Conference Paper

Extraction and Toxicity Assay of Mycotoxin from Entomopathogenic Fungi Isolate of Kusuma Agrowisata Orchard Batu, Jawa Timur, Indonesia

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Abstract

Insect pest control has been a major problem faced by farmers around the world and there has been no perfect solution to handle it. The use of insecticides derived from chemically active compounds has made negative impacts such as the emergence of resistant pest and disruption of ecosystems. In addition, it raised many other negative impacts on the environment and on non-target organisms. Therefore, an alternative is needed to replace chemical insecticides. Entomopathogenic fungus is one of the potential biological control organism to suppress the growth of insect pests. This study aims to extract mycotoxin from entomopathogenic fungi isolated from the apple orchard at Batu, East Java, Indonesia and determine the level of toxicity against Tenebrio molitor larvae. Mycotoxin was extracted using precipitation with ammonium sulphate and dialysis. It was subjected to molecular weight analysis and toxicity test to determine the LC50 value. Results showed that protein bands were found between 20 kDa to 55 kDa. Based on the study, the mycotoxin showed a LC50 at a concentration of 42.78 $\mu g \cdot mL^{-1}$.

Keywords: Entomopathogenic fungi, mycotoxin, *Tenebrio molitor* Linn.

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1. Introduction

Plant pest is one of the important biotic constraints in the cultivation of various crops. Farmers who apply conventional way of farming often use synthetic insecticides to control pest that attacks their crops [1]. Synthetic insecticides are known to have many advantages which are effective at low doses, gives quick result and it can be used in a variety conditions and economical [2]. Besides having many advantages, synthetic insecticides also can lead to various negative impacts, including impact on environmental conservation with the murder of an organism that not the targets which is located within or near the site of application including natural enemies of pests, resistance and resurgence of pests and the emergence of secondary pests and the consumers [3].

One alternative of pest control which can be used to overcome the shortcomings of synthetic insecticides is entomopathogenic organisms [4]. One example of entomopathogenic organisms is fungi capable of infecting various types of pests from several orders by infiltrating into the host insect's body through the skin, gastrointestinal tract, spiracles and other openings [5]. The fungal inoculum which is attached to the body of the host insect will germinate and grow to form a tube sprouts, then enter the body through the skin. Penetration into the body of the insect cuticle is done mechanically and / or chemically with enzymes or toxins. Fungi will develop in the body and invade host tissues throughout the body, causing the insects to die. Fungi mycelia penetrate outside the host body to grow over the host body and produce conidia. These fungi also contain extreme toxic mycotoxin to kill the target insects in just a short time span ranging from 3 d to 5 d after application [6, 7]. These fungi are capable of infecting various stages of insects, including larvae and imago [8]. Some types of fungi entomopathogenic already known to effectively control important pests of plants are *Beauveria* sp., *Metarhizium anisopliae*, *Nomuraea rileyi*, *Paecilomyces fumosoroseus*, *Aspergillus parasiticus*, and *Verticillium lecanii*. *Beauveria* sp. is reported as a highly effective biological agent that can infecting some insect pests, especially of the Lepidoptera, Hemiptera, Homoptera and Coleoptera order [9]. As an insect pathogen, *Beauveria* sp. can be isolated from the crop or the ground. In general, entomopathogenic fungi prefers cold areas with high humidity, such as Malang and Temanggung [10].

Batu, East Java, Indonesia is a plateau area with humid and cold environment. Batu area tends to be at moderate to cold condition with temperature between 22 °C to 26.2 °C This climatic condition is influenced by the altitude of the region Batu ranging from 600 ASL to more than 3 000 ASL. Such a climate is suitable for apple plantation that requires humid and cold condition as well as for the growth of entomopathogenic fungi, one of which is *Beauveria* sp. In previous studies [11]. In this work, mycotoxin from entomopathogenic fungi isolated from apple orchard Batu was extracted and tested for toxicity assay.

2. Materials and methods

2.1. Production of Mycotoxin by Entomopathogenic Fungi

Ammout of 1 mL of hyphae inoculum of fungi in PDA medium with a number of conidia 107 conidia/mlis inoculated aseptically into a sterile, 250 mL erlenmeyer flask containing 100 mL of Potato Broth + Yeast extract, peptone and glucose. The culture was then incubated at 28 °C for 8 d at 110 rpm (mycotoxins produced at stationary phase) (1 rpm = 1/60 Hz).

