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**Research Article** 

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# Antimicrobial activities of *Fusarium* isolated from *Hisbicus rosa-sinensis*

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## ABSTRACT

Fusarium which is the large filamentous fungi group widely found in plants and soil have been not studied much on biological activities. In the study, Fusarium isolated from Hisbicus was used to exploit the biological activities. The antimicrobial activities of Fusarium cultured in modified ISP 4 was against eight different kinds of pathogens including in Candida albicans  $(23.50\pm0.56 \text{ mm})$ , Salmonella typhi  $(17.47\pm0.50 \text{ mm})$ , Pseudomonas aeruginosa  $(16.67\pm0.58 \text{ mm})$ , Staphylococcus sciuri  $(14.43\pm0.55 \text{ mm})$ , Serratia marcescens  $(19.23\pm0.75 \text{ mm})$ , Micrococcus luteus  $(26.67\pm0.58 \text{ mm})$ , Staphylococcus aureus  $(13.57\pm0.51 \text{ mm})$  and Vibrio harveyi  $(15.47\pm0.50 \text{ mm})$  by using the agar diffusion method. Otherwise, Fusarium sp. did not show activity when cultured in ISP 4 even though it could grow very well in this medium. The chloroform and petroleum ether extracts from Fusarium culture were used to test antimicrobial activities. There were at least two antimicrobial compounds produced by Fusarium in modified ISP 4. This study firstly reported Fusarium isolated from Hisbicus rosa-sinensis could produce antimicrobial agents.

Keywords: antimicrobial activities, Fusarium, media, Hisbicus rosa-sinensis

## INTRODUCTION

Scientists currently found that there are over 20 species of genus *Penicillium*. Among of these, *Fusarium solani*, *Fusarium oxysporum*, and *Fusarium chlamydosporum* are the most common species [1]. Some *Fusarium* pathogens in Vietnam such as *Fusarium* vascular wilt diseases causing by *Fusarium oxysporumare* important problems in Vietnam. Cob rots in maize, caused mainly by *Fusarium graminearum* and *Fusarium verticillioides*, are becoming increasingly important in Vietnam. Both species produce mycotoxins that cause toxicity in the grain. Rots of melons and potato tubers are the results of *Fusarium oxysporum*'s infection. Collar rot of legume seedlings such as peas and beans, and root rot of older plants are caused by several strains of *Fusarium solani*. *Fusarium decencellulare* has been found and isolated from branch cankers of Dimocarpus longan in northern Vietnam and from coffee in Dac Lac province.

*Fusarium sp.* also have the ability to produce bioactive compounds such as paclitaxel (Taxol), podophyllotoxin, and vinblastine [2]. Paclitaxel, also known as taxol, is a chemical substance of tetracyclic di-terpene lactam that can be used alone or in combination with other chemotherapeutic agents for the treatment of variable kinds of cancers. Some *Fusarium* strains have the ability to produce paclitaxel. For example, *Fusarium solani* isolated from Taxuscelebica can yields  $1.6 \mu g/l$  of paclitaxel while *Fusarium redolens* was able to produce  $66 \mu g/l$  of paclitaxel in fermentation broth [3,4]. Podophyllotoxin is a non-alkaloid toxin lignan used as a precursor for the chemical synthesis of the anticancer drugs like etoposide, teniposide and etopophose phosphate. These derivatives can also be used in medical applications such as antiviral, antirheumatic, and antitumor agents. The natural resources of this podophyllotoxin are very limited and the synthetic process for production of this lignan are still unacceptable in commercial aspects. Endophytic fungus *Fusarium oxysporum* isolated from the medicinal plant Juniperus recurva can produce podophyllotoxin [5,6]. Vinblastine and vincristine are excellent drugs for cancer treatment, including Hodgkin's lymphoma, breast cancer, non-small cell lung cancer, head and neck cancer, and testicular cancer.

However, their current production using plants is non-abundant and expensive. *Fusarium oxysporum* has been identified to have ability to synthesize these drugs with 76  $\mu$ g of vinblastine and 67  $\mu$ g of vincristine [7].

To study more biological compounds of *Fusarium sp.*, this fungi was isolated from *Hisbicus rosa-sinensis* and tested for antimicrobial activities.

#### **EXPERIMENTAL SECTION**

#### Cultivation and optimization

*Fusarium sp* was isolated from *Hibiscus rosa-sinensis* in Vietnam. The spore stock  $(10^8 \text{ spores})$  of *Fusarium sp*. was kept at -80°C until use. In order to cultivate and optimize the culture conditions for the growth of *Fusarium sp*., ISP No.4 medium [8] and modified ISP4 medium were used.

