Type of the Paper (Article)

The effects of natural biopesticide from *Mirabilis jalapa* toward the immune system of *Spodoptera litura*

Dina Maulina[1,2,*], Sutiman Bambang Sumitro[3], Mohamad Amin[1,*], Sri Rahayu Lestari[1], Muhammad Aziz[4], Tjandra Anggraeni[5]

1Postgraduate Program of Biological Education, State University of Malang, East Java, Indonesia
2Biology Education, Faculty of Teacher Training and Education, Lampung University, Lampung, Indonesia
3Biological Department, Brawijaya University, East Java, Indonesia
4Institute of Innovative Research, Tokyo Institute of Technology, Tokyo, Japan
5School of Life Sciences & Technology, Bandung Institute of Technology, West Java, Indonesia
*Corresponding authors: dina.maulina@fkip.unila.ac.id (D. Maulina); Tel.: +6285321139985; mohamad.amin.fmipa@um.ac.id (M. Amin); Tel.: +6282142262999

Abstract: Biological control provides a safer alternative to reduce the population of agricultural pest. *Mirabilis jalapa* is one of biopesticides containing chemical substances that have a feeding deterrent property against *Spodoptera litura* as folifagus insect pest. This study aimed to analyze the humoral and cellular immunes responses of *S. litura* after exposure to biopesticide extracted from *M. jalapa*. The measured indicator immune responses were activity of hemocyte, lectin, phenoloxidase (PO), and phagocytic activity. The results showed that the average total hemocyte was different significantly depending on the treatment. Exposure to 0.1% and 0.2% (w/v) of *M. jalapa* extract increased the total number of hemocytes as much as 38.08% and 64.15%, respectively. Lectin was quickly formed at 0.1% and 0.2% (w/v) concentrations. The amount of PO enzymes was significantly different at sublethal concentrations compared with control samples ($P < 0.05$). The highest increase in PO activity occurred at 2 h post-treatment and at *M. jalapa* extract concentrations of 0.2% (592.33 IU/mg) and 0.1% (521.33 IU/mg), whereas the highest concentration of the extract (0.8% w/v) caused a decrease in lectin and PO activities. In terms of phagocytic activity, the proportion of phagocytosis cells were 47.62% in control group, and decreased significanlty in both concentrations exposure.

Keywords: immune response, lectin, *Mirabilis jalapa,* phagocytic activity, phenoloxidase, *Spodoptera litura*

1. Introduction

Humans demand high quality and sufficient quantity of food from agricultural production. However, agricultural pests are an obstacle to food production worldwide, and they have become increasingly resistant to a variety of insecticides [1–4]. Unfortunately, most chemical insecticides are very dangerous, and their use is not recommended by environmentalists because an increase in the doses potentially harms non-target organisms. Therefore, botanical insecticides are viewed as a potential alternative to controlling in the pest population.

Botanical insecticides, which are derived from natural substances extracted from plants, are usually safe for the environment [2, 5, 6]. Their applications leave no chemical residues that can harm non-target organisms, such as humans, and the environment [7, 8]. One potential botanical insecticide for insect pests is the plant extract from *Mirabilis jalapa* (four o'clock flower) [9, 10]. This plant contains

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 8 April 2018 doi:10.20944/preprints201804.0089.v1

antiviral and antiviroid compounds belonging to the ribosome inactivating protein family, also known as the Mirabilis antiviral protein (MAP) [11]. However, evaluation of their efficacy is needed to prevent the occurrence of target pest resistance. A laboratory test using *M. jalapa* extract as a biopesticide showed an LD_{50} of 0.8% for *Spodoptera* [10]. The application of sublethal concentration of *M. jalapa* has also been tested with the expectation that the target pest could eventually become resistant to higher doses. The main objective in the application of *M. jalapa* as a biopesticide is to weaken the immune system of the pest.

Spodoptera litura (tobacco cutworm) is one of the most dangerous pests in agricultural crops. It can lead to up to 100% defoliation in crops [12]. The resistance of *Spodoptera* to various chemical compounds needs to be monitored because these pests are quickly spreading across the Asian and South Pacific regions [13]. Therefore, it is extremely urgent to control these pest populations using alternative methods, particularly plant-based biopesticides. To control the pest population, its immune system must be clearly understood. In this study, we evaluated the potential of a biopesticide extracted from *M. jalapa* to control the insect pest *S. litura* by weakening its immune system.

The main role of *M. jalapa* as a biopesticide is to weaken the *Spodoptera* immune system. The immune defense mechanism acts as a barrier to infections when exposed to foreign agents; it biochemically responds when attacked by a foreign agent. Therefore, the state of the immune system can be used as an indicator of the potential for pest mortality.

Generally, insects have both cellular and humoral immune defenses. Humoral and cellular response mechanisms cannot be distinguished; both stimulate each other to exert their activity [14, 15]. Mechanisms of cellular immune systems in insects are always characterized by hemocytic activity. Hemocyte acts as the main subject of cellular immunity to recognize, tolerate and eliminate the presence of foreign in the body. The insect's immune system conditions will be activated when exposed by foreign substances. The hemocyte is working to eleiminate foreign by the phagocytic. However, each type of hemocyte has a different function in insect defense. The whole function of the cells will work together doing the phagocytosis mechanism. Thus, activity of hemocyte and phagocytic are main role in cellular mechanisme as active (amoboid) cells. Therefore, phagocytosis becomes one of the parameters in the cellular defense mechanism. A test of the effectiveness of phagocytosis from insect larvae to determine changes in the immune response that occur in the body of the insect needs to be done when exposed by a toxic substance.

The humoral response is a very crucial part of the insect immune system because it plays a role in activating protective enzymes and stimulating the ability to recognize pathogenic invaders. Therefore, this humoral mechanism acts to stimulate the functioning of the immune system and therefore, the humoral response is one of the parameters of the immune system which can kill the infecting pathogen.

Previous research on pest control using biological agents has been widely conducted, including the study of UTI viruses [16], bacteria [17], natural predators [18] and various natural compounds [19]. These studies mostly evaluated the effectiveness of a biological agent during its application or introduction, especially in determining the magnitude of pest mortality. The lethal concentration of a biopesticide for pest control impacts the time required by a population to become resistant to it, leading to an eventual resurgence of the pest's population once it has acquired resistance. Unfortunately, most biopesticides are considered to be harmful to the ecosystem if applied over a relatively long time period. Therefore, when using a natural compound as a biopesticide, the concentration are very important parameters when trying to deter pest resistance. However, to the best our knowledge, there have been no previous studies examining the use of natural compounds in *M. jalapa* to weaken the pest immune system to decrease immunity cause mortality. We believe that this approach can potentially provide an ecofriendly way to control pest outbreaks because the use of sublethal pesticide concentrations is feasible for farmers.

The aim of this research was to analyze the effect of *M. jalapa* leaf extract on the immune defenses of *S. litura*. Our observations focused on the cellular and humoral immune responses as indicated by acrivity of hemocyte, phenoloxidase (PO), lectin protein concentrations, and phagocytic activity. PO is an enzyme that is directly involved in the melanization sequence which catalyzes the

 doi:10.20944/preprints201804.0089.v1

oxidation of phenols, thus playing a major role in eliminating foreign agents from the body [15, 20, 21] Lectin is a nonenzyme protein (or glycoprotein) that binds or reacts with carbohydrates produced by various foreign agents [21, 22, 23]. Phagocytosis occurs because the activation of the non-cell recognition, the ability of cells to perform phagocytosis is one parameter immune response in insects is running well. PO, lectin and phagocytic are important in the physiological defense mechanism of insects. Immune response was measured by observing the immune response to sublethal concentrations of *M. jalapa* compounds. The results of this research will determine the potential use of *M. jalapa* as a biopesticide to adversely impact the immune system of the pest insect *S. litura*.