2.2. Precipitation with Ammonium Sulphate

Potato Broth + Yeast extract, peptone and glucose medium were separated from the mycelium by filtration using filter paper. The medium is centrifuged at 10 000 g for 30 min at 4 °C, the obtained supernatant was called as crude extract. Then crude extract

gradually was precipitated at a temperature of 4 °C with ammonium sulfate with saturation level of 50 %, 70 % and 90 %. In the precipitation process, ammonium sulphate was added slowly until dissolved using a magnetic stirrer, followed by centrifugation at 10 000 g for 30 min at 4 °C. Supernatant obtained was used for the next precipitation, while the pellet was dissolved in buffer Tris / HCl pH 8 for dialysis.

2.3. Protein determination

Protein concentration incrude extract and each fraction was measured using Bradford method.

2.4. Toxicity assay

Crude extract and each fraction were subjected to toxicity assay against F1 stage 3 *Tenebrio molitor* larvae according to Wartono and Tri Puji [12]. Thirty larvae were placed on a petri dish covered with filter paper. Then, 1 mL suspension of protein samples from crude extract and each fraction were sprayed in the same amount into the petri. Mortality of larvae was observed every day for 48 h. After observation on larvae mortality, the LC50 value was determined. Toxicity testing was conducted using a negative control and a positive control. For negative control, sample was replaced by distilled water at the same volume and for the positive control, sample was replaced with chemical insecticides solution at the same concentration. The test was done with three replications.

2.5. Molecular weight determination (SDS PAGE)

2.5.1. Gel preparation

Separating gel was made by mixing 3 125 mL of 30 % acrylamide stock solution and 2.75 mL of 1 M Tris pH 8.8 into a beaker glass. Then 1 505 mL of double distilled water, 75 µL of 10 % SDS, 75 µL of 10 % APS and 6.25 µL of TEMED were added one by one and homogenized. Stacking gel that made in the same way with the following composition: 0.45 mL of 30 % acrylamide stock solution, 0.38 mL 1 M Tris pH 6.8, 2.11 mL of double distilled water, 30 µL of 10 % SDS, 5 µL of TEMED and 30 µL of 10 % APS.

2.5.2. Sample preparation

Sample solution and loading buffer was mixed at the ratio 1:1. The mixture wasthen heated at a temperature of 100 °C for 5 min.

Figure 1: Purified fungi on Potato Dextrose Agar (PDA) medium.

2.5.3. Gel running, staining and destaining

A volume of 10 to 20 µL sample was loaded into the wells (protein in the sample should be about 20 µg to 40 µg). Voltage of 20 mA was applied. After the running proccess finished, gel was stained with staining solution consisting of 1 g Coomasive Blue R- 250, 450 mL of methanol, 450 mL of distilled water and 100 mL glacial acetic acid. Staining was carried out for 15 min while being shaken. Destaining was done for 30 min in the same way using a mixture of 100 mL methanol, 100 mL of glacial acetic acid and 800 mL of distilled water.

3. Results

3.1. Confirmation of Entomopathogenic fungi isolated from Kusuma Agrowisata apple orchard

Based on previous research, two different colonies of fungi were obtained using insect bait trap. From the morphology of the fungi colonies on PDA medium, the white colonies resembled chalk and the purple colony resembled cotton. Each separate colony was taken and inoculated into PDA medium (incubation at room temperature for 4 d) to obtain pure fungi colony (Figure 1).

To test the ability of the fungi as potential bioinsecticide, ten *Tenebrio molitor* larvae are put in a petri containing the isolated fungi so entomopathogenic fungi can infect the larvae. Based on the results, colony 1 (K.1) caused fungal infection with mortality rate of larvae at 100 % after 72 h. In the entomopathogenic fungal infections, the larvae body was covered with fungi mycelia (Figure 2).

Then, the mortality assay was continued to test the entomopathogenic ability of the colony 2. Based on the results of fungal infections after 72 h, colony 2 (K.2) did not cause death to any of ten *Tenebrio molitor* larvae (mortality rate was 0 % as seen in

Figure 2: Fungal infection of samples of fungi isolates K.1 to *Tenebrio molitor* larvae.

