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Potency and Activity of Secondary Metabolite of *Trichoderma harzianum* AC1(b) J2 inhibitor growth *Colletotrichum capsici* IPBCC 13.1098

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Abstract

Trichoderma harzianum is a fungus that can produce secondary metabolites which able to inhibit the growth of Colletotrichum capsici. a pathogenic fungus causing anthracnose in plants. the Aims of this research were to obtain and analyze the ability of secondary metabolites of Trichoderma harzianum AC1 (b) J2 isolated from Scleria poaeformis. to inhibit the growth of Colletotrichum capsici IPBCC13.1098. The secondary metabolite eluat of Trichoderma harzianum were tested for the antifungal activity toward Colletotrichum capsici IPBCC 13.1098. Fractionation was carried out by Column Chromatography and produced 31 eluat. The secondary metabolite eluat of Trichoderma harzianum with the highest inhibition zone diameter was eluat 1 with 8.4 mm in diameter. The secondary metabolite eluat with the highest value of inhibition zone was carried out by MIC test and thin layer chromatography (TLC). Based on the MIC results, the minimum inhibitory concentration of the secondary metabolite of Trichoderma harzianum to fungus Colletotrichum capsici was 250 ppm with inhibition zone diameter was 0.10 mm. The results of TLC showed orange spots on the TLC plate that indicated alkaloite compounds.

Keywords: Secondary metabolites. Trichoderma harzianum. Colletotrichum capsici. Alkaloite. Scleria poaeformis

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1. Introduction

Colletotrichum capsici is a pathogenic fungus causing anthracnose disease in chili [1]. The control of disease caused by *Colletotrichum capsici* still use on chemical fungicides, which have a negative impact on the environment such as the destruction of soil biota and the death of non-target organisms [2]. Therefore another alternative strategy is needed to control the plant diseases effectively, efficiently, and safely for the environment [3].

Biological control using antagonistic fungi is an alternative which is widely approved and used as a control of plant diseases[4]. One of the antagonistic fungi that can be used as the biological control agent is *Trichoderma harzianum*. *Trichoderma harzianum* able inhibits the growth of disease-causing pathogens in plants through mycoparasitic mechanisms [5].

The mechanism for controlling pathogens using *Trichoderma harzian* is a specific characteristic by colonizing the rhizosphere quickly and protecting the roots from attack by pathogenic fungus[6]. *Trichoderma harzianum* also has the ability to intervene hyphae. Hifa *Trichoderma harzianum* is able to attach hyphal pathogens and then entrap pathogenic hyphae, which results in damage to the hyphae wall resulting in changes in membrane permeability of hyphae, and has the potential to produce secondary metabolites to inhibit the growth of pathogenic fungus[7].*Trichoderma harzianum* can produce secondary metabolites such as azaphilone and butenolide as antifungal agents [8].

This research was conducted to determine the antifungal activity of the secondary metabolite fractions of *Trichoderma harzianum* against the *Colletotrichum capsici*. *Trichoderma harzianum* was isolated from plant *Scleria poaeformis*. *Plate* that grows in swamp areas along the Palembang-Indralaya road. Ogan Ilir Regency. South Sumatra. This research was conducted as the first step in obtain-

ing the secondary metabolite compounds of *Trichoderma harzianum* isolated from *Scleria poaeformis*. in inhibiting the growth of *Colletotrichum capsici.*

2. Materials and Methods

2.1 The Rejuvenation of *Trichoderma harzianum* AC1 (b) J2 and *Colletotrichum capsici* IPBCC 13.1098.

Trichoderma hazianum AC1BJ2 and *Collectotric humcapsici* IPBCC 13.1098 inoculated from the pure isolate stocks into test tubes containing PDA medium, then incubated at room temperature for 7 days. Work culture and stock culture grew on PDA medium [9]..

2.2 Conidia Suspension Making of *Trichoderma harzianum* AC1(b) J2

10 ml sterile distilled water added with Tween 80 (0.05%) solution, poured into a test tube containing the isolate of Trichoderma harzianum 24 hours old, homogenized with vortex mixer. 0.1 mL fungal suspension was taken by volumetric pipette and suspended into a tube containing 10 mL of distilled water then counted the number of conidia using a counting chamber, diluted untill the suspension density was ± 10^7 conidia/ml [10].