2. Materials and Methods

2.1. Insect Culture

S. litura larvae were obtained from the Indonesia Sweetener and Fiber Crop Research Institute (ISFCRI/BALITTAS), Malang, East Java, Indonesia. *S. litura* larvae were reared at 25–26°C and 50%–55% relative humidity. Thereafter, fourth instar larva were placed in plastic rearing jars (diameter of 12 cm; height of 11.5 cm), with each jar containing 50 *S. litura* larvae. Larval rearing jars were cleaned, and the food in them was replaced every 12 h.

2.2. Extract of M. jalapa

M. jalapa leaves were obtained by collecting them from the field in the Lampung Province. *M. jalapa* leaves were dried (without being exposed to light) and dampened using 96% ethanol. The maceration process was carried out for 3 days, after which the crude extract was obtained at the Materia Medica Batu Technical Service Unit (UPT), East Java Provincial Health Office, Indonesia. The extract was concentrated using an evaporation process to form a paste.

2.3. Type of Hemocytes Analysis

Number and composition of hemocyte were observed after 24 h exposure of *M. jalapa* with concentrations of 0.1%, 0.2%, 0.4%, 0.8%, and the control by the microscopic observation. Larvae were anesthetized and injected with 1 mm capillary tube. The hemolymphs was collected and dripped on hemocytometer after being mixed with Turk's solution that served as an anticoagulant (ratio of 1: 1).

2.4. Lectin Analysis

Hemolymphs were collected from *S. litura* after 24 h of exposure to *M. jalapa* at concentrations of 0.1, 0.2, 0.4, and 0.8%. Larvae were then anesthetized and injected into 1-mm capillary tube. The hemolymphs of *S. litura* were collected on Eppendorf tubes that had been filled with phenylthiourea (PTU) crystals. Centrifugation of the solution was conducted for 5 min at a temperature of 4°C and speed of 800 ×g. Samples were separated into pellets and supernatant, which were then placed into separate Eppendorf tubes. The supernatant was used for hemagglutination (HA) inhibition assays. Each pellet was washed using triethanolamine-buffered solution (TBS) at pH 7.4. Then, pellets were resuspended in 50 ml of TBS and centrifuged at a speed of 12,000 ×g for 15 min. The lysates from each sample was used for the HA assay. Next, 2 ml of the hemolymph was homogenized at a pressure of 400 g cm^{-2} for 5 min and centrifuged at a speed of 12,000 ×g for 15 min. This supernatant was used as the source of lectin in the experiment.

HA assay was performed using blood from vertebrate animals containing anticoagulants. The blood sample was washed three times using TBS at pH 7, and its concentration was reduced to 2% (w/v) using TBS. Next, 25 µl of the sample was dropped onto Titertek plate in which 24 µl of lysate with TBS (pH 7.4) had been previously added. This sample was diluted multiple times and incubated at room temperature for 60 min [9].

A protein lectin profile test was performed using electrophoresis, with the supernatant as the sample. In addition, a Bradford protein assay was used to evaluate the analytical profile of proteins

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 8 April 2018 doi:10.20944/preprints201804.0089.v1

using a protein marker; if molecular weight was in the range of 40 kDa, the sample was confirmed as lectin.

2.5. PO Analysis

Hemolymphs from *S. litura* were collected in Eppendorf tubes containing anticoagulant at a ratio of 1:3. The solvent was centrifuged for 15 min at 4°C and speed of 800 ×g. The resulting pellets were washed two times using 2 ml of sodium cacodylate buffer at pH 7 (0.4 M sucrose); then, it was resuspended in 0.2 ml of 0.01 M sodium cacodylate buffer containing 5 mM $CaCl_2$. The mixture was then homogenized using a homogenizing piston, followed by centrifugation at a temperature of 4°C and speed of 1000 ×g for 15 min. The supernatant was used as a sample and loaded onto a 96-well, flat-bottomed plate and incubated for 1, 2, and 3 h. Subsequent color changes were measured using a BioRad 2550 plate reader on A492. The reading result was calculated for further analysis with the following equation:

$$\text{Activity} = \frac{\text{Value from BioRad App.}}{\text{The number of Protein}} \quad (1)$$

$$\text{Enzyme count} = 30\ \mu\text{l fluid size (supernatant)} \times \text{protein concentration} \quad (2)$$

Table 1. Formulation for PO analysis

Blank	Control	Treatment
30 µm distilled water	30 µm supernatant	30 µm supernatant
30 µm buffer cacodylate	30 µm buffer cacodylate	30 µm buffer cacodylate
15 µm L-DOPA	15 µm L-DOPA	15 µm L-DOPA
-	-	30 µm laminarin/trypsin

2.6. PO Data Analysis

The quantity of PO enzyme was analyzed using one-sample of t-test using SPSS version 17.0 (SPSS Inc., Chicago, IL), with $P \leq 0.05$ indicating statistical significance.

2.7. Phagocytic analysis

Phagocytosis assay *in-vitro* requires other foreign objects that are smaller in order to test the ability of cells to perform phagocytosis. To test the *in-vitro* phagocytosis, *Bacillus cereus* cells were used to induce the phagocytosis. *B. cereus* bacteria that have been grown in Nutrient Broth liquid medium was deactivated by heating at 100 °C for 10 min. The separation of supernatant and bacteria pellet from the growing medium was conducted by using centrifuge at 4000 xg for 10 min, then, the pellet was washed with buffer-tris pH 6.5.

The bacterial suspension was made up to a hemocytes to bacteria ratio of 1:50. To increase the activity of hemocytic phagocytosis in vitro, 1 mg/ml laminarin solution (aquabid solvent) was used and mixed on bacterial suspension with pH buffer-tris solvent dissolved at temperature of 38 °C. The hemolymph preparation of *S. litura* larvae that has been infected with *M. jalapa* biopestiside was conducted for 24 h with different concentrations. The hemolymph was drop the hemocytometer and incubated for 10 min. The non-sticking cells were washed slowly with buffer-tris pH 6.5. The available monolayer cells were immediately dripped with bacterial suspensions containing laminarin and incubated for 25 min before microscopic phagocytosis was observed.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 8 April 2018 doi:10.20944/preprints201804.0089.v1

3. Results

3.1. Type of Hemocytes

This study provides data on the expression of the type of hemocyte, lectin, PO, and phagocytic in *S. litura* after exposure to *M. jalapa* extract. The result showed that *S. litura* has five types of hemocytes, there are: plasmatocyte, prohemocyte, oenocytoid, granullar and spherulle (figure 1). Microscopic observation revealed that plasmatocyte were found at 59.98%, prohemocyte 20.73%, granullar 12.74%, oenocytoid 3.33% and spherulle 3.20%. (Figure 2). It were found that the highest percentage are plasmatocyte, prohemosit and granular cell. The high number of these three cells is related to the function and role of the three types of cells in the body (Gillot, 2005). The sub-lethal concentration of *M. jalapa* leaf extract on *S. litura* give the difference in the amount of hemocytes in *S. litura*. The results showed that there was an increase or decrease in hemocytic number of *S. litura* larvae compared with control.