Figure 3: No fungal infection of samples fungi isolates K.2 observed to *Tenebrio molitor* larvae.

Figure 3). This indicated that the colony K.2 does not possess the entomopathogenic ability.

Sample	Volume (mL)	Protein (mg · mL^{-1})	Total Protein (mg)
Crude Extract	6.0	0.95 ± 0.02	5.70 ± 0.11
50 %-Fraction	6.0	0.40 ± 0.28	2.39 ± 0.17
70 %-Fraction	6.3	0.51 ± 0.01	3.23 ± 0.2
90 %-Fraction	6.0	0.28 ± 0.01	1.70 ± 0.06

Table 1: Protein concentration of different fractions.

Sample	Total number of dead insect	Protein concentration (µg · mL^{-1})
Crude Extract	30.00 ± 0.00	200
50 %-Fraction	19.67 ± 0.58	200
70 %-Fraction	30.00 ± 0.00	200
90 %-Fraction	15.33 ± 2.08	200
Supernatant-Fraction	2.33 ± 1.15	183.15

Table 2: Toxicity against *Tenebrio molitor* larvae.

3.2. Fractionation/precipitation and dialysis

Fractionation of toxin from colony fungi 1 (K.1) was done by adding ammonium sulphate at 50 %, 70 % and 90 % saturation degree and the data are presented in Table 1.

3.3. Toxicity assay and lethal concentration$_{50}$

Toxicity assay results are presented in Table 2. The toxicity assay is to demonstrate the existence of insecticidal activity in the mycotoxins.

Based on the results of toxicity assay, it is shown that both mycotoxin from crude extract and dialysed 70 %-fraction caused 100 % mortality of the larvae *T. molitor*. Therefore, crude extract was then selected to be used to determine the LC50 with Range-Finding Test and Definitive Test. In Range-Finding Test as a preliminary test of LC50 was done with a concentration range about 12.5 µg · mL^{-1}, 25 µg · mL^{-1}, 50 µg · mL^{-1}, 100 µg · mL^{-1}. The results are presented in Table 3.

Based on the result as seen in Table 3, dead larvae was found in the negative control, so the mortality data are corrected by using the Abbot formula [13] in order to obtain corrected mortality (Table 4).

Dilution of crude extract	Total insect mortality	Protein (µg · mL^{-1})	Percent (%)
Chemical Insecticide (positive control)	30.00 ± 0.00	-	100
Distilled Water (negative control)	1.00 ± 0.00	-	3.33
2x	28.00 ± 2.64	100.0	93.33
4x	18.67 ± 2.08	50.0	62.22
8x	7.67 ± 0.58	25.0	25.56
16x	4.00 ± 1.00	12.5	13.33

Table 3: Range-finding test.

Protein (μg · mL^{-1})	100	50	25	12,5
Percent mortality (%)	93.33	62.22	25.56	13.33
Corrected mortality (%)	90.00	58.89	22.23	10.00

Table 4: Corrected mortality on range-finding test.

Protein concentration of crude extract (μg · mL^{-1})	Total insect mortality	Percent (%)
25	7.67 ± 0.58	25.56
30	10.00 ± 1.00	33.33
35	13.33 ± 1.53	44.44
40	15.67 ± 1.15	52.22
45	16.67 ± 1.53	55.56
50	18.67 ± 2.08	62.22

Table 5: Narrowed definitive test.

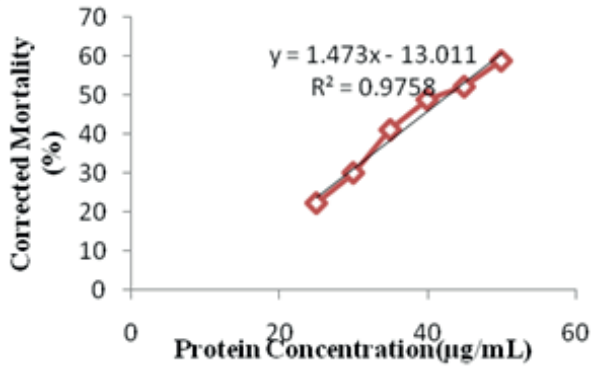

Figure 4: Relationship between protein concentration against corrected mortality.

