from 25 to 100 μg/mL (Fig. 1). Thus, the DNA of RAW 264.7 cells were kept structurally intact when the extract concentrations were gradually increased. In *I. cicadae* biomass, the DNA of RAW 264.7 treated with EA extract had the clearest band (Fig. 1) at 100 μg/mL and EA extract can protect 57.73 ± 4.341% of DNA. Similarly, EA extract of *I. cicadae* fruit bodies had the clearest DNA band and can protect 98.116 ± 0.076% of DNA at 100 μg/mL (Fig. 1).

Zhang et al. (2019) showed that some phytochemicals in *I. cicadae* fruit bodies were found in *Cordyceps* spp. such as Cordycepin (Zhang et al., 2019). Cordycepin can protect neuron cells against oxidizing agents like 6-hydroxydopamide (Olatunji et al., 2016). Therefore, both the EA extract of *I. cicadae* biomass and fruit bodies are two potential extracts containing many secondary metabolites that can protect the DNA of RAW 264.7 cells, especially the EA extract of *I. cicadae* fruit bodies.

Table 8. Percentage of DNA protection after treatment with extracts from the biomass and fruit bodies of *Isaria cicadae* F0004

μg/mL		Percentage (%) survival of DNA			
		0	25	50	100
BM	EA	26.153 ± 0.019^{d}	41.364 ± 0.22^{c}	37.003 ± 0.64^{b}	57.73 ± 4.341^{a}
	EtOH	26.153 ± 0.019^{g}	28.908 ± 0.9^{f}	45.748 ± 0.96^{e}	44.593 ± 0.098^{e}
FB	EA	31.547 ± 0.008^{k}	52.171 ± 0.5^{j}	80.799 ± 3.196^{i}	98.116 ± 0.079^{h}
	EtOH	31.547 ± 0.008^{o}	41.85 ± 0.093^{n}	45.901 ± 0.504^{m}	57.703 ± 0.139^{l}

Notes: BM (Biomass); FB (Fruit body); EtOH (ethanol); EA (Ethyl acetate); The letters a, b, c, d, e, f, g, h, i, j, k, l, m, n and o in the same column represent the difference between the IC_{50} values of the extracts at $p < 0.05$ level.