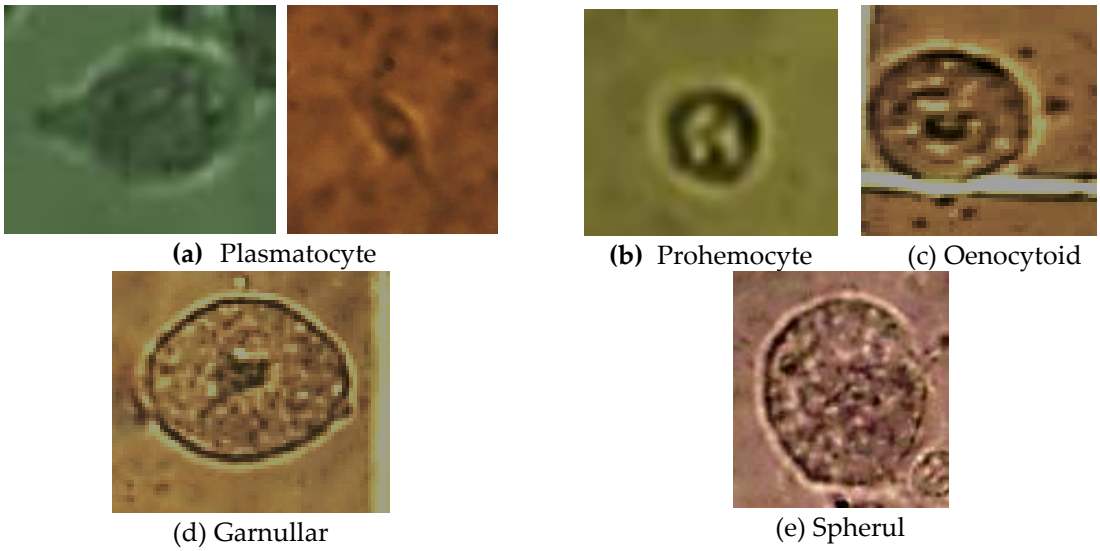


Figure 1. The type of *S. litura* hemocyte

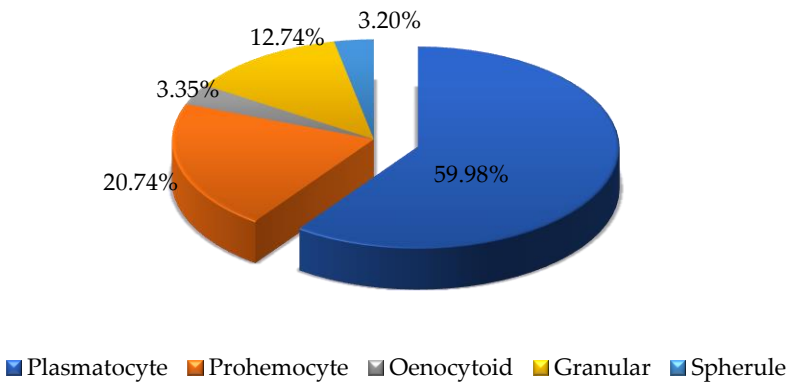


Figure 2. Concentration of total hemocyte types in *S.litura*

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 8 April 2018 doi:10.20944/preprints201804.0089.v1

198 **Table 2.** The average of hemocyte exposured by *M. jalapa*

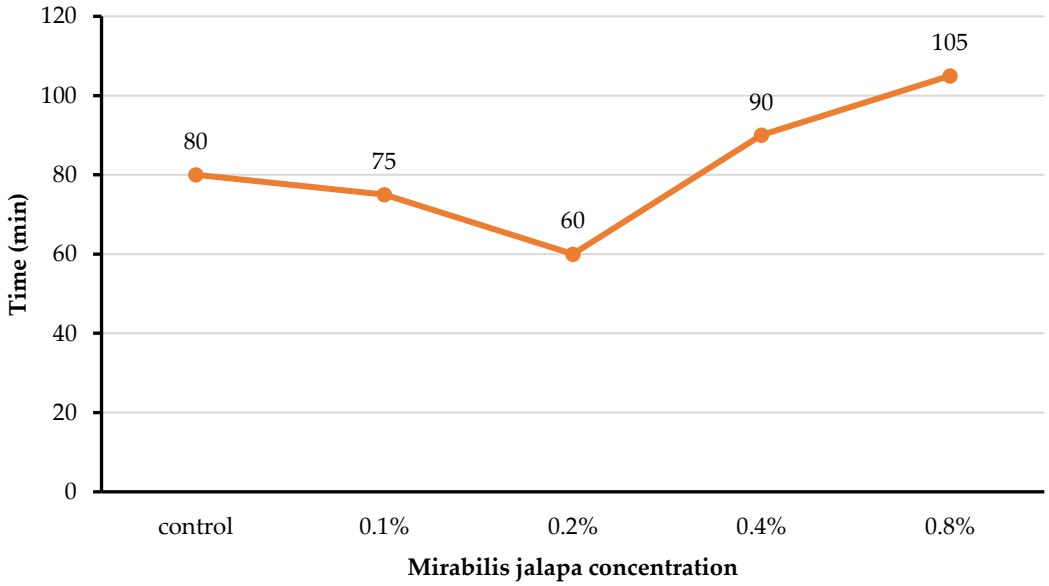
Concentration	The average of hemocyte ± SD (sel/ml x 10^6)				
	Plasmatocyte	Prohemocyte	Granullar	Oenocytoid	Spherulle
Control	350,75 + 43,30a	121,25 + 29,80a	74,50 + 13,77a	19,50 + 5,69a	18,75 + 10,05ab
0,1 %	329,25 + 149,27a	169,50 + 47,00ab	123,50 + 78,42ab	75,75 + 14,77b	32,50 + 3,11a
0,2 %	467,00 + 161,13a	272,75 + 86,73b	197,50 + 53,53b	47,25 + 20,04b	17,50 + 9,39ab
0,4 %	343,50 + 235,36a	120,25 + 134,69ab	100,75 + 141,52b	17,00 + 11,52a	10,00 + 5,66bc
0,8 %	79,25 + 13,65b	25,25 + 5,0c	24,00 + 5,35c	7,50 + 1,73c	4,75 + 1,29c

199 Note: numbers follow by different alphabeth in the same column show a significant difference $P \geq 0.05$.

200 Table 2 lists the comparison of the average hemocytic type of *S. litura* larvae. The results
201 explained that plasmatocyte cells had the highest average number of other hemocytic cells.
202 Concentration of 0.2% (w/v) led to significant increase in prohemocytes, granullar cells, and
203 oenocytoids (P <0.05). A concentration of 0.8% resulted significantly decreased to all types of *S. litura*
204 hemocyte compared with the control (P <0.05). The average of total hemocyte was differ significantly
205 in the treatment group, exposure to 0.1% and 0.2% (w/v) of *M. jalapa* extract increased the total
206 number of hemocytes as much as 38.08% and 64.15% respectively. In contrast, exposure to 0.4% and
207 0.8% (w/v) reduced the number of hemocytes to 37.02% and 51.04% respectively.

208 *3.2 Lectin*

209 The rate of lectin formation was determined using titration HA assay and lectin profile testing.
210 A concentration of 0.2% (w/v) resulted in fastest lectin formation (60 min), whereas 0.8% resulted in
211 the slowest lectin formation (105 min). The profile of lectin in the insect bodies in the supernatant of
212 hemolymphs was already within the range of 40 kDa molecular weight, indicating an immune
213 response (Figure 4). Lectin presents in the supernatant at all *M. jalapa* extract concentrations studied
214 (Figure 3).