2.3 Cultivation and Extraction of the Secondary Metabolites of *Trichoderma harzianum* AC1(b)J2

The ± 10^7 conidia/ml suspensions of *Trichoderma harzianum* were transferred under a sterile condition to the PDB medium into 290 mL of Potato Dextrose Broth (PDB) medium. The cultures were incubated at room temperature for 30 days. Sterile PDB medium was used as a control. The fungal biomass and dried biomass were weighed [11].

The growth medium (PDB) of *Trichoderma harzianum* mixed with ethyl acetate solvent then formed 2 layers. Secondary metabolites layer concentrated using a rotary evaporator at 73 °C to obtain the thick extract of *Trichoderma harzianum* [12]

2.4 The Isolation of Secondary Metabolites of *Trichoderma harzianum* AC1(b)J2

Isolation of the secondary metabolites were carried out by column chromatography, silica gel powder 60F 254 merck poured into the column, stirred to avoid the air cavity in the middle of the column. The silica gel powder in the column reached three-quarters of the column height. Then added the Trichoderma harzianum extract.The n-hexane: ethyl acetate solvent with ratio of 100: 0, 95: 5, 90:10, 85:15, 80:20, 75:25, 70:30, 65:35, 60 : 40, 55:45, 50:50 as much as 100 mL were poured into the chromatography column in stages, with the column faucet opened, each separate unit stored in a vial per 10 mL, then evaporated using hair dryer until thick fraction was obtained. Each isolate will be analyzed for antifungal activity [13]

2.5 Secondary Metabolite Isolation Test *Fungi Trichoderma harzianum* AC1(b)J2 againts Colletotrichum capsici IPBCC 13.1098.

The *Colletotrichum capsici* suspension poured into a petri dish as much as 0.1 ml, then poured 10 ml Potato Dextrose Agar (PDA) and homogenized until the medium became solid. Paper discs with a diameter of 6 mm were saturated with 15µl solution of isolate secondary metabolites of *Trichoderma harzianum* placed on the surface of the medium, and incubated at room temperature for 7 days, observations carried out every day for 7 days. The parameter observed was the growth diameter of pathogenic fungi [14]

2.6 Determination of Minimum Inhibitory Concentration (MIC)

The secondary metabolite fraction of *Trichoderma harzianum* with the highest inhibition zone was selected to obtain the MIC determination. MIC determination was carried out by the diffusion method using paper discs with diameter of 6 mm. Fraction solution was made with concentrations of 2000, 1000, 500, 250, 125, 100, 75, and 50 ppm used DMSO as solvent, the control was dropped on paper discs and placed on the Potato Dextrose Agar (PDA) medium. The suspension of pathogenic fungi was poured into a petri dish as much as 0.1 ml, then poured 10 ml of Potato Dextrose Agar (PDA), the petri dish was shaken to be mixed perfectly. The saturated paper discs were placed into the top of culture medium and then incubated [15].

2.6 Thin Layer Chromatography

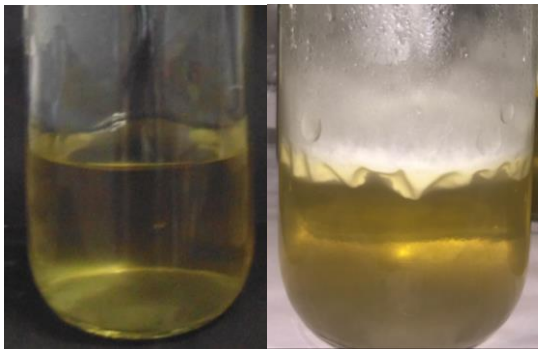
Secondary metabolites isolate of *Trichoderma harzianum* AC1 (b) J2 which used on the determination of MIC dissolved with ethyl acetate. The chromatogram was made a point at the end to drip extracts of secondary metabolites. The secondary metabolite extract was bottled at the point on the chromatogram, then moved by the mobile phase using eluents with various comparisons of ethyl acetate and n-hexane 2: 3, 3: 2, 1: 4, 4: 1 for 10 minutes. Eluent should not be higher than the point that is extracted from the extract on the chromatogram. Then the chromatogram was lifted and sprayed with 10% H2SO4, heated on a hot plate. The spots seen by using UV light 366 nm, and the Rf value was determined using this formula [16]

$$Rf = \frac{Distance\ travelled\ by\ component}{Distance\ travelled\ by\ solvent}$$

