

RESEARCH ARTICLE

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Detection of Antimicrobial, Antioxidant and Cytotoxicity Activities of *Fusarium oxysporum* F01 Isolated from *Catharanthus roseus* Collected in Vietnam

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Abstract

The ability of endophytic fungi to produce valuable bioactive compounds when surviving in medicinal herbs. Finding out endophytic fungi originated from *Catharanthus roseus* with antimicrobial, antioxidant and cytotoxicity activities is important for pharmaceutical development. The isolation was based on the morphology of fungi. Identification was performed by sequencing 18S rRNA that was compared with known genes using Blast search in the combination of phylogenetic analysis by using Clustal W and PhyML in GenomeNet. For the antimicrobial test, the agar diffusion method was used. DPPH scavenging assay for the antioxidant activity determination by using spectrophotometry. The cytotoxicity test was carried out by Sulforhodamine B method. LC-MS was applied for predicting components. We isolated the *Fusarium oxysporum* F01 strain originated from *Catharanthus roseus*. The aqueous extracts showed inhibition against *Staphylococcus aureus* ATCC 25923, *Pseudomonas aeruginosa* ATCC 27853 (15.00 ± 0.50 mm), *Serratia marcescens* ATCC 14756 (9.00 ± 0.87 mm), *Vibrio parahaemolyticus* ATCC 17802 (15.50 ± 0.87 mm), *Escherichia coli* ATCC 25922 (12.50 ± 0.50 mm). The antioxidant activity of the extract obtained from the supernatant was determined with an IC50 value of about 11 µg/mL. The extract also showed cytotoxicity effect on liver cancer cell line (HepG2) and breast cancer cell line (MCF-7) with

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Abbreviations: ANOVA: Analysis of variance, ATCC: American Type Culture Collection, BLAST: Basic Local Alignment Search Tool, DMEM: Dulbecco's Modified Eagle Medium, DPPH: 1,1-diphenyl-2-picrylhydrazyl, ETE: Environment for Tree Exploration, IC50: half maximal inhibitory concentration, LB: Luria-Bertani, LC-MS: Liquid chromatography mass spectroscopy, OD: Optical density, PDB: Potato dextrose broth, SRB: Sulforhodamine B, TCA: trichloroacetic acid, YEME: Yeast Extract-Malt Extract.

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the percentage of inhibition of 84.47 ± 3.18 and 94.69 ± 1.59 , respectively. LC-MS was used to point out the presence of pratol, a melanogenesis agent. The study provided more interesting information about *Fusarium oxysporum* F01 isolated in *Catharanthus roseus* grown in Vietnam, contributing to pharmaceutical sources in the world.

Keywords: *Fusarium oxysporum* F01, *Catharanthus roseus*, antimicrobial and antioxidant activity, cytotoxicity, LC-MS

INTRODUCTION

Microorganisms produced many biological compounds such as bacteriocin, conjugated linoleic acid, gamma buryric acid, lactic acid from lactic acid bacteria¹⁻⁴, prodigiosin production from *Streptomyces coelicolor*⁵, penicillin from fungi⁶. Defined as an organism growing, existing within the plant causing no harm to its host, the endophyte is known to generate no apparent symptoms of disease and some of natural plant compounds⁷. Endophytic fungi are symbiotically associated with their host plant, mimic their chemistry and synthesize the same natural products and bioactive compounds as these originated from their host, and thus are screened for the production of valuable compounds. There were Taxol and taxane production by endophytic *Taxomyces andreanae* isolated from pacific yew⁸. Endophytic *Cladosporium* from *Boerhaavia diffusa* Linn showed *in-vitro* antioxidant activity⁹.