215

216 **Figure 3.** Formation rate of hemagglutination titer

217

218

219

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 8 April 2018 doi:10.20944/preprints201804.0089.v1

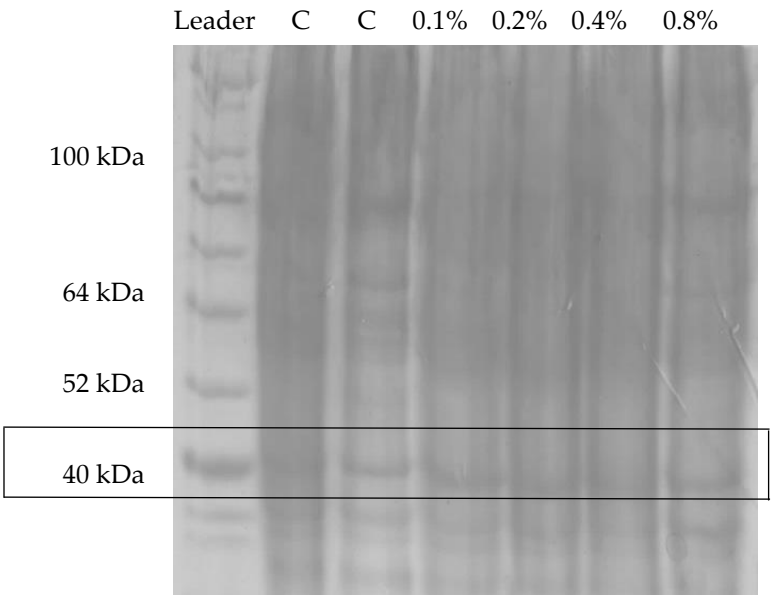


Figure 4. Profile of lectin protein

3.3 PO (Phenoloksidase)

PO activity was measured by the amount of enzymes present at time intervals following applications of sublethal concentrations of *M. jalapa* (0.1, 0.2, 0.4, and 0.8%) (w/v) (Figure 5). Although PO was also formed at all concentrations, its activity showed differences across different concentrations and exposure periods (Figure 5). The relationship between *M. jalapa* concentration and PO formation ($P < 0.05$) and amount (or activity) of PO was dependent on the extract concentration and incubation time (exposure) in the bloodstream.

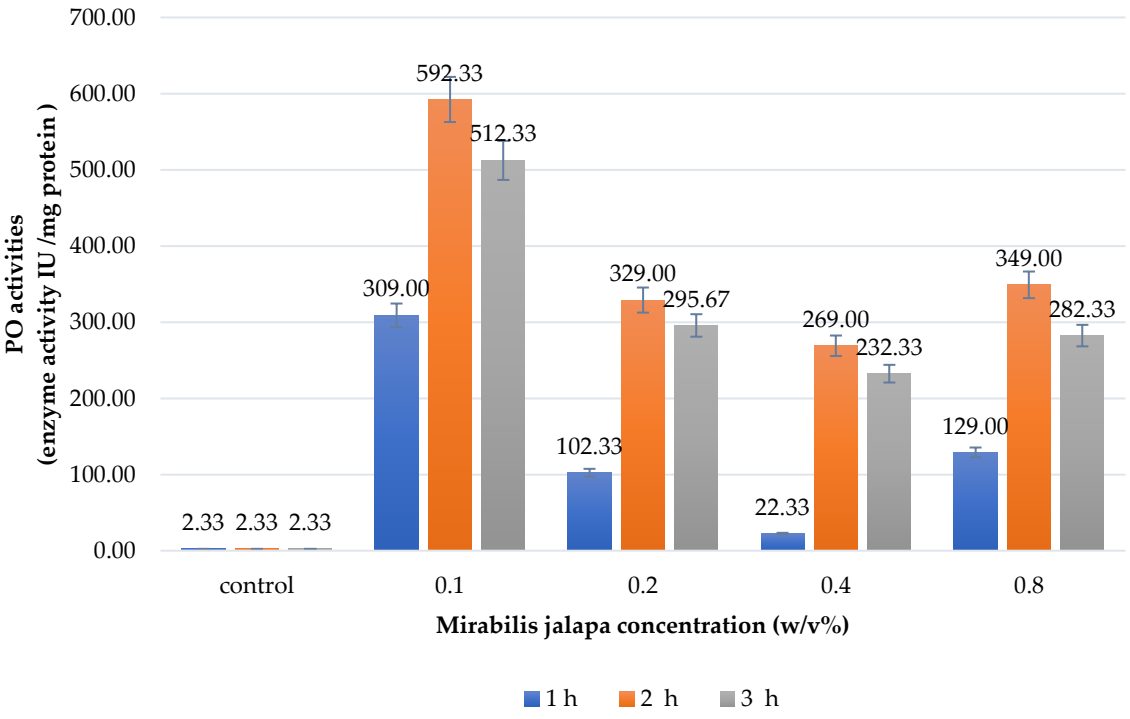


Figure 5. PO activities

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 8 April 2018 doi:10.20944/preprints201804.0089.v1