Range-Finding Test result indicated that the potential value of LC50 is at concentration between 25 μg · mL^{-1} and 50 μg · mL^{-1}. Therefore, the range of concentrations used for narrowed Definitive Test between 25 μg · mL^{-1} and 50 μg · mL^{-1}. The result of narrowed Definitive Test is presented in Table 5.

Due to the larvae mortality found in the negative control amounted to 3.33 %, the mortality data was corrected using Abbot formula [13] in order to obtain corrected mortality (Table 6). The data are then analysed using linear regression analysis.

The regression equation derived from the analysis is Y = 1,473X + 13,011. From the regression equation, the protein concentration of a toxin able to cause mortality of larvae as much as 50 % (LC50) is predicted to be 42.78 μg · mL^{-1}.

Protein concentration (μg · mL^{-1})	25	30	35	40	45	50
Percent mortality (%)	25.56	33.33	44.44	52.22	55.56	62.22
Corrected mortality (%)	22.23	30.00	41.11	48.89	52.23	58.89

Table 6: Corrected mortality on definitive test.

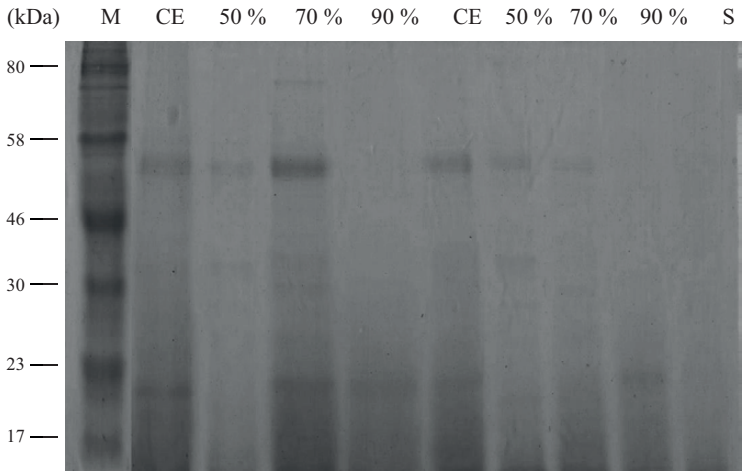

Figure 5: SDS PAGE Result: Protein Marker (M), Crude Extract (CE), 50%-Fraction (50 %), 70 %-Fraction (70 %), 90 %-Fraction (90 %), and Supernatant (S).

3.4. Determination of Protein Molecular Weight by SDS PAGE

SDS PAGE results can be seen in Figure 5. Crude extract and 70 %-Fraction showed a protein band of the same size (55 kDa). It is also indicated that the 50 %-Fraction, which also has a protein band 55 kDa, provided toxicity effect with larval mortality greater than the 90 %-Fraction, which did not show the protein band of 55 kDa.

4. Discussion

4.1. Confirmation of fungi Entomopathogenic isolated from Kusuma Agrowisata apple orchard

In this study, fungus used in the production of mycotoxins was isolated of the Kusuma Agrowisata Apple Orchard, Batu, East Java, Indonesia, with temperatures around 24 °C. Soil samples were used to infect the larvae of *Tenebrio molitor* at room temperature and dark condition by means of insect baiting traps where 10 T. *molitor* larvae buried in soil samples and incubated at humid conditions for 2 wk. Infected larvae died with the characteristics of the body covered with fungi mycelia. This is in accordance with the characteristics of the larvae infection by *Beauveria* sp. [14]. Enrichment was done by inoculating these fungi in Potato Dextrose Agar (PDA) as general medium for fungi. After incubation at room temperature for 4 d, two different colonies were observed to grow with different morphology.

Each colony of fungi is inoculated back to the new PDA medium in order to obtain pure colonies. This is done to further testing of the colony which is entomopathogenic. The colonies were labeled with K.1 and K.2, respectively. Furthermore, 10 *T. molitor* larvae were put in petri dish containing each type of colony and it was observed that only colony K.1 caused significant mortality of *T. molitor* larvae where the larval body covered mycelia. In addition, the morphology of fungi K.1 has the appearance of white with chalk texture similar to that as reported by Utami [15], where the

entomopathogenic fungi *Beauveria bassiana* isolated from Malang and Magetan districts were found able to infect *P. xylostella*.