There were many plants showing biological activities such as *Plumbago indica* L.¹⁰, *Alpinia conchigera* Griff¹¹, *Achillea millefolium* Linn¹², *Murraya paniculata* (L.) Bark¹³, *Strobilanthes kunthianus*¹⁴ and others. *Catharanthus roseus* or *Vinca rosea*, which is also commonly known as the Madagascar periwinkle or rose periwinkle is a plant species belongs to Apocynaceae family. It is used as an ornamental as well as medicinal plant. *Catharanthus roseus* is well known for production of copper nanoparticle¹⁵, antibacterial activities¹⁶, anti-cancerous agents such as vinca alkaloids which have effect on pain-relieving or contain anticancer properties. Vinblastine and vincristine of *Catharanthus roseus* have been developed and applied in anticancer drugs as prescriptions¹⁷. As a matter of fact, an endophyte called *Fusarium oxysporum* of rosy periwinkle plant, *Catharanthus roseus* was discovered to produce vincristine – an anticancer drug¹⁸⁻²⁰. In term of *Fusarium oxysporum*, an ascomycete fungus of Nectriaceae family including many species that was discovered

by Wollenweber and Reinking²¹. *Fusarium oxysporum*, which is dominant in active soil, can be harmless to plants or be a pathogenic to its host. In contrast, it is beneficial in form of endophytes – having a mutual relationship to plants, for example, some of these bioactive compounds have been found and extracted vincristine¹⁸⁻²⁰. Moreover, antibiotic resistance in microorganisms is increasing with a high rate nowadays such as *Salmonella* sp.²², *Escherichia coli* and *Klebsiella*²³, *Staphylococcus aureus*²⁴. Additionally, antioxidant activities that can help human in preventing some diseases were studied much on plant extracts²⁵⁻²⁸ whereas microbial extracts were not investigated in both the depth and width.

Therefore, the study isolated *Fusarium oxysporum* 01 from *Catharanthus roseus* collected in Binh Thuan (Vietnam). The strain was used to prove an excellent endophyte for many valuable substances. The antimicrobial assay was carried out to present the power of *Fusarium oxysporum* F01 isolated from *Catharanthus roseus* against *Staphylococcus aureus* ATCC 25923, *Pseudomonas aeruginosa* ATCC 27853, *Serratia marcescens* ATCC 14756, *Vibrio parahaemolyticus* ATCC 17802, *Escherichia coli* ATCC 25922 when using agar diffusion method and screened antioxidant activity and cytotoxicity. Moreover, the production of valuable compounds in a specific amount was also screened by liquid chromatography-mass spectrum (LC-MS). The results contributed to the evidence of endophytic fungi showing activities when having a mutual life with medicinal herbs.

MATERIALS AND METHODS

Specimens

Catharanthus roseus trunk was collected in Binh Thuan (Vietnam).

Pathogenic bacteria including *Staphylococcus aureus* ATCC 25923, *Pseudomonas aeruginosa* ATCC 27853, *Serratia marcescens* ATCC 14756, *Vibrio parahaemolyticus* ATCC 17802,

Escherichia coli ATCC 25922 were purchased from American typed culture Collection (USA).

Cancer cell lines (MCF-7 and Hep-G2) were purchased from Sigma (USA).

Chemicals and reagents

Culture media and medium components for media including Potato dextrose broth (PDB), Potato dextrose agar (PDA), Luria-Bertani (LB) broth, Yeast Extract, Malt Extract were purchased from Bio Basic (Canada).

1,1-diphenyl-2-picrylhydrazyl (DPPH) and Sulforhodamine B (SRB) were purchased from Sigma (USA).

Antibiotics were purchased from Institute of Drug Quality Control (Ho Chi Minh City, Vietnam).

DNA isolation kit was purchased from Qiagen (Germany).

Solvents were purchased from Merck (USA).

Other analytical chemicals were purchased from Thermo Fisher Scientific (USA).

Isolation and Identification

The trunk (100g) of *Catharanthus roseus* collected in Binh Thuan (Vietnam). Immediately, it was cut into small pieces, cultured in potato dextrose broth (PDB) in 5 days. The culture was spread on potato dextrose agar (PDA) to select the single colonies with mycelium. The colony was used to culture in PDB in 5 days for DNA extraction using DNA extraction kit. After checking the purity and concentration of DNA, DNA was used for sequencing. The sequence was compared to well-known strains in gene bank using Blast search²⁹. The sequence was aligned with the related sequence and reconstructed phylogenetic tree using the function "build" of the Environment for Tree Exploration (ETE3) v3.1.1³⁰ as implemented on the GenomeNet. ML tree was inferred using PhyML v20160115 ran with model and parameters according to Guidon et al³¹.