3.4 Phagocytic

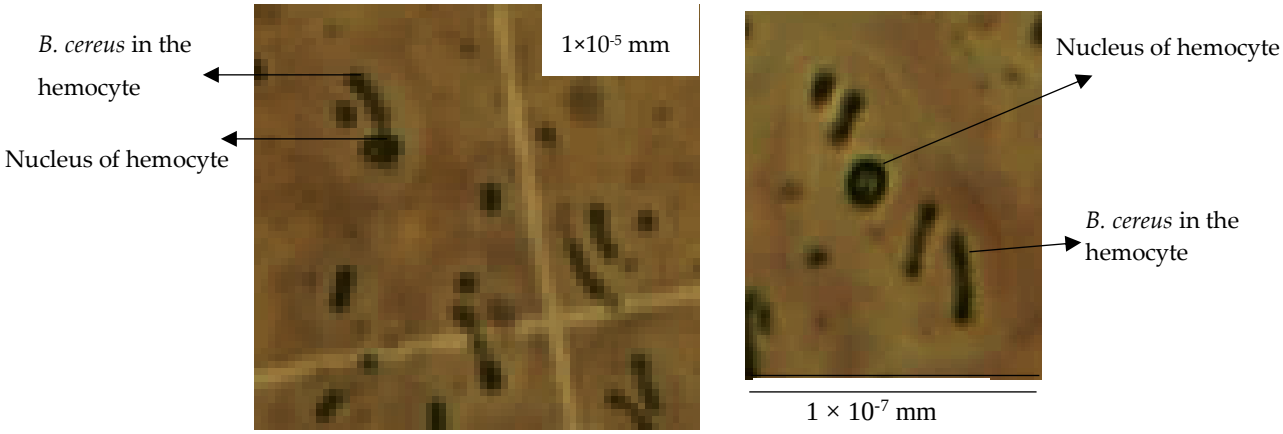


Figure 6. Phagocytosis of *S. litura* hemocyte against *Bacillus cereus*

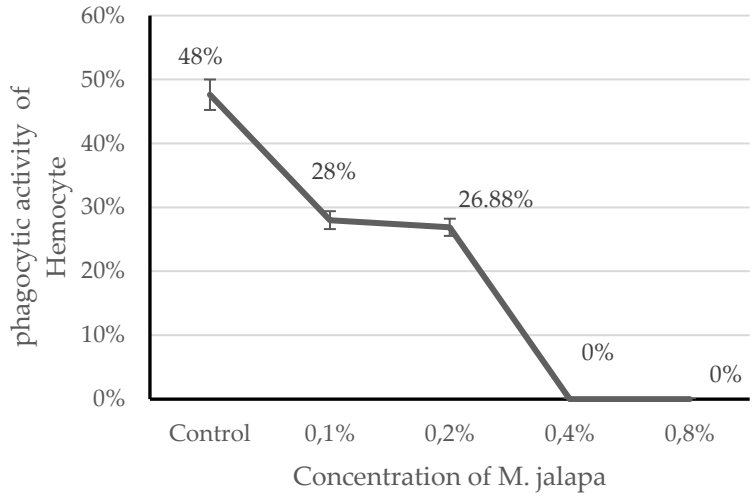


Figure 7. Phagocytic activities of *S. litura* hemocyte

Phagocytosis activity is one of the parameters of immune response in insects. The activity occurring in *S. litura* hemocytic cells after induced by *B. cereus* bacteria. Figure 6 shows the activity of hemocyte that phagocytate *B. cereus* cells. The one of hemocyte can phagocytes more than one of bacterial cell. Figure 7 shows that in the ability of hemocytic *S. litura* cells to be able to phagocytic, in the control conditions resulted 47.62% cells was done and it was decreased in concentrations 0.1% and 0.2% (w/v) as much as 28.00% and 26.88%. The phagocytic activity does not occur when the concentrations of *M. jalapa* 0.4% and 0.8%. The activity of phagocytosis in hemocytic cells of *S. litura* on *M. jalapa* is inversely proportional to the amount of hemocytes. The total amount of hemocytes in *S. litura* larvae is increasing when giving *M. jalapa* extract at 0.1% and 0.2% concentration, but the ability of cell phagocytosis decreases.

4. Discussion

Insect immune systems consists of cellular and humoral mechanisms. Their work to induce each other. Cellular activity is very important to eliminating pathogens. Indications of cellular defense are seen in hemocytic activity. Generally, type of insect hemocyte are: granullar, plasmatocytes, oenocytoids, prohemocytes, coagulocytes, spherulle and adipohemocytes [15]. Each types of hemocyte has a different function in insect, which influenced by species, feed intake, and

 doi:10.20944/preprints201804.0089.v1

environment. Feed supplements also influence the different types and amounts of hemocytes in a species, when *Apis mellifera* given dietary feed intake, by administering different amounts and types of nutritional intake affecting the diversity of hemocytes and concentrations [24]. Figure 1 shows that *S. litura* have five types of hemocytes: Type A (plasmatocyte), Type B (oenocytoid), Type C (prohemocyte), Type D (granullar) and Type E (spherulle).

Identified hemocytes from *S. litura* have different amounts of composition. There are many factors to caused different composition among others: eclosion patterns on insects, age and stadia, sex, and the date of mating that indicate an increase in the amount of hormone during mating. It was seen in the larval stadia hemocyte of *Danaus plexippus* female is higher than males [25]. During the development, the insect has a diversity of hemocyte, in the *Papilio demoleus* in instar 5 stage is the highest amount of hemocyte [26]. Hormone is another factor causing changes of insect hemocytes. Concentration of hormones affect the anatomical and physiological changes of the insect body [14]. The hemocytes changes aims to maintain physiological balance and endurance thier body. Therefore, *S. litura* larvae have variations in the number of hemocytes.

Figure 2 shows that *S. litura* were three hemocytic cells the highest percentage, there are: plasmatocyte, prohemocyte and granullar cell. The high number of these cells is related to the function and role of the three types of cells for the body defense [15]. Plasmatocyte is the highest cell count compared to the other hemocytic cells that was 59.98%. Plasmatocyte plays a major role in the activity of insect phagocytosis. Prohemocyte cells have an amount of 20.74%. Prohemocyte is celll that actively to mitosis and differentiate into other hemosit cell form. Prohemocyte increases in number when induced by foreign substances, these cells perform high mitotic activity [27]. The percentage of cells possessed by granular cells is 12.74%, the function to recognizing the presence of foreign pathogens that enter the insect body. The high concentration of these cells causes the activation of immune mechanisms in the insect.

M. jalapa may induce changes in the amount of *S. litura* hemocytes. The lowest concentration (0.1% and 0.2%) (w / v) was increasing oenocytoid, granullar and prohemocyte ($P < 0.05$). The increase in these three cells occurs due to a reaction to the body's defenses. Oenocytoids is related to the function of cells that play a role to introduce of foreign compounds in the body. It was produce pro-phenoloxidase enzymes and function in the melanization process. Granullar is a derivative of amoboid plasmatocyte cells that contribute to phagocytic activity [15]. Physiological, the granular cells have a function as a marker of the presence of foreign substances in the body by removing the material content of the cell. The compounds of the granular cell cytoplasmic material will be recognized by plasmatocyte as a command to perform phagocytosis. Granular cells also play a role in other immunological mechanisms such as activation of pro-phenoloxidase, plasma gel formation, capsule formation and the introduction of foreign particles. The number of granular cells in some Lepidoptera classes can reach 60% of the total hemocytic population.

Prohemocyte works to inceasing a number of cells in the body by the mitotic [27]. The hemocyte cells typically specialize the form into plasmatocyte cells. Thus, the increase in prohemocyte cells in particular will have an impact on the increase in the number of plasmatocytes and both of these cells will be involved in the mechanism of the insect's immune system. Overall, the comparison of the average hemocytic type of *S. litura* larvae when given the extract of *M. jalapa* occurs differently. The results showed a difference in the average type of hemocytes with different concentrations of *M. jalapa*. The result of calculation of difference of mean of hemocyte is seen in table IV.1 exposure of the five extract concentrations of *M. jalapa* plasmatocyte cells has the highest average number of other hemocytic cells. Plasmatocytes are cells that perform phagocytosis by having a rich cell content of the golgi complex and the cytoplasmic reticulum making it possible to actively move the cells. The large number of lysosomes with high enzyme content on plasmatocytes serves as a catalyst for foreign substances [15]. An overall increase in hemoglobin in the five types of hemocytes occurs when the concentration of *M. jalapa* extract is 0.2%. Concentration of 0.2% led to significant increase in prohemocytes, granular cells, and oenositoids ($P < 0.05$). It was induced an increasing in cell activity on toxic biopesticide *M. jalapa*. Increasing the number of cells was occur to eliminate, destroy, lyse or even make toxic substances extract *M. jalapa* that has entered into the body become tolerant to it self.

 doi:10.20944/preprints201804.0089.v1

Granullar and spherulle cells was significantly decreasing in concentration of 0.4% (P <0,05). The decline both of cells indicates that the introduction of cells to foreign matter has been disrupted. The conditions will lead to a decrease in the S. litura cellular immune system. The concentration of 0.8% was significantly decreasing all off types of hemocyte compared with the control (P <0.05). It has an effect on the attenuation of the highest physiological ability of larvae compared to other concentrations.

The humoral immune response in insects plays a major role in the immune system by activating various enzymatic and nonenzymatic reactions used by the body for the recognition of foreign agents and developing resistance to them. The mechanism depends on the ability of the cells to recognize foreign agents through receptors on their membranes. There are eight receptors involved in the humoral immune mechanism: immulectins, thioester-containing proteins (TEPs), LPS-binding protein, peptidoglycan recognitions proteins (PGRPs), gram-negative bacteria binding proteins (βGRPs), hemolin (immunoglobulin superfamily), and *Bombyx mori* multibinding protein. The introduction of foreign agents perceived by these receptors impact the cell's response by stimulating the induction and secretion of antimicrobial peptides and initiating the melanization process [21].

Receptors are an important part of the immune defense mechanisms in organisms. Lectin receptors, which are proteins that bind carbohydrates [23], are the main factors activating phenol oxidation in the hemolymph plasma [21–23]. Immulectin in granular cells and eonocytoids increase encapsulation activity [28]. A receptor's ability to recognize a foreign agent (nonself glycoprotein or glycolipid), induces the primary receptor to initiate an immune response. Therefore, lectins, which are capable of inducing cellular and humoral sequences in the immune system, can be used as an indicator for the recognition of foreign agents/pathogens and subsequent signal transductions.