3. Results and Discussion

The research that has been done on the antifungal test of the metabolites of the secondary fungi *Trichoderma harzianum* AC1 (b) J2, against *Colletotrichum capsici* IPBCC 13.1098. beginning with the cultivation of *Trichoderma hazianum* AC1 (b) J2 fungi on the PDB medium (Potato Dextrose Broth). Cultivation it is carried out to produce secondary metabolites produced by the *Trichoderma hazianum* fungi. Secondary metabolites are compounds that are'nt directly involved in the process of growth and development of an organism [17]. Secondary metabolites play a role in a self-defense of an organism against pathogens, this is because secondary metabolites are generally toxic to several types of pathogens. The results of the cultivation of *Trichoderma hazianum* AC1 fungi (b) The initial medium volume is 300 mL after being cultivated for 30 days to become 267 mL with an extract weight of 11.2071 grams. The Reduced volume of medium because the fungus has carried out an adaptation process so that it can use the nutrients contained in the medium used. Adaptation can also harmonize the physiological position of fungi on the environmental conditions of the medium. The increase in the spread of fungal mycelium in the medium used can be caused because the fungus has been able to cope with the condition of the medium [18].

Production of secondary metabolites by fungi in the medium of PDB is characterized by changes in the color of the GDP medium. The change in the medium of PDB will be increasingly visible along with the increase in fungi cultivation found in the figure 1.



a b

Figure 1. Cultivation of *Trichoderma harzianum* AC1 fungi (b) J2 in PDB (Potato Dextrose Broth) medium (a) 1day, (b) 31 day

The change in the medium are caused because the fungi cultivated in the medium produces various kinds of secondary metabolites as a result of their metabolic processes, and secondary metabolites are produced by fungi at the end of the stationary phase. Secondary metabolite synthesis will begin when there is limited nutrition in the growth medium. then performed extraction of secondary metabolites using ethyl acetate solvents [19]. Ethyl acetate is a semi-solvent that can dissolve polar and non- polar compounds [20].

The results of the fractions obtained were 31 fractions with different solvent ratios. Fractionation was carried out aimed at separating compounds based on their level of polarity [21]. The results of the comparison of 31 fraction eluents are shown in Table 1.

Table 1. The comparison of eluen results in fractination of *Trichoderma hazianum* AC1(b)J2

Fraction	Solvent (mL)			Description (Color)
	N-Hexana	Ethyl acetate	Methanol	
1	100	0	0	Brown
2	95	5	0	Brown
3	90	10	0	Brown
4	85	15	0	Brown
5	80	20	0	Brown
6	75	25	0	Beige
7	70	30	0	Beige
8	65	35	0	Beige
9	60	40	0	Brown
10	55	45	0	Brown
11	50	50	0	Brown
12	0	0	100	Brown

Based on the Table 1. The eluent used in fractionation was started from an eluent which had a low polarity level then the eluent polarity level was increased slowly. The antifungal test results of the metabolites secondary to the *Trichoderma harzianum* AC1(b)J2 fungi against Colletotrichum capsici IPBCC 13.1098, . and Positive control test in the form of antibiotic nystatin and negative control were presented in figure 2.

The biggest inhibition zone diameter is seen in fraction 1 which is 8.4 mm, while inhibition zone diameter in fraction 2 has 1.8 mm diameter inhibition zone, fraction 3-21 does not have inhibitory zone diameter, fraction 22 has inhibitory zone diameter of 4.1 mm., and the fraction 23-31 does not have a diameter of the inhibition zone. the diameter of the inhibitory zone formed on the test medium

was due to the diffusion of active compounds from secondary metabolites on paper disks to the medium. The inhibition zone is a zone that is not overgrown by test fungi, this is because secondary metabolites are tested that can inhibit the growth of the tested fungi. Antifungal compounds can cause damage to fungal cell walls, change the membrane membrane stability, inhibit fungi cell protein synthesis which causes inhibition of the growth of these fungi or even cause death in these fungi. The compounds in fraction 1 have the highest antifungal effectiveness when compared with other secondary metabolites .

