# Identification of bioactive compounds from *Mirabilis jalapa* L. (Caryophyllales : Nyctaginaceae) extracts as biopesticides and their activity against the immune response of *Spodoptera litura* F. (Lepidoptera: Noctuidae)

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**ABSTRACT**

Biopesticide are biological agents sourced from the natural products to control the pest population. Mirabilis jalapa extract is one of biopesticides that contain the repellence of insects compounds to reduce the *Spodoptera litura* F. insect pest. This study aims to identify the bioactive compound in the *M. jalapa* that potentially for the biopesticide and the effect of immune response of *S. litura* after exposure of *M. jalapa* extract. This research was conducted using Liquid Chromatography-Mass Spectrophotemeter (LC-MS) analysis to the identify the bioactive compounds from *M. jalapa* extract. The measured indicator of the immune response wasthe average of hemocyte and phenoloxidase (PO) enzyme analysis. The results showed that *M. jalapa* has 30 types of specific compounds. Three of the best compound with abundant composition of *M. jalapa* more than 6.00% (g/dry weight) were mirabijalone B, vulgxanthin I and miraxanthinI. The compounds were identified as potential rapellents to *S. litura*. Additionally, concentration of 0.2% *M. jalapa* extract induce the average of hemocytesof *S. litura* ($P<0.05$) as muchas23.98x106 cells/ml. The PO activity occurs after 1h treatment of M. jalapa extract in concentrations of 0.1% (309.00 IU/mg) and 2h in concentration 0.1% (592.33IU/mg), whereas the concentration of 0.8% (w/v)caused decrease in the PO activities. Therefore, *M. jalapa* has the potential to induce the immune mechanism, their active compound has been potentially used as a biological insecticide.

**Key words:** Biopesticide, Immune defenses, LC-MS, *Mirabilis jalapa, Spodoptera litura*.

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## INTRODUCTION

The quality and quantity of good agricultural products is a reason to satisfy the human food demands. However, pests become a major problem for the success of agricultural crops in many countries. *Spodoptera litura* F. (Lepidoptera: Noctuidae) is a dangerous polyphagic pest that causes damage to the plants. It has a high reproductive capacity with a wide distribution covering Indonesia, India, Japan, China and Southeast Asia (Sinti *et al*., 2009) and the South Pacific (Sparck, TC, 2014). *S. litura* is a widely distributed crop pest that has a significant impact on the productivity of economic crops (Meagher *et al*., 2008). *S. litura* has a host range in soybean, peanut, cabbage, sweet potato, potato, and mustard green. It's caused considerable economic damage to multiple agro-crops annually. As part of an ecosystem, *S. litura* exists and will be sustainable in the future able in the nature. Unfortunately, the resistance of *S. litura* to chemical insecticides make it more difficult to control the pest (Rehan &Freed, 2014 ). Therefore, we need an safer alternative to control their population.

The use of chemical insecticides to reduce the pests has a negative impact in the environment and non-target organisms (Kumar, 2015). This causes resistance in large population which results in damage to the nature (Bai *et al*., 2011; Leng, 2011). Therefore, biopesticide may be an alternative to preserve the organisms that are resistant to chemicals compounds. Botanical pesticides act as synergistic component in several IPM strategies (Srinivasan, 2012) because they come from natural products (Kandagal, 2011; Nathan, 2004; Leng, 2011). They have no effect to the chemical residues that harm non-target organisms, humans and the environment (Horne and Page, 2008; Vega and Kaya, 1993). The natural compounds from secondary metabolites often many benefits, one of them being botanical insecticides (Rattan, 2010). The effectiveness of the use of pesticides resulting from the natural synthesis of plants tends to be safer and user friendly (Owen, 2004).