Cultivation for antimicrobial activity

Fusarium oxysporum F01 isolated from *Catharanthus roseus* in Vietnam was cultured in modified yeast extract- malt extract (YEME) as medium for the fermentation process in 14 days old cultures. The culture of *Fusarium oxysporum* F01 were centrifuged at 10,000 rpm for 10 minutes at 25°C to separate the mycelia from the culture

broth as pellet and supernatant. The supernatant was concentrated and fractionated with distilled water in an appropriate proportion for further analysis.

For the preparation crude pellet extract, the pellet (1 g) was sonicated in water before centrifugation. The crude pellet extract was collected for further analysis.

Antimicrobial activity test

Pathogenic organisms for testing were *Staphylococcus aureus* ATCC 25923, *Pseudomonas aeruginosa* ATCC 27853, *Serratia marcescens* ATCC 14756, *Vibrio parahaemolyticus* ATCC 17802, *Escherichia coli* ATCC 25922. From stock, the pathogens were inoculated and cultured in LB broth until OD600 of 0.5 was achieved for further tests. Antibiotics including ampicillin, ciprofloxacin, gentamycin were used as control.

Agar well diffusion technique was used to evaluate the antimicrobial activity of aqueous extracts. As described by previous study³², the LB agar plate was inoculated by spreading 20 µL of pathogen culture onto the agar plate by sterile swabs, then four 5-mm diameter holes were aseptically punched by sterile tips and then 80 µL extracts from supernatant and intracellular extract obtained from pellet extracts was introduced to the wells. Antibiotics were used as positive controls whereas the negative control was modified YEME. The agar plates were incubated under 37°C and such antimicrobial agents diffused into the agar and inhibit the growth of microbes. After 10 - 12 hour incubation, the inhibition zones around the wells were measured. Table 1 showed the reference and the concentration of antibiotics corresponding to type of organisms.

Similarly, the SE samples were used to screen for antimicrobial activity. The antimicrobial compounds diffused into the agar and inhibited microbial growth. Consequently, the inhibition growth zones were measured.

Antioxidant by 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging test The radical scavenging activity of different concentration of fungal crude extracts was determined by using DPPH assay³³. DPPH (1,1-diphenyl-2-picrylhydrazyl) solution was prepared by dissolving DPPH with methanol. The DPPH solution was kept out of light. The blank was

set up with 189 µL of methanol along with 9 µL of DPPH. Ascorbic acid was used as standard sample which was prepared by diluting stock solution with distilled water into different concentrations (1, 2, 3, 4, 5, 6 µg/mL). The working solution of extracts was prepared with different concentrations (1, 2.5, 5, 7.5, 10, 12.5 µg/mL) with distilled water. Then, 9 µL DPPH solution was mixed with 180 µL of standard solution or diluted extracts. All mixtures were incubated in the dark at room temperature for 30 minutes. The absorbance of the mixture was then recorded at 517 nm. The experiment was triplicate.

Cytotoxicity test

Sulforhodamine B (SRB) assay was a colorimetric method to determine sensitivity and cytotoxicity of a substance³⁴. The anionic dye binds electrostatically with the positive-charged parts of the proteins. The amount of binding dye correlates with the number of total cellular proteins. The cells were cultured to attain 70-80% coverage of culture flasks and passed into 96-well plate with a density of 104 cells per well. The plates were incubated at 37°C in 5% CO₂ for 24 hours. Next, the medium of sample was added into the wells and incubated at 37°C in 5% CO₂ for 48 hours. Then, the cells were fixed with trichloroacetic acid (TCA) and stained with SRB (0.2%) and left at room temperature (25°C) for 5 to 20 minutes. After SRB was discarded, the cells were washed gently with acetic acid (1%) for four times. Let the cells

air-dry at room temperature (25°C) from 12 to 24 hours. The results were obtained by loading 200 µL tris base into each well, then shaking for 10 minutes until dissolved completely for measuring the optical density (OD) at 492 nm and 620 nm. In the test, the positive control was camptothecin (0.07 µg/mL) and the negative control was DMEM (0.25%).

Liquid Chromatography – Mass Spectroscopy (LC-MS)

The predicted compounds containing in samples were determined by LC-MS. Begin with the ionization, the ions were separated according to their mass to charge (m/z) ratio; followed by the transfer of ions to mass analyzer. Then, the spectra were recorded in the positive ionization mode.