#### Antimicrobial test

Pathogens used for antimicrobial test are *Candida albicans* ATCC 10231 (P1), *Salmonella typhi* ATCC 19430 (P2), *Pseudomonas aeruginosa* ATCC 27853 (P3), *Staphylococcus sciuri* ATCC 29061 (P5), *Serratia marcescens* ATCC13880 (P6), *Micrococcus luteus* ATCC 10240 (P8), *Staphylococcus aureus* ATCC 29061 (P9), and *Vibrio harveyi* ATCC 14126 (VA). Except yeast cultured in yeast medium, all bacteria were cultured in Luria-Bertani (LB) broth medium and gram-stained to check the purity.

*Fusarium sp.* spores ( $10^8$  spores) were cultured in 100mL modified ISP4 medium and in 100 mL ISP4 medium in the Erlenmeyer flask for 24h, 48h, 72h, 96h, 120h and 240h. After each period, the fractions of culture were transferred into 50mL falcons. The supernatant fractions were harvested after centrifuging at 10000 rpm for 10 minutes at 25°C. Both pellets and supernatant were collected for antimicrobial test.

The antimicrobial test will be performed by Agar well diffusion method [9]. Modified ISP4 medium and ISP4 medium were used as the negative control for the test. Amount  $(10^6 \text{ CFU/mL})$  of pathogen cultures (*Candida albicans, Salmonella typhi, Pseudomonas aeruginosa, Staphylococcus sciuri, Serratia marcescens, Micrococcus luteus, Staphylococcus aureus* and *Vibrio harveyi*) was spread onto the plate, using the sterile swab. Applying aseptic techniques, wells were formed in the LB agar plates using a small sterile metal stick. The supernatant was pipetted into the wells. All the plates were incubated at room temperature. After ten to twelve hours, the inhibition zones around the wells were measured and analyzed.

#### **Preliminary purification**

To purify the supernatant, chloroform, petroleum ether, methanol were used. The supernatant was added into three sterile falcons. Petroleum ether was used to extract the non-polar compounds with the alternative ratios (petroleum ether: extract = 1:1; 1:2). The mixtures were mixed vigorously. After collection of the petroleum ether fractions, the remaining extract was alternatively added with chloroform with the suitable ratio of chloroform: supernatant (2:1; 1:1; 1:2). Repeating the same steps as petroleum ether, the mixtures were mixed vigorously. The collected chloroform fractions were evaporated and then the powder was dissolved in distilled water for antimicrobial activity.

#### **RESULTS AND DISCUSSION**

#### Cultivation and optimization

In order to study the biological activities, the growth of *Fusarium sp.* was studied in different media (ISP4 and modified ISP4) to determine whether fungi grew well or not before testing. After growing in two media in different time, the cultures were measured the optical density. The OD values were obtained (Table 1). Noticeably, *Fusarium sp.* grew in modified ISP4 better than in ISP4 although *Fusarium sp.* grew the growth rate similarly when the incubation time reach to 24h (Table 1). However, *Fusarium sp.* grew very fast after 48h incubation in modified ISP4, determined by measuring optical density (OD=1.004). In ISP4, *Fusarium sp.* gave the small change in optical density values (OD= 0.125-0.643) when culturing in ISP4 from 24h to 240h.

The figure 1 illustrated antimicrobial activies of *Fusarium sp*. The weakest antimicrobial activity against *Candida albicans* was expressed after culturing *Fusarium sp*. in 24h that the inhibition zone was smallest and measured about  $4.83\pm0.29$  mm (Table 2). The activity increased until culturing in 10 days, showing the highest activity. The inhibition zone was measured about  $23.50\pm0.56$  mm (Table 2).

In *Salmonella typhi*, the weakest antimicrobial activity was expressed after culturing *Fusarium sp.* in 24h that the inhibition zone was smallest and measured about  $5.17\pm0.76$  mm (Table 2). The activity increased until culturing in 10 days, showing the highest activity. The inhibition zone was measured about  $17.47\pm0.50$  mm (Table 2).

OD value	24h	48h	72h	96h	120h	240h
modified ISP No.4	0.188	1.004	1.099	1.341	1.661	1.683
	0.188	1.004	1.099	1.339	1.661	1.684
	0.188	1.004	1.099	1.340	1.661	1.684
ISP medium No. 4	0.124	0.134	0.221	0.350	0.445	0.642
	0.125	0.134	0.222	0.350	0.446	0.643
	0.126	0.135	0.222	0.351	0.446	0.644

Table 1: The OD value of *Fusarium sp.* when cultured in different culture

#### Antimicrobial activity test

When culturing in modified ISP4 medium, *Fusarium sp.* had the antimicrobial activity to all strains of pathogens that were tested (Figure 1).