Our results showed that lectin was present in *S. litura* when it was exposed to *M. jalapa* biopesticide. The rapidity of the lectin response was measured by the formation of HA titers, which showed different results for each concentration. The HA test was performed to observe the lectin response; i.e., its binding to vertebral blood cell membranes (carbohydrates). When *S. litura* larvae was exposed to four sublethal concentrations of *M. jalapa* (0.1, 0.2, 0.4, and 0.8%), lectin binding with carbohydrates and vertebrate erythrocyte cells occurred more rapidly in samples treated with 0.2% concentration than in control samples. In addition, the formation of HA titers in the control sample occurred at 80 min, whereas samples treated with 0.1% and 0.2% (w/v) extracts led to faster HA formation (60 min).

The formation rate of the titer was influenced by the number of hemocytes produced by cells, because the immulectin receptors on the surface of the cell (supernatant) responds to an increase in the number of hemocytes, which in turn leads to an increase in the number of lectin receptors. Therefore, the degree to which lectin binds a foreign agent can be promptly recognized and its intensity can be quantified. A concentration of 0.2% is thus considered as the optimum concentration to stimulate the immune response, as indicated by the rapid increase in the number of hemocytes produced ($P < 0.05$) at this concentration [9]. The adduction of *M. jalapa* at 0.4% and 0.8% concentrations produced a longer response time (at 90 and 105 min, respectively) than did the control sample (80 min). This signifies that there are fewer hemocytes produced at higher concentrations *M. jalapa* biopesticide exposure. This suggests that hemocyte cells are not able to proliferate at higher concentrations. Toxicity at high concentrations causes an alteration in an insect's enzymatic and coordination systems; hence, the cells that induce the cell mitotic processes become inhibited [29]. The activation of PO is the main enzymatic reaction important to the humoral response sequence. This enzyme plays an important role in melanogenesis in invertebrates. PO is the key player in the encapsulation of multicellular pathogens and recovery of defense tissues for use against pathogens, such as bacteria (gram positive and negative), fungi, viruses, and other foreign agents [30, 31, 32]. PO and DOPA decarboxylase are the main mediators in the process of melanization. Thus, PO is the main mediator (tool) used by insects to fight some pathogens [33].

PO is an enzyme responsible for the immune response, which melanization formation, encapsulation, and nodulation. PO induction begins with the recognition of a foreign agent via its receptors, which then activates the serine protease pathway that produces phenylalanin, which then

 doi:10.20944/preprints201804.0089.v1

leads to the activation of ProPO to PO. The extent and rapidity of this activity is related to sex, life cycle, temperature, season, and species of the host. [20].

The induction of a foreign agent increases the blood's PO concentration [34]. Our results showed that the adduction of *M. jalapa* biopesticide was also able to induce *S. litura* to activate the PO enzyme. (*M. jalapa* acts as a foreign substance that induces the PO activity on *S. litura*.) In fact, biopesticide from *M. jalapa* increased the PO at all introduced concentrations ($P < 0.05$) relative to control samples. This increase proves that there was an immune system response to the boipesticide. The highest increase of PO occurred 2 h after induction of 0.1% concentration of biopesticide, leading to the highest PO activity value ($P < 0.05$). A 3 h induction time also showed a higher PO activity relative to the 1 h induction time and the control samples ($P < 0.05$). Our results indicate that there is an immune reaction to the treatments concentrations we provided. The lowest concentration (0.1%) increased PO activity, while higher concentrations tended to lead to a decrease in the activity.

Sublethal concentrations of *M. jalapa* extract induced the humoral immune defense system through the increase of lectin and PO. Concentrations of 0.4% and 0.8% (w/v) resulted in lower lectin and PO activity than did lower concentrations. This indicates that the immune reaction is highest at sublethal concentrations. In contrast, at the lowest concentrations (0.1% and 0.2%), *M. jalapa* actually stimulates an increase in the humoral response relative to both lectin and PO. The same thing happened in the cellular response, in which the 0.2% was the optimal concentration for Spodoptera to activate its immune system response to defend against the biopesticide.

Figure 6. showed that in vitro activity of phagocytosis occurring in *S. litura* hemocytic cells after induced by *B. cereus*. Hemocyte of *S. litura* recognizes *B. cereus* as pathogen a part of non-self. Plasmatocyte cells play a role in the activity of phagocytosis [15]. Plasmtocyte cells perform phagocytic activity in some types of insects when induced by B. rossius [35]. However, in certain types of insects the granular cells play a role in the activity of phagocytosis, ie *A. subalbatus* has very active granular cells in the cellular defense of granular cells that perform phagocytic activity [36]. Another study described that granular cells in *Galleria mellonela* insects play a role in the process of phagocytosis [37]. The phagocytic event begins when the pathogenic chemical signals are recognized by the receptors of the hemocytes. Plasmatocyte cells will move amoeboid approaching the pathogen source, and then adhesi bonding between *B. cereus* cells that are pathogenic with receptors of plasmatocyte cells. The bond between the pathogen and the plasmatocyte cell becomes very strong, and it will form a base in area according to *B. cereus* size by activating the filamentous actin which is the cytoskeleton to form pseudupodia cells [38].

The next stage of phagocytosis is the endocytosis of *B. cereus* bacteria into the plasmatocyte cells. The plasmatocyte cytoplasm contains many lysosomes that are responsible for catalyzing the alien pathogens entering the body. The enzyme catalyzing from lysosomes is used to degrade *B. cereus*. Exposure to toxic substances from M. jalapa leaf extract resulted in weakening of hemocytic cells to perform phagocytosis. The results of phagocytosis ability of hemocytic larvae of *S. litura* that have been infected by *M. jalapa* extract on *B. cereus* can be seen in figure 6. Figure 7. showed that the phagocytic ability of hemocytic cells from *S. litura* larvae to *B. cereus* decreased after infection with toxic *M. jalapa*.

Control conditions resulted that the ability of hemocytic cells from *S. litura* larvae to be able to phagocytic *B. cereus* by 47.62%. Decreased phagocytosis activity occurred in the extract of *M. jalapa* with concentrations of 0.1% and 0.2% because the communication ability between hemosit cells cannot run properly. The effects of toxic substances from M. jalapa extract resulted in disturbance and damage to communication between cell response and humoral response to *S. litura* larvae. Saponin, tannin and flavonoid compounds contained in M. jalapa plant are larvicidal for larvae. The tannin compound which is a polymer of flavonoids in the larval body will bind to the salivary proteins and the digestive enzymes trypsin and Chymotripsin which impact on the inactivation process of these proteins by converting the protein conformation to be coagulated [39]. While Saponin is a compound that serves to disrupt the conformation of the membranes of insect cells, namely by binding to the cell membrane sugar groups in insect larvae. Bounding the phenolic compound of *M. jalapa* with the sugar group in the hemocyte cell membrane *S. litura* will disturb the chemical signals from B. cereus which will bind to the hemocytic cell membrane. The lectin bond that should occur

 doi:10.20944/preprints201804.0089.v1

between hemocytic cells and *B.cereus* cells has been replaced by saponin compounds. Thus, inhibition by saponin compounds results in no bonding between the hemocytes and *B. cereus* which causes chemotaxis in the hemocytic cells to be inhibited. This results in phagocytosis events not working well and not even happening. The failure of the larval body response to perform phagocytosis is seen in the administration of *M. jalapa* concentrations of 0.4% and 0.8%. This condition causes the ability of the immune system to become very weak resulting in the hemocyte cells are unable to recognize and eliminate the foreign pathogens that enter the body.

