Histological changes in the midgut of *Spodoptera litura* larvae exposed by the extract of *Mirabilis jalapa* leaves

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**Abstract.** *Mirabilis jalapa* in Indonesia is better known as Bunga Pukul Empat (the four o'clock flower) which has the potential a natural insecticide to overcome pests in agricultural crops, such as *Spodoptera litura* insects. If *S. litura* larva is infected by toxic compounds, it will cause larval mortality which can be observed in the midgut (middle intestine). This research used a Completely Randomized Design of 4 treatments with 1 control and 4 times of iteration in each treatment using 0% (control), 0.2%, 0.4%, 0.8% and 1.6% (b/v) of *M. jalapa* leave extract concentration. The observation result of *S. litura* midgut histological structure showed that the higher concentration of *M. jalapa* leaf extract, the more damage occurred in its midgut tissue. Microphotography at a concentration of 0.2%; 0.4%; and 0.8% showed that some cells have undergone swelling, disintegration, and lysis. The worst damage was found at the concentration of 1.6%, in which the basal of epithelial cells undergone a wide range of disintegration, peritrophic membrane disappeared, all epithelial cell lysis, muscular layer became thin, the structure of basement membrane was indistinct, the form of goblet cells became irregular, and the longitudinal muscle disappeared. Based on the results, extract of *M. jalapa* has potential as a botanical biopesticide.

1. **Introduction**

The first paragraph after a heading is not indented. Indonesia is an agricultural country in which agriculture plays an important role in the entire national economy. From several sub-sectors, the growth of food crops sub-sector is the smallest as it only reaches 2.10% per year. The productivity of agricultural production factors decreases due to various problems such as climate, pests, and diseases [1]. One of the major causes of agricultural production problems is plant pest’s attack, which is mostly from insects. One of the most disadvantageous insects which are often encountered in the production of crops is *Spodoptera litura*. The insect is harmful to various plant species as it is polyphagous and has a wide range of hosts.

Pest control alternatives in the field can be conducted by developing biological products containing narrow-spectrum chemicals that are specific for the target organisms. One of the alternatives is to utilize the toxic compounds found in plants known as natural insecticides. Plant-based natural insecticides are generally interpreted as pesticides whose active ingredients are obtained from plants that are toxic to pests (Plant Disturbing Organisms) as it has secondary metabolites containing various bioactive compounds [2].
Mirabilis jalapa in Indonesia is better known as Bunga Pukul Empat (the flower of four o’clock). The plant is originated from South America and has been used as a traditional medicine for various diseases. It has the ability to survive in extreme climates, such as in areas experiencing a long dry season. M. jalapa is one of the plants that have the potential to be a natural insecticide, as the leaves of its flowers have been found to contain bioactive components, such as flavonoids, alkaloids, tannins, and saponins [3]. S. litura is belongs to the order of Lepidoptera which will be perished when exposed to toxic compounds. One of the causes of its death can be seen in its midgut (middle part of intestine), since it is the area which is not covered by chitin layer, while the foregut and hindgut is coated by chitin. Accordingly, toxic compound can be harmful for the larvae through its midgut.

The testing of various crops with various larvae pests have been conducted by researchers. However, the histology of the midgut part of S. litura larvae has not been published. Hence, it is considered as necessary to conduct a research about the effect of M. jalapa leaf extract on the histological structure of midgut in gastrointestinal tract of S. litura larvae, and also to investigate the life cycle length of S. litura in laboratory condition.

2. Materials and Methods

2.1 Experimental design
The study used Completely Randomized Design which consists of 4 treatments with 1 control, each with 4 replications. The tests were conducted by following the order of concentrations: 0% (control), 0.2%, 0.4%, 0.8% and 1.6% [4]. The sample of S. litura was gathered from the Lempake area of Sidodadi Village. Propagation of S. litura was performed in the Anila Ecology and Systematics of Universitas Mulawarman. The materials used in this study including M. jalapa, S. litura instar III, wild spinach leaves, honey solution 10%, NaCl 0.9%, Neutral Buffered Formalin (NBF) 10%, alcohol (70%, 90%, and 95%), absolute, paraffin, entellan, xylol, aquades, water, eosin, hematoxylin, and tissue.