One of the potential botanical insecticides to reduce the population of *S. litura* is *Mirabilis jalapa* L. (Caryophyllales : Nyctaginaceae) extract. It has benefits as an insect repellent (Maulina and Anggraeni, 2014; Suryani and Anggraeni, 2014). Previous studies showed that *M. jalapa* contains alanine which functions as an inhibitor of glutamate in the body of insects which disturbs the nervous and muscular systemic mechanism (Maulina *et al*., 2018). This plant contains antiviral and antiviroid activity compounds in the form of Ribosome Inactivating Protein (RIP) known as Mirabilis Antiviral Protein (MAP) (Vivanco, 1999).The activity and determination analysis of the concentration of *M. jalapa* plant extract to find the LD50 is concentration of 0.8% against *S. litura* (Maulina, 2014). The sub lethal concentrations of *M. jalapa* extract to treat the pest aims to prevent resistance. Kogan and Ortman (1978) classify the system of crop resistance to herbivorous insects into three, namely antisenosis, antibiosis, and tolerance. Therefore, *M. jalapa* biopesticide provides an opportunity to control *S. lituraalso*.

The immune system becomes the main role mechanism that occurs in the insect body. *M. jalapa* biopesticide provides an opportunity for weakening the immune system from *S. litura*. The potential of *M. jalapa* as a biological control agent for pest is not widely known. The results of previous studies revealed the effect of *M. jalapa* extract on the physiology effects of *S. litura* in the cellular immune response. This study aims to identify the bioactive compound in *M. jalapa* that potentially acts as biopesticide. The immune system is an indicator coming decrease of the body's defence mechanism. This happens because the defence mechanism becomes the body's barrier to exposure to foreign objects and its response. Mode of action of *M. jalapa* as a biopesticide is to damage the body defense of *S. litura*.

Generally, the insect has cellular and humoral defenses. The mechanism of humoral and cellular responses is to stimulate each other to perform their roles (Gillot, 2005; Chapman, 2009). Hemocyte acts as the main subject of cellular immunity to recognize, tolerate and eliminate the presence of foreign substance in the body The phenoloksidase (PO) enzymehas a crucial mechanism, because it plays a role in activating enzymes and stimulation of the introduction of foreign objects. It acts to stimulate the cellular immune system to run. Therefore, the humoral response in the form of PO enzyme is one of the parameters causing the death of larva. The effect of the immune response on insect death is the initial knowledge of compounds that have the ability as biopesticides. Finally, the results of this research can explain the potential use of *M. jalapa* as a biopesticide to adversely impact the immune system of *S. litura*.

## MATERIALS AND METHODS

### Extract of *M. jalapa*

*M. jalapa* as biological agents were obtained from the Lampung-Indonesia Province. The extraction process of *M. jalapa* leaves used maceration method. Initially, *M. jalapa* leaves washed and dried without exposure to the light for three weeks. Furthermore, dried leaves were mashed to become a powder weighting

45.08g. Next, the leaf powder was macerated using 96% ethanol. This process was carried out for three days. We replaced the maceration solution of *M. jalapa* leaves every 24 hrs. The ratio of the amount of leaf powder and solvent (ethanol 96%) was 500g of dried leaf powder extracted in six litters of ethanol solvent. The maceration solution was collected for further separation of solvent and dissolved compounds derived from *M. jalapa* leaves by using an evaporator.

Evaporation process obtained to achieve a paste as much as 48.08g, then it's assumed to 100% concentration. The multilevel dilution technique of *M. jalapa* was conducted to determine the concentration with the following calculation:

V1 x N1 = V2 x N2

Where V is volume and N is concentration of *M. jalapa*

This study used the sub lethal concentrations of 0.8, 0.4, 0.2 and 0.1% (w/v) of *M. jalapa* extract. The percentage of concentration was defined as the percent of *M. jalapa* extract weight (in grams) in the total volume of solution (100 mL ethanol).

**LC-MS Analysis**

LC-MS analysis of *M. jalapa* leaf extract used the LC-MS 2010 EV, Shimadzu with a reverse phase LC system, the ratio of a mobile phase of acetonitrile distillation water is 7: 3 and 10 mmol ammonium acetate. The technique of separating the LC pump gradient system (LC-10 ADVP) and the vacuum pump flow velocity were 1 mL/min. C-18 Shimadzu shimpack column volume in column dimensions: 150 mm x 2.1 mm with column temperature 40°C. The injection volume of the test material is 10 µL used a nitrogen generator at a gas temperature of 250°C. Mass spectrometry detector (SPD-10 AVP) was a positive ion Electrospray Ionization (ESI) technique.