Figure 1: Illustrations for antimicrobial tests of *Fusarium sp.* using the supernatant extracted from *Fusarium sp.* culture against *Candida* albicans (P1), Staphylococcus sciuri (P5), Vibrio harveyi (VA) and Micrococcus luteus (P8)

In *Pseudomonas aeruginosa*, the weakest antimicrobial activity was expressed after culturing *Fusarium sp.* in 24h that the inhibition zone was smallest and measured about  $4.57\pm0.40$  mm (Table 2). The activity increased until culturing in 10 days, showing the highest activity. The inhibition zone was measured about  $16.67\pm0.58$  mm (Table 2).

In *Staphylococcus aureus*, the weakest antimicrobial activity was expressed after culturing *Fusarium sp* in 24h that the inhibition zone was smallest and measured about  $1.50\pm0.50$ mm (Table 2). The activity increased until culturing in 10 days, showing the highest activity. The inhibition zone was measured about  $14.43\pm0.55$ mm (Table 2).

In *Serratia marcescens*, the weakest antimicrobial activity was expressed after culturing *Fusarium sp.* in 24h that the inhibition zone was smallest and measured about  $2.83\pm0.29$ mm (Table 2). The activity increased until culturing in 10 days, showing the highest activity. The inhibition zone was measured about  $19.23\pm0.75$ mm (Table 2).

In *Micrococcus luteus*, the weakest antimicrobial activity was expressed after culturing *Fusarium sp* in 24h that the inhibition zone was smallest and measured about  $4.00\pm0.00$ mm (Table 2). The activity increased until culturing in 10 days, showing the highest activity. The inhibition zone was measured about  $26.67\pm0.58$ mm (Table 2).

In *Satphylococcus aureus*, the weakest antimicrobial activity was expressed after culturing *Fusarium sp.* in 24h that the inhibition zone was smallest and measured about  $2.17\pm0.29$ mm (Table 2). The activity increased until culturing in 10 days, showing the highest activity. The inhibition zone was measured about  $13.57\pm0.51$ mm (Table 2).

In *Vibrio harveyi*, the weakest antimicrobial activity was expressed after culturing *Fusarium sp.* in 24h that the inhibition zone was smallest and measured about  $3.33\pm0.58$ mm (Table 2). The activity increased until culturing in 10 days, showing the highest activity. The inhibition zone was measured about  $15.47\pm0.50$ mm (Table 2).

From the results of antimicrobial activities determined in different incubation time that was summarized in Table 2, the analysis of the effects of incubation time on the antimicrobial activities of *Fusarium sp.* was done (Figure 2).



Figure 2: Inhibition zone around the well resulted from antimicrobial test with different strains of bacteria

Table 2: Mean and Standard deviation of the inhibition zones of the antimicrobial tests

	Candida albicans (P1)	Salmonella typhi (P2)	Pseudomonas aeruginosa (P3)	Staphylococcus sciuri (P5)	Serratia marcescens (P6)	Micrococcus luteus (P8)	Staphylococcus aureus (P9)	Vibrio harveyi (VA)
24h	4.83±0.29	5.17±0.76	4.57±0.40	1.50±0.50	2.83±0.29	4.00±0.00	2.17±0.29	$3.33 \pm 0.58$
48h	13.17±0.76	9.50±0.50	9.03±0.15	6.33±0.76	7.83±0.29	13.67±0.58	5.33±0.58	5.93±0.31
72h	$18.00 \pm 0.00$	13.40±0.56	11.10±0.36	8.47±0.55	11.47±0.55	15.50±0.50	6.83±0.31	8.33±0.58
96h	19.50±0.50	14.33±0.58	14.17±0.76	9.73±0.25	12.50±0.50	19.10±0.46	8.00±0.00	11.67±0.58
120h	22.00±0.50	15.67±0.58	15.70±0.10	12.33±0.31	$16.00 \pm 0.00$	20.23±0.68	9.70±0.36	12.60±0.53
240h	23.50±0.56	17.47±0.50	16.67±0.58	14.43±0.55	19.23±0.75	26.67±0.58	13.57±0.51	15.47±0.50

From Figure 2, the results exhibited that antimicrobial activity of the *Fusarium sp.* continuously increased from 24 hours to 240 hours of culturing. This species showed the best antimicrobial activity at 240 hours with all types of pathogens.

### Antimicrobial test of the purified substance

Because *Fusarium* extract showed biological activity, the study tried to perform the preliminary purification for understanding about characteristics of the biological agents. Petroleum ether and chloroform fractions were collected, evaporated and dispersed into distilled water. The powders obtained in petroleum ether and chloroform fractions could be soluble in water. As a result, petroleum ether and chloroform fractions could contained certain compounds which were soluble in both polar and non-polar solvents.

In order to test which fraction contained the antimicrobial agent, the antimicrobial tests were done.