The activity of phagocytosis in insects is very closely linked with a series of humoral immune responses. It appears that Drosophila mutant non-humoral responses do not indicate the presence of phagocytic events when enzymed with *Escherchia coli* [38]. Phagocytosis becomes inhibited when the humoral response is removed from Drosophila's body. Similarly, Spodoptera humoral activity with lectin content parameters when given the extract of biopestisida *M. jalapa* showed that the lectin content was lower in number along with the increasing of sub-lethal concentration from the extract of *M. jalapa* [9]. Thus, the linkage between humoral and cellular defense responses runs synergistically ie the decrease in lectin content is directly proportional to phagocytosis events. Lectin serves as opsonin in the process of phagocytosis. Lectins mediate the bonding of sugar chains in phagocytes and other cell surfaces, so that the lectin as a humoral message increases the opsonization ability of phagocyte cells against foreign substances. Thus, it can be explained that a series of cellular immune responses that occur in insects include the work synergism of the humoral response [40].

In principle, the application of *M. jalapa* biopesticide induces an immune response reaction by decreasing overall physiological functions, rather than killing *S. litura* outright. This sublethal application of the biocide is aimed at preventing a biological resistance in the target pest. Prevention of resistance is necessary for easy and sustained control of pests. In the event of resistance, evidence of resurgence can be confirmed as a result of multiplication of insecticide dose [41, 42]. Therefore, the use of the biopesticide extracted from *M. jalapa* can prevent resistance and resurgence from occurring if the biocide is applied in accordance with the accepted standards for Integrated Pest Management (IPM) [2, 6, 43]. The results of our study can be used as a basic reference for the optimal application rate of *M. jalapa* biopesticide on agricultural crops. Determining the population size of Spodoptera pests in a given field is required for one to establish the amount of biopesticide needed to be applied. The magnitude of the impairment of the immune system observed in this study was enough to kill the target pests. Therefore, it is has great potential for controlling insect pest populations.

5. Conclusions

M. jalapa leaf extract has a great potential for becoming an important bioinsecticide against *S. litura* because it stimulates the cellular and humoral immune system response in *S. litura* larvae. The adduction of *M. jalapa* leaf extract induced concentration total hemocyte, lectin, PO, and phagocytic activities in *S. litura* larvae. The average of total hemocyte was differ significantly in the treatment group, exposure to 0.1% and 0.2% (w/v) of *M. jalapa* extract increased the total number of hemocytes as much as 38.08% and 64.15% respectively. In contrast, exposure to 0.4% and 0.8% (w/v) reduced the number of hemocytes to 37.02% and 51.04% respectively. Lectin activity quickly formed at 0.1% and 0.2% (w/v) concentrations. The number of PO enzymes induced was significantly different at sublethal concentrations compared with control samples. The highest increase in PO activity occurred after 2 h of induction time and at concentrations of 0.2% (592.33 IU/mg) and 0.1% (521.33 IU/mg). Higher concentrations induced lower lectin and PO activities. In term of phagocytic activity, the proportion of phagocytosis cells were 47.62% in control group, and decrease in 0.1% and 0.2% (w/v) *M. jalapa* treatment respectively. Our results suggested that the *M. jalapa* extract could potentially be used as a biopesticide to decrease their immune system to resulting in death of the pest.Our results indicated that *M. jalapa* extract is a biopesticide capable of inducing lectin and PO activities in insect pests. Concentrations of 0.8% (w/v) *M. jalapa* extracts lead to their mortality by a weaker immune response in *Spodoptera* .

 doi:10.20944/preprints201804.0089.v1

Acknowledgments: We would like to thanks Hibah Penelitian Disertasi Doktor, Direktorat Riset dan Pengabdian Masyarakat, Direktorat Jendral Penguatan Riset dan Pengembangan Kementerian Riset, Teknologi, dan Pendidikan Tinggi Indonesia which has provided funding for this research.

Author Contributions: D.M. performed analysis and wrote the paper; S.B.S, S.R.L., and T.A. provided several advice and assistance during analysis; M. Amin supervised the research: M. Aziz checked and corrected the paper.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Bai, Y., Yan, R., Ke, X., Ye, G., Huang, F., Luo, Y., Cheng, J., Effects of transgenic Bt rice on growth, reproduction, and superoxide dismutase activity of *Folsomia candida* (Collembola: Isotomidae) in laboratory studies. *Journal of Economic Entomology* ***2011***, *104*, 1892–1899. DOI: 10.1603/EC11095.
2. Leng, P., Zhang, Z., Pan, G., Zhao, M. Applications and development trends in biopesticide. *African Journal of Biotechnology*, ***2011***, *10*, 19864–19873. DOI: 10.5897/AJBX11.009.
3. Romeis, J., Bartsch, D., Bigler, F., Candolfi, M.P., Gielkens, M.M.C. Assessment of risk of insect-resistant transgenic crops to nontarget arthropods. *Enthomology Publications*, ***2008***. IOWA State University 26, 203–208. DOI: 10.1038/nbt1381.
4. Shapiro, M. Enhancement in activity of homologous and heterologous baculoviruses infectious to beet armiworm (Lepidotera: Noctuidae) by an optical brightener. *Journal of Economic Enthomology*, ***2000,*** *93*, 572–576. DOI: 10.1603/0022-0493-93.3.572
5. Kandagal, A.S., Khetagoudar, M.C. Study on larvicidal activity on weed extract against *Spodoptera litura*. *Journal of Environmental Biology* ***2011,*** *34*, 253–257. DOI: 10.1007/s00436-008-1142-x.
6. Nathan, S.S., Chung, P.G., Murugan, K. Effect of botanical insecticides and bacterial toxin on the gut enzyme of the rice leaf folder *Cnaphalocrocis medinalis*. *Journal of Phyparasitica* ***2004***, *35*, 433–443. DOI: 10.1007/BF02980437
7. Horne, P.A., Page, J. Integrated pest management for crops and pastures, ***2008***. Victoria: Landlink Press. pp. 136. ISBN: 9780643092570.
8. Tanada, Y., Kaya, H.K. Insect Pathology. Academic Press, ***1993,*** INC-Harcourt Brace Jovanovich, Publishers. eBook ISBN: 9780123849854
9. Suryani, I., Anggraeni, T. The effect of leaf biopesticide *Mirabilis jalapa* and fungi *Metarhizium anisopliae* to immune response and mortality of *Spodoptera exigua* Instar IV. *AIP Conference Proceeding: Bandung*. ***2014.*** DOI: 10.1063/1.4868808.
10. Prasetyo, D.B. Effect of *Mirabilis jalapa* leaves ethanol extract to the consumptions, weight and larva growth, pupation, and the percentage of *Spodoptera exigua* hubner imago. ***2006.*** (In Indonesia) Bacelor degree Institut Teknologi Bandung
11. Vivanco, M.J. Antviral and antiviroid activity of MAP-containing extracts from *Mirabilis jalapa* roots. *Plant Diseases*, ***1999,*** *83*, 116–121. DOI: 10.1094/PDIS.1999.83.12.1116
12. Suharsono. The sensitivity of water-tolerant soybean strains against grayak caterpillar *Spodoptera litura* F (In Indonesia). ***2011***. Plant Research Institute for Pulses and Tubers
13. Schreiner, I. Cluster caterpillar (*Spodoptera litura* [Fabricius]). Agricultural Pest of the Pacific ADAP. ***2000***. http://hdl.handle.net/10125/33420.
14. Chapman, R.F. The Insect Structure and Function 5thEdition: Stephen J. Simpson, the University of Sydney, Australia, Angela E. Douglas, Cornell University, New York, USA. 2009. New York Cambridge University Press. Cambrigde University. ISBN: 9780521113892.
15. Gillot, C. Entomology Third Edition. University of Saskatchewan-Springer Netherlands. ***2005***. pp: XVIII, 832. DOI: 10.1007/1-4020-3183-1.
16. Lacey, L.A., Grzywacz, D., Shapieo-ilan, D.I., Frutos, R., Brownbridge, M., Goettel, M.S. Insect pathogens as biological control agent: back to the future. *Journal of Invertebrate Pathology*, ***2015,*** *132*, 1–41. DOI: 10.1016/j.jip.2015.07.009.
17. Taszlow, P., Vertyporokh, L., Wojda, I. Humoral immune response of *Galleria mellonela* after repeated infection with *Bacillus thuringiensis*. *Journal of Invertebrate Pathology*, ***2017*** *149*, 87–96. DOI: 10.1016/j.jip.2017.08.008.
18. Charnate, E., Sarmento, R.A., Venzon, M., Pedro-Neto, M., Junior. D.F.F., Santos. F.A., Pallni. A. Suitability and nutritional requirements of the predatory mite *Typhlodromus transvalensis*, a potential biological control agent of physic nut pest mite. *Biological Control*, ***2017,*** *115*, 165–172. DOI: 10/1016/j.biocotrol.2017.10.008.
19. Sigh, B., Kaur, A. Control of insect pest in crop plants and stored food grains using plant saponins: a review. *LTW-Food Science Technology*, ***2017***, *87*, 93–101. DOI: 10/1016/j.lwt.2017.08.77.
20. González-Santoyo, I., Córdoba-Aguilar, A. Phenoloxidase: a key component of the insect immune system. *Entomologia Experimentalis et Applicata*, ***2012,*** *142*, 1–16. DOI: 10.1111/j.1570-7458.2011.01187.x
21. Marmaras, V.J., Lampropoulou, M. Regulators and signalling in insect hemocyte immunity. Cellular Signalling, **2009**, 21, 186–195. DOI: 10.1016/j.cellsig.2008.08.014.