**Culture of *S. litura* larvae**

*S. litura* insect larvae were collected from the Sweet and Fiber Crops Research Institute (BALITTAS) Malang-East Java. The fourth instars larvae used in this study larva were given a feed of green mustard leaves during their rearing and acclimated to the laboratory. *M. jalapa* extract is given by spraying on the entire leaf surface of each larvae measuring 5x5 cm2/larvae.

**Average of hemocyte analysis**

The average hemocytes analysis in *S. litura* larvae was carried out after being infected by *M. jalapa* leaf extract for 24 hrs. The hemolimph was taken using the technique of cutting the prolegs of larvae. Moreover, the hemolimph was collected on an ependorf microtube tube using a hematocrit capillary pipette. The turk solution as an anticoagulant mixed into the hemolimph solution in a ratio of 1: 1. A counting of hemocytes replicated five different larvae for each condition. It was analyzed using F test by the SPSS 20.0 software. Means were divided using the Tukey test with the significant difference test at $P \leq 0.05$

**Phenoloxidase (PO) analysis**

The collection of *S. litura* hemolimph mixed into a microtube followed by an anticoagulant solution in a ratio 1: 3. The anticoagulant composition was NaOH 3.92g; NaCl 10.87g; EDTA 6.33g; Citric acid 3.23g; and distilled water 1000 mL. Next, the solution was centrifuged for 15 minutes (4$^{o}$C; 800xg), to obtain pellet and supernatant.

The pellets obtained were washed using 2ml sodium cacodylate buffer (0.4 M sucrose), then suspended in 0.2 ml 0.01 M sodium cacodylate buffer containing 5 mM $CaCl_2$ and homogenized with piston homogenizer. The ingredients to make a cacodillate buffer (0.4 M sucrose) are sodium cacodillate (21.40g); sucrose (136.8 g); hydrochloric acid (HCl) to a regulate acidity to7; distilled water (1000mg); and $(CH_3)_2AsO_2H_{27}$ (6g). The composition of cacodillate buffer pH 7 ($CaCl_2$) was sodium cacodillate (21.40 g); $CaCl_2$ (0.55 g); HCl and distilled water 1000 ml. Next, the solution was centrifuged at a temperature of 4$^{o}$C (1000 x g) for 15 minutes to obtain pellet and supernatant.

The supernatant was used as a source of phenoloxidase enzyme analysis. Quantification of color changes for phenoloxidase is prepared with blank provisions, controls and treatments. The blank

consists of: 30µm distilled water, 30µm cacodylate buffer, 15µm L-DOPA; control consisted of 30µm distilled water, 30µm cacodylate buffer, 15µm L-DOPA. The treatments consisted of: 30µm distilled water, 30µm cacodylate buffer, 15µm L-DOPA, and 30 µm laminarin (heated using distilled water at 100 o C). L-Dopa used as a substrate that binds enzymes. Each solution was incubated for 1 hr, 2 hrs and 3 hrs. Furthermore, the absorbance of color changes was measured using a spectrophotometer on the A492 scale with the following calculation:

PO enzyme (IU/mg) = (Value from BioRad app)/(The number of protein)

PO = size of 30 µl (supernatant) x concentration of protein (Anggraeni, 2011).

## RESULT

### LC-MS of *M. jalapa* analysis

The results of LC-MS analysis show that *M. jalapa* has 30 specific types of compounds shown in Fig. 1. The whole compounds contained in *M. jalapa* plants is seen at the top of the data curve. Representation of the results of these compounds is stated in table 1. The data represent