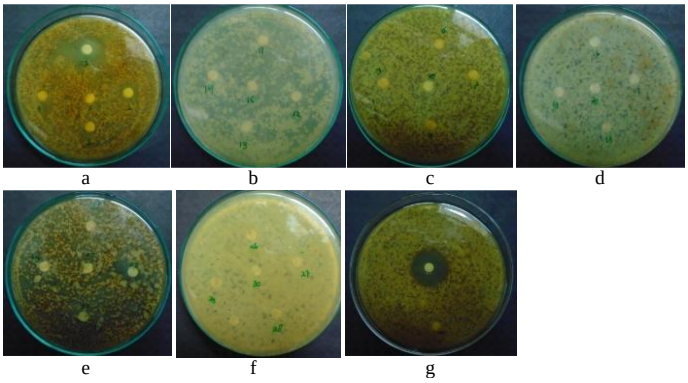


Figure 2. Test of the activity of compounds Secondary Metabolites Fungi *Trichoderma harzianum* AC1 (b) J2 against *Colletotrichum capsici* IPBCC 13.1098. and Positive control test in the form of antibiotic nystatin and negative control.(a) fraction 1-5, (b). fraction 6-10, (c). fraction 11-15, (d). fraction 16-20, (e). fraction 21-25, (f).fraction 26-31, (g). Positive control test in the form of antibiotic nystatin and negative control

The biggest inhibition zone diameter is seen in fraction 1 which is 8.4 mm, while inhibition zone diameter in fraction 2 has 1.8 mm diameter inhibition zone, fraction 3-21 does not have inhibitory zone diameter, fraction 22 has inhibitory zone diameter of 4.1 mm., and the fraction 23-31 does not have a diameter of the inhibition zone. the diameter of the inhibitory zone formed on the test medium was due to the diffusion of active compounds from secondary metabolites on paper disks to the medium. The inhibition zone is a zone that is not overgrown by test fungi, this is because secondary metabolites are tested that can inhibit the growth of the tested fungi. Antifungal compounds can cause damage to fungal cell walls, change the membrane membrane stability, inhibit fungi cell protein synthesis which causes inhibition of the growth of these fungi or even cause death in these fungi. The compounds in fraction 1 have the highest antifungal effectiveness when compared with other secondary metabolites .

The diameter of inhibition zone formed correlates with the effectiveness of a compound as an antifungal. The greater the inhibition zone diameter of a secondary metabolite compound, the higher the activity of the secondary metabolites as antifungal. While in the positive control in the form of antibiotic nystatin, the diameter of the nystatin inhibition zone against the *Colletotrichum capsici* IPBCC 13.1098. is 12.8 mm. given the symbol + (positive) and is located in the middle of the test medium.

The biggest inhibition zone diameter is seen in fraction 1 to *Colletotrichum capsici* IPBCC 13.1098. which is 8.4 mm. Based on these results, the secondary metabolites of Trichoderma harzianum isolates in fraction 1 were selected for MIC testing.

The results of determining the MIC of metabolites of the secondary Trichoderma harzianum fungus against Colletotrichum capsici IPBCC 13.1098. presented in Table 2. It shows a decrease in the diameter of the inhibition zone along with a decrease in the concentration of the compound.

Table 2. Inhibitory Zone Diameter and Minimum Inhibitory Concentration of MIC Secondary Metabolic Compounds Fungi *Trichoderma harzianum* Against *Colletotrichum capsici* IPBCC 13.1098.

No	Concentration (ppm)	Inhibitory Zone Diameter (mm)
1	2000	0,76 mm
2	1000	0,40 mm
3	500	0,20 mm
4	250	0,10 mm
5	100	0
6	75	0
7	50	0

Testing of the metabolites of the secondary fungi *Trichoderma harzianum* against Colletotrichum capsici IPBCC 13.1098. carried out with the highest concentration of 2000 ppm. The concentration is diluted to half to a concentration of 50 ppm πg/mL. The diameter of the inhibition zone formed at a concentration of 2000 ppm was 0.76 mm, the concentration of 1000 ppm formed a inhibition zone of 0.40 mm, a concentration of 500 ppm was 0.20 mm, the concentration of 250 ppm was 0.10 mm. The concentration of compounds lower than 250 ppm cannot produce a zone of inhibition of the *Colletotrichum*