Statistical analysis

In antimicrobial assay, data were reported and presented as mean ± standard deviation. The statistic was analyzed by SPSS software and Microsoft Excel, especially Two-way ANOVA was used for checking significant difference between two factors: stains and treatments; then Tukey test was applied as Post Hoc test for multiple comparisons of groups on treatment factor.

RESULTS

Identification of fungi

By using Blast search, the strain had 100% identity to *Fusarium oxysporum* strain F-H.6.5-030318-02 by comparing its 18S, 5.8S, 28S

Table 1. Organisms with corresponding positive controls

Test organisms	Positive control	Concen. (µg/mL)
<i>Staphylococcus aureus</i>	Gentamycin	64
<i>Pseudomonas aeruginosa</i>	Ciprofloxacin	2
<i>Serratia marcescens</i>	Ciprofloxacin	4
<i>Vibrio parahaemolyticus</i>	Ampicillin	32
<i>Escherichia coli</i>	Ampicillin	128

Table 2. Inhibition zone diameter of water extracts to organisms

Test organisms	Positive control	Aqueous extract
<i>Staphylococcus aureus</i>	18.50 ± 0.50	17.83 ± 0.58
<i>Pseudomonas aeruginosa</i>	30.33 ± 0.58	15.00 ± 0.50
<i>Serratia marcescens</i>	40.33 ± 0.29	9.00 ± 0.87
<i>Vibrio parahaemolyticus</i>	44.50 ± 0.50	15.50 ± 0.87
<i>Escherichia coli</i>	22.33 ± 0.58	12.50 ± 0.50

Table 3. Cytotoxicity (% inhibition) of the crude extract *Fusarium oxysporum*

Cancer cell lines	Fungal extract	Camptothecin (Positive control)	DMEM medium (Negative control)
Hep G2	84.47 ± 3.18	98.15 ± 2.05	0
MCF-7	94.69 ± 1.59	97.62 ± 2.80	0

ribosomal RNA sequences to partial gene of 18S ribosomal RNA at the internal transcribed spacer 1, and complete sequence of 5.8S ribosomal

RNA gene with the internal transcribed spacer 2. The sequence was deposited in DNA Data Bank of Japan (DDBJ) with accession number

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JN232157.1	AACCCCTCAAGCACAGCTTGGTGTGGGACTCGCGTTAAATTCGCGTTCCTC
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MH864441.1	ATATGTA---ACTTCTGAGTAAACCATAAAATAAATCAAAACTTTCAACA
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Fig. 1. Multiple sequence alignment. (EU364859.1:14-657: *Fusarium oxysporum* strain F-X.1.7-030520-12; AY669122.1: *Fusarium oxysporum* strain F-W.6.2-030304; AY669125.1:10-653: *Fusarium oxysporum* strain F-X.1.7-030520-12; EU364842.1: *Fusarium oxysporum* strain F-H.6.5-030318-02; JN232157.1: *Fusarium oxysporum* isolate 152; LR535806.1: *Fusarium oxysporum* 247; EU364844.1: *Fusarium oxysporum* strain F-H.6.5-030318-01; CP052041.1:22857-23499: *Fusarium oxysporum* Fo47 chromosome IV; MH864510.1: *Fusarium oxysporum* strain CBS 127297; MH864441.1: *Fusarium oxysporum* strain CBS 127149; MH864510.1:9-651: *Fusarium oxysporum* strain CBS 127297; AY669124.1: *Fusarium oxysporum* strain F-H.6.5-030318-J1; MW789355.1: *Fusarium oxysporum* isolate F3-2018; EU364842.1: *Fusarium oxysporum* strain F-H.6.5-030318-02; JN232157.1: *Fusarium oxysporum* isolate 152; AY669122.1: *Fusarium oxysporum* strain F-W.6.2-030304; LR535806.1: *Fusarium oxysporum* 247; seq: the sequence for isolated strain).

(LC645215). The sequence was aligned with the sequences of other *Fusarium oxysporum* strains (Fig. 1). Moreover, after phylogeny analysis using neighbor joining evaluation, the strain was closed to *Fusarium oxysporum* strains (Fig. 2). As a result, *Fusarium oxysporum* 01 originated from *Catharanthus roseus* was identified.