4.2. Extraction, toxicity assay and determination of Lethal Concentration (LC50) of Mycotoxins from Entomopathogenic fungi

Mycotoxin production was done by inoculating the entomopathogenic fungi in suitable medium (Potato Broth with the addition of 4 % of glucose, 4 % of yeast extract and 3 % of peptone). Glucose is the optimal carbon source and peptone is the optimal organic nitrogen source to increase the production of mycotoxins [16]. Separation of mycotoxin from the mycelia was done with centrifugation, leaving the mycotoxin dissolved in the supernatant. This supernatant is called crude extract. The harvested crude extract was then subjected to precipitation by ammonium sulfate addition with saturation level of 50 %, 70 % and 90 % and finally was subjected to the subsequent dialysis to remove any remaining salt ammonium.

Toxicity assay is done by spraying mycotoxins to 30 *Tenebrio molitor* larvae placed on a petri dish. The results showed that the crude extract showed comparable toxicity as the supernatant from 70 % saturation degree of ammonium sulphate precipitation did, giving the highest mortality of the larvae (100 % mortality). Therefore, LC50 will be determined from the crude extract. Larvae experiencing mortality at 48 h after treatment was characterized by changes in body color to black and also shriveling of the larval body, probably due to lysis process [17].

Toxicity assay was also done for the supernatant aimed to confirm that insecticidal activity was really due to the protein and not the micromolecule or secondary metabolites. Based on the results obtained, the supernatant did not show insecticidal activity nor larval mortality. According Vey et al. [18], mycotoxin from entomopathogenic fungi showing insecticidal effects are mostly proteins or peptides cyclic. Moreover, according to Quesada-Moraga and Vey [19], purified mycotoxin from entomopathogenic fungi resulted in protein macromolecules with insecticidal activity.

Range-finding test showed that potential LC_{50} value of the mycotoxin is between 25 $\mu g \cdot mL^{-1}$ and 50 $\mu g \cdot mL^{-1}$. Definitive test that narrows the range of the results of the range-finding test finally resulted in an LC50 value to be 42.78 $\mu g \cdot mL^{-1}$. According to Genthner et al. [20], LC_{50} mycotoxin produced from entomopathogenic fungi *Beauveria* sp. was amounted to 84.2 $\mu g \cdot mL^{-1}$. Murugesan et al. [21], also reported that mycotoxin fungi *Trichophyton mentagrophytes* showed an LC50 value of 110 $\mu g \cdot mL^{-1}$. Although LC50 value of the chemical insecticide used in this work was less than 1 $\mu g \cdot mL^{-1}$, the use of mycotoxins as bioinsecticide is still potential because it has advantages over chemical insecticides as it is safer for humans and other non-target organisms [22].

According Natawigena [23], the mechanism of resistance in insects may be given by the morphological, physiological and biochemical aspects. Morphologically and physiologically, insects possess different cuticles or barriers such as fur and have different speeds in deciphering insecticide. Biochemically, the enzyme in the body of the insect is capable of performing the proccess of inactivation of the active substance.

4.3. Determination of Protein Molecular Weight by SDS PAGE

In Figure 5, it appears proteins in the extract are of around 20 kDa to 55 kDa. Based on the results of toxicity assay, most likely mycotoxin size is about 55 kDa. Since that fraction showed the highest mortality of *T. molitor* larvae.

Many researchers have worked on the activity study of insecticidal entomopathogenic fungi and found diverse compounds, among others: Beauvericin - MW 28 kDa [24], destruxins - MW 22 kDa) [25], bassianin - MW 18 kDa; bassianolide – MW 390 kDa; beauverolides - MW 72 kDa [18], tenellin - MW 60 kDa [26], and oosporein - MW 83 kDa [27]. There are still other mycotoxin from entomopathogenic fungi that has not been identified by name as researched by Meng et al. [28], with a size of about 50 kDa derived from *Beauveria* sp.

Based on the results of SDS PAGE, we found protein bands around 20 kDa, 36 kDa, and 55 kDa. The optimal insecticidal activity is observed by the band of around 55 kDa. Similar finding was published by Cheong [29] where *Beauveria bassiana* produced mycotoxins which MW of 21 kDa to 55 kDa and the protein with a band measuring approximately 55 kDa had been identified to have potential insecticidal activity.

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