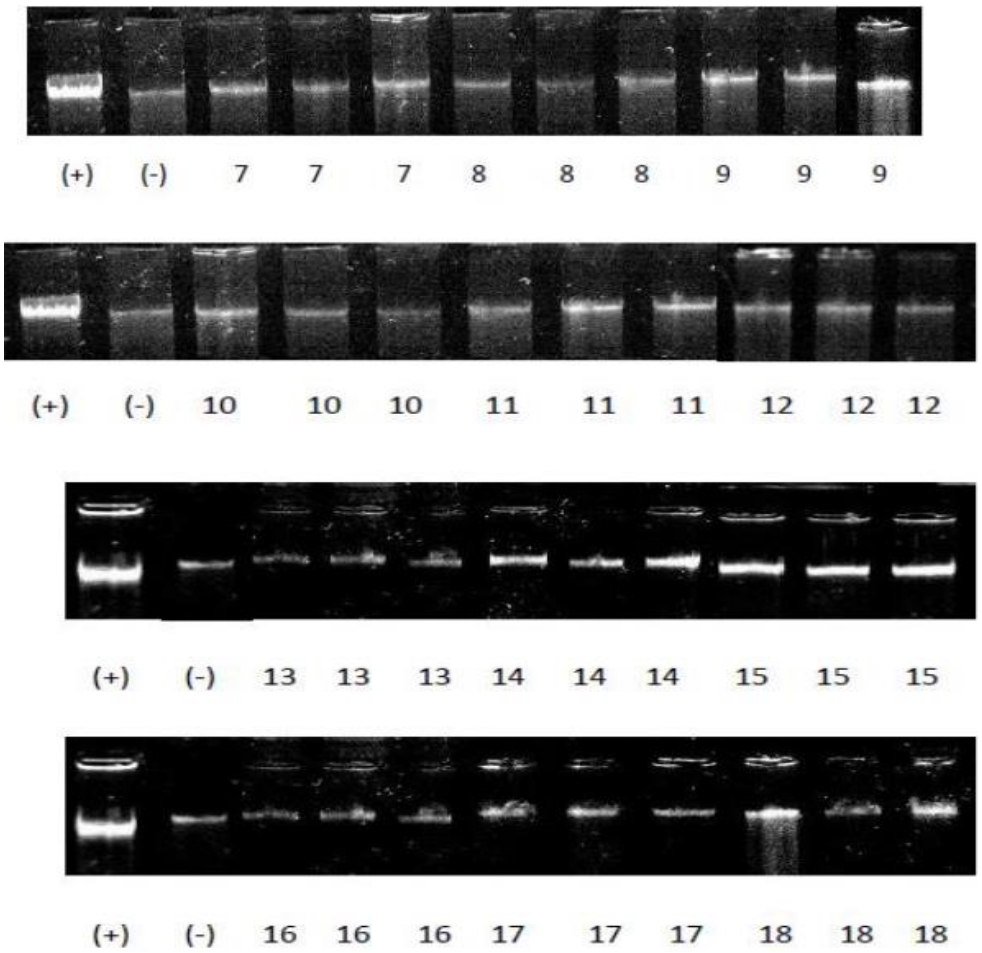

Figure 1. Agarose gel electrophoresis for DNA of RAW 264.7 after treatment with extracts from biomass and fruit body of Isaria cicadae F0004. Lane (7) to (9): EA extract of biomass in 25, 50, 100 µg/mL; (10) to (12): EtOH extract of biomass in 25, 50, 100 µg/mL; (13) to (15): EA extract of fruit body in 25, 50, 100 µg/mL, (16) to (18): EtOH extract of fruit body in 25, 50, 100 µg/mL; (+): Blank; (-): Negative control

DISCUSSION

Insect parasitic fungi such as *C. militaris* and *C. sinensis* have been widely investigated, but it is encouraged to examine new strains to increase the number of bioactive materials, especially in Vietnam. *I. cicadae* F0004 was isolated, cultured, and extracted, and its activity was determined in different ways, showing effective antioxidant activities. The extract has many substances, and each substance shows antioxidant activity in different metabolic pathways. Based on their molecular structures, the substances in *I. cicadae* extracts are mainly categorized as organic acids, amino acids, lipids and phospholipids, nucleosides, and carbohydrates. Some compounds have functional or bioactive components-for example, cordycepin, cordycepic acid, ergosterol, adenosine, guanosine, uridine, inosine, thymidine, and other nucleosides (Zhou et al., 2009). They have multiple bioactivities, such as anticancer, antimetastatic, antidepressant, immunoregulati on, and antioxidant properties (Olatunji et al., 2016; Nakamura et al., 2015). Among them, N-(2-hydroxyethyl) adenosine was isolated

and proven to exhibit antioxidant activity by measuring its radical scavenging effect on DPPH radicals (Ahn et al., 2008). In addition, cordycepic acid (D-mannitol) exhibited diuretic action and prophylaxis against postoperative acute renal failure, as well as relieving cough and asthma and possessing activities against free radicals (Lin et al., 2016).

Some other active ingredients detected in other stages (mycelia, primordia, and stroma) from *I. cicadae* (Lu et al., 2017) exhibited a potential inhibitory effect on HepG2 and HepG2/ADM cells, with IC_{50} values ranging from 2.40 to 14.48 mM Oosporein also exhibits a broad spectrum of in vitro antimicrobial, antioxidant, and cytotoxic activities (Alurappa et al., 2014). Our study contributes more information on the biological activities of *I. cicadae* F0004 isolated in Vietnam, including the ability to protect cell tissues such as protein membranes, lipid membranes, and DNA (which are easily damaged molecules under oxidizing agents) and the ability to inhibit enzymes that activate oxidation reactions such as XO. Another remarkable result is that the cultured biomass has higher activities than the fruit body. As it is easier to collect active substances from biomass than from fruit bodies, this result may encourage the development of active biomass cultures.

CONCLUSION

Based on our results of investigating antioxidant activity in vitro (DPPH radical scavenging assay, ABTS radical scavenging assay, reducing power, XO inhibition) and in the RAW 264.7 cell line (protection of proteins, DNA, and cell membranes), as well as our contribution to the knowledge of the bioactivities of *I. cicadae*, the EA fraction extracts of both samples from *I. cicadae* F0004 showed better antioxidant activity than the other extracts. However, it is necessary to qualify and quantify the phytochemicals present in the EA extracts and to investigate the antioxidant activity in animal models and humans.

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