Antimicrobial activity

The antimicrobial activity of *Fusarium oxysporum* F01 on pathogens was demonstrated in Table 2. As shown in Table 2, the extract collected from supernatant of culture had effect on five organisms. The extract had the strongest inhibitory effect on *Staphylococcus aureus* (17.8 ± 0.58 mm)

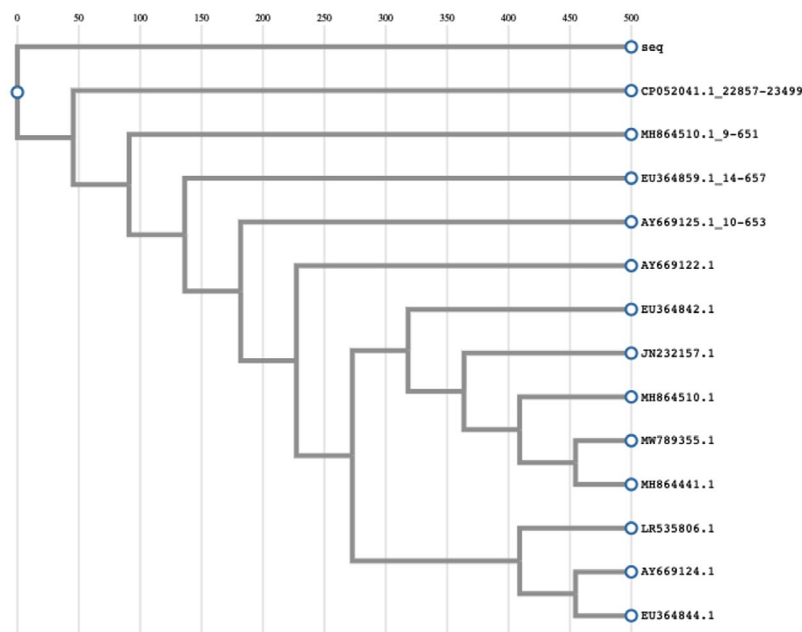


Fig. 2. Phylogeny analysis. (EU364859.1:14-657: *Fusarium oxysporum* strain F-X.1.7-030520-12; AY669122.1: *Fusarium oxysporum* strain F-W.6.2-030304; AY669125.1:10-653: *Fusarium oxysporum* strain F-X.1.7-030520-12; EU364842.1: *Fusarium oxysporum* strain F-H.6.5-030318-02; JN232157.1: *Fusarium oxysporum* isolate 152; LR535806.1: *Fusarium oxysporum* 247; EU364844.1: *Fusarium oxysporum* strain F-H.6.5-030318-01; CP052041.1:22857-23499: *Fusarium oxysporum* Fo47 chromosome IV; MH864510.1: *Fusarium oxysporum* strain CBS 127297; MH864441.1: *Fusarium oxysporum* strain CBS 127149; MH864510.1:9-651: *Fusarium oxysporum* strain CBS 127297; AY669124.1: *Fusarium oxysporum* strain F-H.6.5-030318-J1; MW789355.1: *Fusarium oxysporum* isolate F3-2018; EU364842.1: *Fusarium oxysporum* strain F-H.6.5-030318-02; JN232157.1: *Fusarium oxysporum* isolate 152; AY669122.1: *Fusarium oxysporum* strain F-W.6.2-030304; LR535806.1: *Fusarium oxysporum* 247; seq: the sequence for isolated strain).

than *Vibrio parahaemolyticus* (15.50 ± 0.8 mm) and *Serratia marcescens* (9.00 ± 0.87 mm) and *Escherichia coli* (12.50 ± 0.50 mm) *Pseudomonas aeruginosa* (15.00 ± 0.50 mm). Specially, when the effect against *Staphylococcus aureus* was compared to Gentamycin ($64 \mu\text{g/mL}$), the extract gave inhibition zone that was not significant to gentamycin by statistical analysis. This fungus could be an ideal source of antimicrobial agent with high potency in *Staphylococcus aureus* treatment while *Staphylococcus aureus* is resistant to antibiotic highly.