The partial purified substances in chloroform fraction had the antimicrobial activity with *Candida albicans (P1)* and *Salmonella typhi (P2)* (Figure 3). However, it showed no zone of inhibition with *Pseudomonas aeruginosa (P3)*, *Staphylococcus sciuri (P5)*, *Serratia marcescens (P6)*, *Micrococcus luteus (P8)*, *Staphylococcus aureus (P9)*, and *Vibrio harveyi (VA)*. The substance purified from petroleum ether did not have the antimicrobial activity with any pathogens when it showed no inhibition zone. Moreover, the supernatant after purification still had the antimicrobial activity when it exhibited the inhibition zone with all pathogens (Figure 4).



Figure 3: The zone of inhibition around the well exhibited the antimicrobial activity of purified substances against *Candida Albicans* (P1) and *Salmonella typhi* (P2)



Figure 4: Illustrations of the results of antimicrobial test of *Fusarium* using purified subtance (b) and the remaining supernatant (a<sub>1</sub>, a<sub>2</sub> and a<sub>3</sub>) after purification against *Staphylococcus aureus* (P9) and *Vibrio harveyi* (VA)

To easily observe the antimicrobial activities in different chloroform fractions, the analysis was summarized in figure 5.



Figure 5: Antimicrobial activity of purified substance and the supernatant after purification against *Candida albicans* and *Salmonella typhi* with different purification ratio using chloroform

From figure 5, it was seen that the inhibition zones which exhibited the antimicrobial activity of the purified substances became larger when the ratio chloroform: supernatant increase from (1:2) to (2:1). In contrast, the inhibition zones of the supernatant after purification become smaller when the ratio chloroform: supernatant increase with the same ratio.

Modified ISP4 medium was chosen for culturing *Fusarium sp.* because it is rich in nutrients. Modified ISP4 provided fungus with two important nutrient sources, carbon source (in dextrose, beef extract) and protein source (in beef extract and yeast). In addition, the pH of medium is 6.5, nearly equal to the pH required for culture fungi medium. *Fusarium sp.* showed the better growth in the modified ISP4 medium than ISP4. The modified ISP4 medium might be a good medium for *Fusarium sp.* growth that could be affected on the biological activities. Modified ISP4 medium was suitable for culturing *Fusarium sp.* in large scale.

From figure 1 and 2, it could be concluded that *Fusarium sp.* was able to produce substances with antimicrobial activity when cultured in modified ISP4 medium.

*Fusarium sp.* could not produce antimicrobial substances when culturing in ISP4. Probably, ISP4 was not a good medium for *Fusarium sp.* growth that affected on the secondary metabolites as well as antimicrobial agents. As showing in figure 2, more than one antimicrobial agents in *Fusarium* supernatant because the activities did not form a straight curve when culturing from 24h to 240h. Otherwise, with the curve shape, the antimicrobial activities were higher at 48 h, but lower at 72h in case of *Micrococus luteus*. In *Serratia*, the activity increased from 24h to 72h, then the activity looked stable until 120h, but sudden increased until 240h. It was meant that there was a complexity of antimicrobial activities in *Fusarium*. The similar phenomenon was also observed in other pathogen effects. To identify the antimicrobial compounds, more studies should be done so far.

Because the supernatant showed antimicrobial activity, it could be concluded that the purified substance was polar compound. During preliminary purification, after using petroleum ether extraction, the antimicrobial activity was not detectable. The supernatant layer stayed below the petroleum ether layer and above the chloroform layer. It could conclude that molecular weight of the supernatant lays between 86.16 (g/mol) and 119.38 (g/mol) because molecular weight of the supernatant is smaller than molecular weight of chloroform and larger than petroleum ether. Molecular weight of petroleum ether and chloroform alternately is 86.18 (g/mol) and 119.38 (g/mol). From figure 5, the results indicated that there are more than one substance with having antimicrobial activities against all pathogens even though the chloroform gave the antimicrobial activities against *Candida albicans* and *Salmonella typhi*. The more amount of chloroform, the more substances were purified. The inhibition zones were also greater. The inhibition zone of the supernatant became smaller each time the amount of chloroform solvent increase. It means the supernatant has lost substance during the purification using chloroform and that substance has laid in the chloroform layer.

#### CONCLUSION

This Fusarium sp. has strong antimicrobial activity when it exhibited the inhibition zone to all the pathogens that was tested (*Candida albicans, Salmonella typhi, Pseudomonas aeruginosa, Staphylococcus sciuri, Serratia marcescens, Micrococcus luteus, Staphylococcus aureus* and Vibrio harveyi). It can be a good source to produce antimicrobial agents.

With the results of purification step and antimicrobial step for purified substance, it can be concluded that the supernatant extracted from *Fusarium sp.* had more than one substance with antimicrobial activity.

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