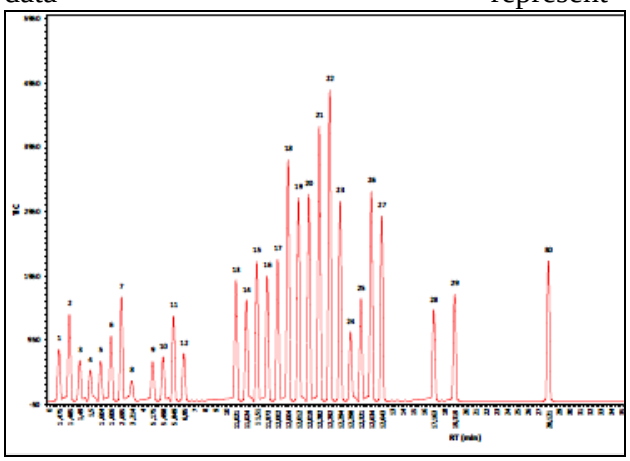

**Figure 1**.The chromatogram result of *M. jalapa* extract

the compound content of *M. jalapa* plant which is sorted by the composition percentage of the compound content from the highest to the lowest percentage. Mirabijalone compounds, vulgaxantin and miraxanthin are compounds with the highest percentage of content more than 6.00% (g/dry weight). Mirabijalone-B has 8.55% of the total compound content of other *M. jalapa*. Specifically, the mirabijalone compound was identified as a compound that is only present in the genus Mirabilis. Furthermore, vulgaxantin I compounds were 7.57% and miraxanthin was 6.64% having other high compound content sequences.

### Average of hemocyte analysis

Table 2 shows that the concentration 0.2% of *M. jalapa* was given the effect of increasing the total number of hemocytes. The amount of *S. litura* larval hemocytes was 0.2% at 23.98 x $10^6$ cell /mL compared to controls ($P < 0.05$). Hemocytes have changed after the being exposed of *M. jalapa* extract which shows the immune mechanism that works. The results of this study indicate that *M. jalapa* was able to induce a cellular immune system of *S. litura* where the dose concentration determines the number of cells that work.

### PO analysis

The humoral immune response works when exposed by *M. jalapa.* Figure 2 shows the average number of PO enzymes formed by *S. litura* after exposure to *M. jalapa* biopestsides ($P < 0.05$) compared to controls. In normal conditions the amount of PO in the body of *S. litura* is as much as 2.33 IU/mg of protein (Fig. 2).The interval of second and third hour after exposure, concentration of 0.1% *M. jalapa* had PO activity values of 592.33 and 512.33 IU/mg of protein ($P$ <0.05). The sub lethal concentration of *M. jalapa* which is 0.8% occurs at the second and third induction time intervals decreases PO activity, which is 349.00 and 282.33 IU/mg protein compared to given concentrations of 0.1% and 0.2%.

## DISCUSSION

Mirabijalone, miraxanthin and vulgaxanthin are compounds that are contained in large quantities and abundant in the leaves of the *M. jalapa* plant. The three of specific compounds are derivatives of compounds in the group of protein groups. Mirabijalone is a compound that is only found in plants with genus *Mirabilis*. Previous study showed that mirabijalone activated the caspase-3 pathway in the bodies of mammals (Linghu *et al*., 2014). Spodoptera has a functional similarity in caspase pathway insects; additionally the recipient receptors have similarities in the

caspase-3 pathway with mammals (Cooper *et al*., 2009).