For *Pseudomonas aeruginosa*, *Serratia marcescens*, *Vibrio parahaemolyticus* and *Escherichia coli*, the activities against these strains were not as strong as antibiotics used as control samples. However, the results showed the extract could contain many potential agents. To improve the activity, the concentration of extract should be increase. Moreover, all the extracts obtained from pellet did not show antimicrobial activity. To predict which compound in the pellet

originated extract, LC-MS was presented in Fig. 3. The molecular mass ($[M^+ H]^+$) was 269.0748. The detection showed pratol in the extract. Pratol is an melanogenesis³⁵. Therefore, the pellet extract did not show activities tested in the study.

Antioxidant by 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging

The evaluation of the antioxidant activity of the extract was determined by measuring its scavenging ability against DPPH in the stable radical assay. Based on the curve of ascorbic acid ($y = 8.0796x + 39.646$, $r^2 = 0.9899$), the antioxidant activities of the extract prepared with different concentrations were determined. All the inhibitory percentage in antioxidant assay built up the straight curve ($y = 8.0796x + 39.646$, $r^2 = 0.9899$) (Fig. 4). It meant that the obtained results were relative and reliable. The IC50 of crude extract is $11.03 \mu\text{g/mL}$ that was higher than ascorbic acid ($\text{IC}_{50} = 8 \mu\text{g/mL}$). The crude extract showed the weaker antioxidant activity than ascorbic acid.

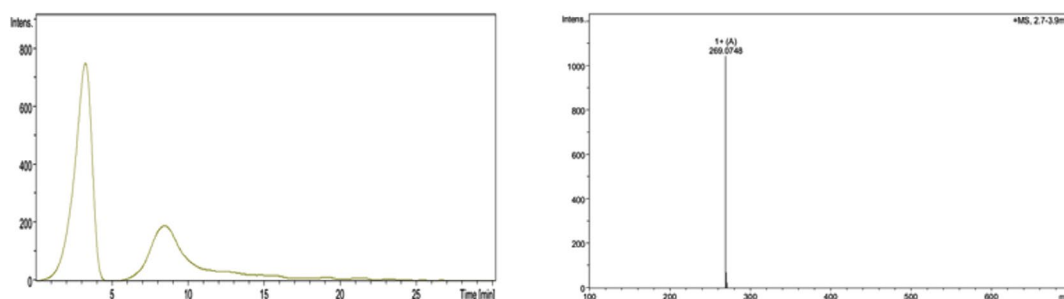


Fig. 3. LC-MS analysis of the extract obtained from pellet.

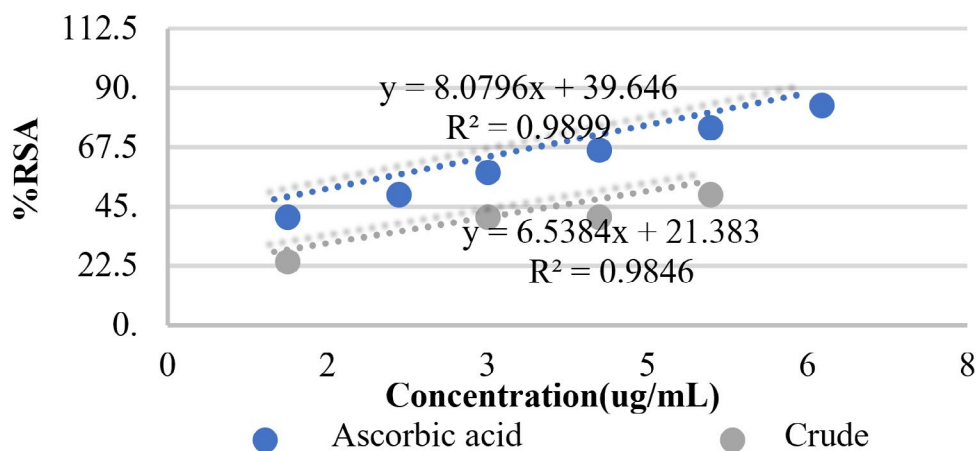


Fig. 4. Percentage radical scavenging activity of ascorbic acid and crude extract.