capsici IPBCC 13.1098 fungus and is presented in Picture 3

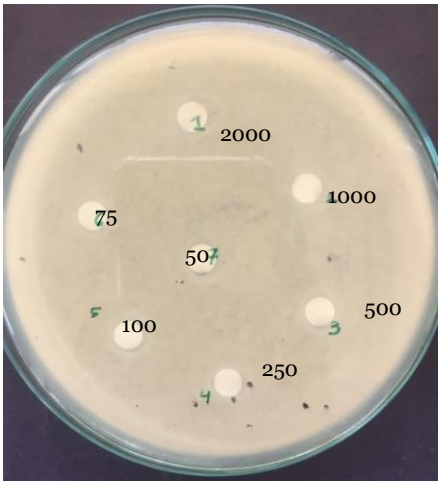


Figure 3. KHM Test Results of secondary metabolites of *Trichoderma harzianum* against *Colletotrichum capsici* IPBCC 13.1098. with a concentration of 2000 ppm, 1000 ppm, 500 ppm, 250 ppm, 100 ppm, 75 ppm, 50 ppm.

The smallest concentration of the secondary metabolite compound of the *Trichoderma harzianum* which still formed an inhibition zone to *Colletotrichum capsici* was 250 ppm with diameter of 0.10 mm. MIC value of the secondary metabolite compound of Trichoderma harzianum against *Colletotrichum capsici* was 250 ppm. Minimum Inhibitory Concentration is the lowest concentration of a secondary metabolite compound that still inhibits the growth of *Colletotrichum capsici* IPBCC 13.1098.

Decreasing the concentration of compounds will affect the diameter of inhibition zone formed. The lower the concentration of secondary metabolites used, the smaller the inhibition zone formed. Based on [22] the decrease in concentration of a compound influences the diameter of the inhibitory zone formed.

Fraction 1 with the largest inhibitory zone diameter was tested by Thin Layer Chromatography (TLC). The results of TLC is shown in Figure 4.

Based on the results of the TLC showen in Figure 4. Secondary metabolite compounds fraction 1 Trichoderma harzianum has 1 brown stain spot after sprayed by H_2 SO_4and heated by hot plate. Thin Layer Chromatography (TLC) used to determine the group of compounds contained in the fraction [23]. In addition, TLC was also conducted to obtain the purity of compounds by looking at the spots on the TLC plate [24]. The fraction 1 had brown spot color indicated tannin compound, tannin can change the permeability of fungal membrane cells, the change of membrane cell permeability will inhibit the metabolic process of these fungal cells. Based on [25] tannins have antifungal activity by inhibiting chitin synthesis used for wall formation cells in fungi and damage cell membranes so that the growth of fungi is inhibited.

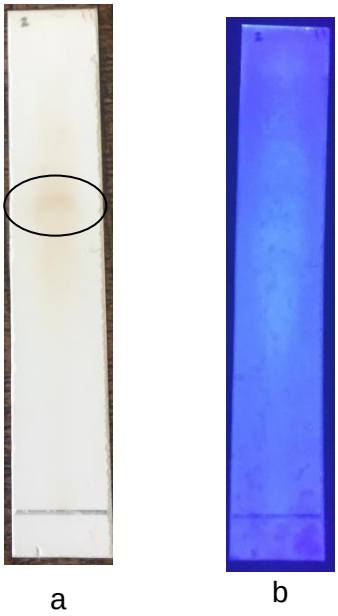


Figure 4. TLC result of secondary metabolites of fraction 1 has 1 brown stain spot, (a) on the hotplate, (b) in the UV light

Based on the results of the TLC, there was only one spot color on TLC of fraction 1. The stain color that appears on the TLC plate shows the class of compounds found in the analyzed fraction. The Rf value and spot color of the fraction 1 of the secondary metabolite compound of *Trichoderma harzianum* presented in Table 3. According to [26] the tannin compound browned on the TLC plate.

Table 3. Rf value of secondary metabolite compound of fraction 1

No	Fungi	Fraction	Rf value	spot color	compound
1	*Trichoderma harzianum*	1	0,75	brown	Tannin[26]

4. Conclusion

Based on the results of research that has been done, it can be concluded that :

1. *Trichoderma harzianum* AC1 (b) J2 compound has anti-fungal activity against *Colletotrichum capsici* IPBCC 13.1098, with the highest inhibition zone of 8.4 mm in fraction 1.
2. The minimum of inhibitory concentration (MIC) test results in fraction 1 showed that fraction 1 could inhibit up to a concentration of 250 ppm.

3. The secondary metabolite group *Trichoderma harzianum* AC1 (b) J2 which acts as an antifungal in fraction 1 are a tannin compound with brown spots.

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