**Table 1.**The content of the compound *Mirabilis jalapa* leaves

| Peak number | RT (min) | Similarity index (%) | Curve Area | Compotition (%) | Compound Analysis |
|---|---|---|---|---|---|
| 1 | 1.475 | 92 | 809.55417 | 1.42877 | β Ocimene |
| 2 | 1.485 | 92 | 1359.86614 | 2.40001 | Trigonelline |
| 3 | 1.49 | 92 | 639.71629 | 1.12903 | Methyl benzoate |
| 4 | 1.5 | 92 | 489.61825 | 0.86412 | Myrcene |
| 5 | 1.604 | 92 | 629.70016 | 1.11135 | Benzyl acetate |
| 6 | 1.606 | 92 | 1026.53717 | 1.81172 | Methyl salicylate |
| 7 | 2.695 | 92 | 1629.60916 | 2.87608 | Jasmone |
| 8 | 3.214 | 92 | 318.54432 | 0.56220 | Benzyl butyrate |
| 9 | 5.175 | 92 | 622.06971 | 1.09788 | Dihydroxyphenylanaline |
| 10 | 5.498 | 92 | 695.32162 | 1.22717 | α Farnesene |
| 11 | 5.849 | 92 | 1328.55812 | 2.34476 | Betalamic acid |
| 12 | 6.95 | 92 | 749.59172 | 1.32295 | Nerolidol |
| 13 | 11.021 | 92 | 1889.65828 | 3.33504 | Kaempferide |
| 14 | 11.024 | 92 | 1582.69376 | 2.79328 | Rhamnocitrin |
| 15 | 11.51 | 92 | 2179.47989 | 3.84654 | Indicaxanthin |
| 16 | 11.973 | 92 | 1957.79925 | 3.45530 | Velutin |
| 17 | 12.002 | 92 | 2217.84792 | 3.91426 | Isorhamnetin |
| 18 | 12.004 | 92 | 3759.76614 | 6.63557 | Miraxanthin II |
| 19 | 12.012 | 92 | 3168.58241 | 5.59220 | Boeravinone F |
| 20 | 12.019 | 92 | 3228.99448 | 5.69882 | Miraxanthin III |
| 21 | 12.282 | 92 | 4287.01551 | 7.56611 | Vulgaxanthin I |
| 22 | 12.292 | 92 | 4846.70152 | 8.55389 | Mirabijalone B |
| 23 | 12.294 | 92 | 3115.83299 | 5.49910 | Mirabijalone D |
| 24 | 12.298 | 92 | 1086.52727 | 1.91760 | 9 O Methyl 4 hydroxyboeravinone B |
| 25 | 12.321 | 92 | 1609.68192 | 2.84091 | Miraxanthin V |
| 26 | 12.634 | 92 | 3265.71592 | 5.76363 | Mirabijalone A |
| 27 | 12.643 | 92 | 2886.11927 | 5.0938 | Miraxanthin I |
| 28 | 17.163 | 92 | 1427.97152 | 2.52021 | β Sitosterol |
| 29 | 19.319 | 92 | 1665.11725 | 2.93875 | β Amyrin |
| 30 | 28.121 | 92 | 2186.58293 | 3.85908 | Mirabijalone C |

Note: The grey column shows the compound containing abundant compotition (¬> 6%)

**Table 2**. The average of hemocyte analysis in *M. jalapa*

| Concentration of *M. jalapa* (%) | The average of hemocyte ± SD (cell/ml x $10^6$) |
|---|---|
| Control | 11.78 ± 3.80 [a] |
| 0.1 | 14.86 ± 1.38 [a] |
| 0.2 | 23.98 ± 3.21 [b] |
| 0.4 | 8.14 ± 0.94 [c] |
| 0.8 | 2.81 ± 1.02 [d] |

Note: the following number with the different alfabethin the same coloum means the variable has a significant effect ($P$ <0.05).

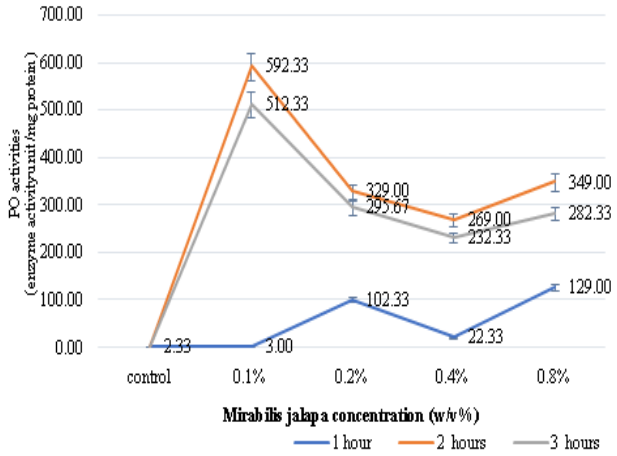


**Fig. 2**.The average phenoloksidase enzyme exposure *M. jalapa*

Caspase-3 is a metabolic pathway to induce apoptotic reactions. Apoptosis is a programmed cell death process carried out by cells of the body of an organism that aims to body defend itself. Apoptosis in insect naturally occurs by pathogens infected, therefore the cell will turn off itself dead and the pathogens in the body die too. When *S. litura* was exposed to mirabijalone compounds, the induced caspase-3 pathway led to increased apoptotic activity causing mass cell death. This mechanism has an impact on the weakening of the body of *S. litura* to decreasing the tissue and organs damage.