Cytotoxicity activities

The samples were prepared at the concentration of 50 mg/mL for cytotoxicity test on MCF-7 and Hep-G2 cells. All the samples had cytotoxicity on these cell line (Table 3). In the study, we couldn't detect vincristine and vinblastine in *Fusarium oxysporum* F01 by LC-MS. The cytotoxicity was caused by others compounds in *Fusarium oxysporum* F01.

The biological activities were due to this strain had symbiotic effects with *Catharanthus roseus* which had well-known activities. *Fusarium oxysporum* originated from environment commonly causes diseases, however, *Fusarium oxysporum* F01 showed biological activities such as antimicrobial and antioxidant activities together with cytotoxicity. The study brings out the importance of microorganisms showing pathogenic or beneficial effects depending on their host.

DISCUSSION

BLAST search is the tool for comparing biological sequence information. A BLAST search help to identify database sequences which are similarity to the unknown sequence. In the study, *Fusarium oxysporum* F01 had partial sequence of 18S, 5.8S and 28S rRNA with 100% identity to the gene information of strain F-H.6.5-030318-02. the reconstruction, analysis, and visualization of phylogenetic trees and multiple sequence alignments were analyzed simplify by ETE that supports in comparative genomics and phylogenetics. In the combination of phylogeny analysis, *Fusarium oxysporum* F01 originated from *Catharanthus roseus* was identified obviously.

Table 2 showed the antimicrobial activity of *Fusarium oxysporum* F01 extract collected from supernatant of culture on five organisms. The extract showed inhibition against the growth of *Staphylococcus aureus*, *Vibrio parahaemolyticus*, *Serratia marcescens* and *Escherichia coli* and *Pseudomonas aeruginosa* in different manners, leading to the different effects. Interestingly, the potency of the extract was not significant to gentamycin (64 µg/mL) by statistical analysis when inhibited *Staphylococcus aureus* growth. Therefore. Exploiting this strain metabolites to

treat these bacteria is important to inhibit the tested bacteria that had risks of resistance such as *Staphylococcus aureus*. The extract obtained from cultural supernatant showed the weaker cytotoxicity activity than ascorbic acid due to it contained the mixture of antioxidant agents and non-antioxidant agents that interfere the activity. However, the antioxidant agent existed in the extract after antioxidant activity screening. Moreover, the extract at the concentration of 50 mg/mL for cytotoxicity test on MCF-7 and Hep-G2 cells. By the comparison to the controls, the extract gave the significantly lower activity than camptothecin on HepG2, but insignificant difference from camptothecin on MCF-7. In the study, we couldn't detect vincristine and vinblastine in *Fusarium oxysporum* F01 by LC-MS analysis due to low concentration or the unsuitable cultivation. The cytotoxicity was caused by others compounds in *Fusarium oxysporum* F01. The pellet extract could not show strong antimicrobial and antioxidant activities in the study. However, the pellet extract contained pratol that is a melanogenesis. Obviously, *Fusarium oxysporum* F01 isolated from *Catharanthus roseus* showed the benefit activities, strengthening that symbiotic endophyte could produce useful activities when it may be harmful when living in the other sources.

CONCLUSION

Extract from cultural supernatant of *Fusarium oxysporum* F01 had inhibitory activities against *Staphylococcus aureus* ATCC 25923, *Pseudomonas aeruginosa* ATCC 27853, *Serratia marcescens* ATCC 14756, *Vibrio parahaemolyticus* ATCC 17802, *Escherichia coli* ATCC 25922. *Fusarium oxysporum* F01 will contribute to develop antimicrobial activities against *Pseudomonas aeruginosa*, *Serratia marcescens*, *Staphylococcus aureus*, *Vibrio parahaemolyticus* and *Escherichia coli* when these strains were resistant to antibiotics besides antioxidant and cytotoxicity activities. The pellet extract was screened to predict pratol used for melanogenesis prevention although it did not show antimicrobial and antioxidant activities. The mechanisms and purified compounds will be exploited to understand well and apply for future.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTIONS

NHKT proposal investigator, ideas, design experiment. NHKT, NTTTT, DTTV and HDL performed the experiment. NHKT, NTTTT, DTTV and HDL performed the data analysis. NPQA design figures and tables. NHKT, NTTTT and NPQA wrote the manuscript. All authors read and approved the manuscript for final publication.

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DATA AVAILABILITY

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

ETHICS STATEMENT

Not applicable.

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