22. Yu, Q.X., Kanost, M.R. Immulectin-2, a lipopolysaccharide-specific lectin from an insecta, *Manduca sexta*, is induced in response to gram negative bacteria. Journal of Biological Chemistry, **2000**, 275, 37373–37381. DOI: 10.1074/jbc.M003021200.
23. Yu, Q.X. A novel C-type immulectin-3 from *Manduca sexta* is translocated from hemolimph into the cytoplasma hemocyte. Journal of Insect Biochemistry and Molecular Biology, **2005**, 35, 285–295. DOI: 10.1016/j.ibmb.2005.01.004.
24. Mohandes, S. S., Nafea, E.A., & Fawzy,A.M. Effect of Diferent Feeding Diets on The Haemolimph of The Newly Emerged Honeybee Works *Apis melliefera* L. Egypt Acad, *Journal Biology andScience,*, ***2010*, 3**, 213 - 220.
25. Lindsey, E. & Altizer, S. Sex differences in immune defences and responses to paratism in Monarch butterflies, *Journal of Evolution & Ecology*, **2008**, 9258, 23-39.
26. Jalali, J. & Salehi, R. The haemocyte types, differential and total count in *Papilio demoleus L.*(Lepidoptera : Papillonidae) during post-embryonic development. *Journal of Entomology-Zoology*, ***2008*,** 3, 199 - 206.
27. Qamar, A. & Jamal, K. Differential hemocyte counts of 5th instar nymps and adults of *Dysdercus cingulatus* Fabr (Hemiptera: Pyrrhocoridae) treated with acephate an organophosphorus insecticide, *Biology and Medicine*, **2009,** 1, 116 - 121.
28. Yu, Q.X. Immulectin-2, a pattern recognition receptor that stimulate hemocyte encapsulation and melanization in the tobacco hornworm, *Manduca sexta*. *Journal of Developmental and Comparative Immunology*, ***2004*,** 28, 891–900. DOI: 10.1016/j.dci.2004.02. 005.
29. *Mirabilis jalapa* and their mortality when treated with *Bacillus thuringiensis* (In Indonesia). Magister Degree. Bandung: ITB.
30. Ashida, M., and Brey, P. Recent advances in research on the insect prophenoloxidase cascade. Molecular Mechanisms of Immune Responses in Insects (ed. by P Brey and D Hultmark), Chapman & Hall, London, UK, ***1997***, pp. 135–171.
31. Boman, H.G. Antibacterial immune proteins in insects. Immune mechanisms in invertebrate vectors (ed. by AMLackie), Symposia of the Zoological Society, London, UK, ***1986***, pp. 45–58. DOI: 10.1007/978-3-642-70768-1_6.
32. Nappi, A.J., Christensen, B.M. Melanogenesis and associated cytotoxic reactions: applications to insect innate immunity. *Insect Biochemistry and Molecular Biology*, ***2005***, 35, 443–459. DOI: 10.1016/j.ibmb.2005.01.014.
33. Cerenius, L., Soderhall, K. The prophenoloxidase-activating system in invertebrates. *Immunological Reviews*, ***2004***, 198,116–126. DOI: 10.1111/j.0105-2896.2004.00116.x.
34. Anggraeni, T., Melanie, Putra, R.E. Cellular and humoral immune defenses of Oxya japonica (Orthoptera: Acrididae) to entomopathogenic fungi Metarhizium anisopliae. *Entomological Research*, ***2011*,** 41, 1–6. DOI: 10.1111/j.1748-5967.2010.00311.x
35. Scapigliati, G. & Mazzini, M. In Vivo and In Vitro Phagocytosis by Hemocytes of the Stick Insect *Bacillus rossius, Journal of Boll-Zoology*, ***1994*,** 61, 115 - 120.
36. Hillyer, J. F., Schmid, S. L., & Chistensen, B. M. Hemocyte-mediated phagocytosis and melanization in the mosquito *Armigeres subalbatus* following immune challenge by bacteria. *Cell Tissue.* ***2003*.** 313, 117 - 127.
37. Tojo, S. Naganuma, F. Arakawa, K., & Yokoo, S. Involvement of Both Granular cells, and Plasmatocytes in Phagocytic Reaction in the Greater Wax Moth, *Galleria mellonella, Journal of Insect Physiology*, ***2000***, 46, 1129–1135.
38. Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K., & Walter, P. *Molecular Biology of The Cell. **2008*** Garland Science. Taylor & Fancis Group.
39. Freeman, B.C. & Beattie, G. A. An overview of plant defenses againts pathogen & herbivors, *Journal of The Plant Health Instructur, 2008*, 226, 1–6. DOI: 10.1094/PHI-I-2008-0226-01
40. Erickson, M. E., Mishra, S. & Schneider, D. Interaction between the cellular and humoral immune responses in Drosophila. *Journal of Current Biology*, **2000**, 10, 781–784. DOI: 10.1016/S0960-9822(00)00569-8
41. Dutcher, J.D. A review of resurgence and replacement causing pest outbreaks in IPM. General Concept in Integrated and Disease Management, Entomology Departement, University of Georgia, Tifton GA, USA. **2007.** pp. 27–43. DOI: 10.1007/978-1-4020-6061-8_2.
42. Sparck, T.C., Nauen, R. IRAC: mode of action classification and insecticide resistance. *Pesticide Biochemistry and Physiology*, ***2014***, 121, 122–128. DOI: 10.1016/j.pestbl.2014.11.014.
43. Kumar, S., Singh, A. Biopesticide: present status and future prospect. *Journal of Fertilizer and Pesticide*, ***2015***, 6, 1–2. DOI: 10.4172/jbfbp.1000e129.