Vulganxanthin is a group of betaxanthin compounds in clumps of glutamine and amino acid derivatives. Groups in this class of compounds are beneficial for plant defence, because naturally these compounds protect plants from insect attacks. This compound is toxic so it is identified as a repellent compound (Harborne *et al*., 1999).

Miraxanthin is a yellow to orange alkaloid compound (orange) which is specifically found in plants with Cryophillales (Harborne *et al*., 1999). This alkaloid group of compounds is known to have insecticidal, antifeedan and inhibitor properties for insect developmental stages (Guo and Yang, 2013). The results revealed that the larvae of *S. litura* experienced physiological disorders that cause larvae to fail to move instar (molting) (Ge *et al*., 2015). This shows that alkaloid compounds cause developmental inhibition for larvae. The antifeedant properties of alkaloids cause malnutrition which leads to death with weakening characterization in immunity (Maulina, 2014).

The compounds above showed that *M. jalapa* contains highly larvacidal compounds. The results of LC-MS analysis have shown that the content of compounds with a high percentage and abundance of *M. jalapa* shows that this plant is functionally indicated as biopesticide (vegetable insecticide). Therefore, this plant is an alternative to controlling the *S. litura* insect pest.

Average of *S. litura* hemocytes changes when *M. jalapa* infected. It means the body defence works toward the toxins (pathogens) (Sanjayan *et al*., 1996). Increasing of hemocyte indicatedthe immune response of *S. litura* works (Pathak, 1993). The hemocyte produces a signaling to activate regeneration of new cells. Hemocyte will proliferaterapidly when the immune system is activated (Pathak, 1993). It was proven that the immune system in *Puspereus chrysocoris* was activated when induced by penfluront compounds which caused an increase in total hemocytes (Pugazhvend and Soundararajan, 2009). Additionally, *Dysdercus cingulatus* hemocytewas increased too when induced by achephate chemical compounds which are organophosporus insecticides (Qamar and Jamal, 1999).

Concentration of 0.2% *M. jalapa* is the optimum concentration to induce an immune response of *S. litura*. The concentration 0.4% and 0.8% gave the effect of decreasing the number of hemocytes compared to the control ($P<0.05$). This happens because the physiological conditions of *S. litura* larvae are already weak. It has resulted in the the proliferation of hemocyte decreasing ($P < 0.05$). Signaling of cells between hemocyte cells to be able to induce mitotic cell events is inhibited, this is caused by toxic compounds from *M. jalapa*. The disruption of the coordination and enzymatic systems inhibition of cells response which does not even occur because hemocytes do not recognize the foreign compounds that come to them.

The enzymatic reaction in the important humoral response series is activation of PO. This enzyme plays an important role in the mechanism of melanogenesis in invertebrates. This enzyme is the key in the formation of encapsulation in multicellular pathogens, repair of defence tissues against pathogens such as bacteria (grams + and -), fungi, viruses and other foreign body agents (Gonzales, 2011; Nappi and Christensen, 2005; Ashida and Brey, 1997; Boman, 1986). PO and dopa decarboxylase are the main mediators in the formation of melanization. Thus, PO is the main tool used by insects to fight several pathogens (Cerenius and Soderhall, 2004).

PO induction begins when the pathogens penetrate to the insects body, next the receptors activates the serine protease pathway to produces PAPs (Phenylalanine). It leads to mechanisms process of Pro-PO to PO. This activity is also depending on gender, life cycle, temperature and season, and species differences (Gonzales, 2011). The results of study showed that *M. jalapa* was able to induce *S. litura* to activate PO enzymes. It was means that *M. jalapa* extract acts as a foreign substance which induces PO activity in *S. litura*). The increase in PO enzymes proves that an immune response occurs in the body of the insect.

*M. jalapa* leaf extract has great potential to become an important bioinsecticide against *S. litura* as it stimulates cellular and humoral immune response in *S. litura* larvae. *S. litura* undergoes physiological changes after exposure to *M. jalapa* biopesticides . The results of this study prove that the sub-lethal concentration of *M. jalapa* has potential to be used as biopesticide to decrease the immune system of *S. litura* based on IPM, due to its capability to prevent the resistance from *S. litura* pest.

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