2.2 Leaf Extraction of M. jalapa
M. jalapa leaves of 3 kg wet weight were washed and cut into small pieces before being exposed to wind in order to make it dry. After drying, the leaves were then milled by using a blender until the form changed into powder. The fine powder was then macerated with an ethanol solution of 96% for 72 hours, filtered, and then immersed with the same solvent until the solution is clear. The filtrate was then evaporated through a rotary evaporator in approximately 40°C until it became a thickened filtrate and the leaf extract of M. jalapa could be obtained. The testing was conducted through the leaf dipping method [5].

2.3 Preparation of S. litura midgut (Paraffin Method)
Dead S. litura larvae were subjected to a surgical process to remove its midgut. The midgut was then washed with a solution of NaCl 0.9% until it is free from blood and impurities. After washing, the midgut was inserted into the sample bottle containing the Neutral Buffered Formalin (NBF) 10% for the fixation process. The midgut was then cut with a thickness of 3-5 mm, placed into the slotted cassettes, and then inserted into the Tissue Processor spinning tool with the following stages: (1) re-fixation with NBF 10% for 3 hours, then (2) dehydrated with stratified alcohol solution from 70%, 90%, and 95%, respectively for one hour and absolute alcohol for 3 hours, followed by (4) immersion with xylol for 3 hours, then continued with (5) waxing for 2 hours. The next step is (6) embedding paraffin by using Paraffin Embedding Center EC-350. After the paraffin embedding stage is completed, the subsequent process is cutting by using a semi-automatic microtome with a thickness of 3 μ. Following the cutting process is Affixing, for the attachment of the sample on the object-glass. Paraffin deparaffinization can then be conducted by using a hot plate, followed by a staining process (tissue staining with Hematoxylin-eosin) by using the Slide Stainer tool HMS 70.
2.4 Staining process
The subsequent process was staining by using Slide Stainer HMS 70 with immersion stages of xylol, xylol, xylol, each for 5-10 minutes. It was then followed soaked in a stratified alcohol solution of 95% for 5 minutes, followed by 95% and 100% every 3 minutes, then soaked in aquades for 3 minutes, Hematoxylin for 3 minutes, eosin for 5 seconds, and then soaked with aquades for 3 minutes. The next step was soaking in stratified alcohol concentrations of 95%, 95%, absolute, and absolute alcohol, each for 3 minutes. It was then followed by soaking in the solution of xylol, xylol, and xylol, each for 3 minutes. After the staining process finished, the organ preparation was dried. The preparation was then inserted into xylol then spilled with entellan, and covered with a cover glass. The next stage was to dry the preparation from entellan, in order that it can be observed through microphotography technique. The organ histology gained from the observation through Zeis Tube microscope 2012 would then be scrutinized for the damage, based on the reference from books and research journals.

3. Results and Discussions

3.1 Results
Examination of M. jalapa leaf extract in this research was conducted through the leaf dipping method. The direct immersion method of feeding will cause the attachment of the botanical pesticide compound on the leaf which will then be given to the target insects as food [6]. The botanical pesticide residue attached to the leaf will be eaten and entered the body of targeted insects, thus the active ingredient of the pesticide also goes into the body of the insect. The working mechanism of the botanical insecticides is included in the group of stomach poison. Eaves that have been eaten will enter the S. litura intestine, in which the whole poisoning process will occur. The histological structure of S. litura midgut in the control treatment showed that the midgut structure was not damaged since the peripheral membrane and the epithelial cells were under normal circumstances. The growing regenerative cells indicate that the larvae will continue to grow (Figure 1). Midgut observations were performed using the 2012 Zeis Tube Microscope with 1024x1024 resolution.

The histological structure of control S. litura midgut showed that the midgut structure was not damaged during 48 hours of observation. Its constituent cells which consist of peritrophic membranes are intact, exist and firmly constrict the surface layer of midgut epithelial cells, the epithelial cells are arranged tightly adjacent to each other in the form of a high columnar column (cylindrical) with a blue-purple nucleus arranged in basement membrane side, a collection of regenerative cells attached to the surface of the basement membrane, muscular layers, microvilli, and goblet cells. In Lepidoptera, the midgut epithelium consists of four types of cells which are involved in the absorption and secretion of enzymes (columnar cells), ion homeostasis (goblet cells), endocrine function (endocrine cells) and the replacement of new epithelial cells (regenerative cells). Regenerative cells are undifferentiated and responsible for midgut epithelial regeneration that replaces damaged and lost cells during digestion [7]. Microvilli help in increasing surface area and nutrient uptake for the maximum absorption process.
This condition confirms that the integrity of the midgut structure enables metabolic activity to continue running smoothly due to the function of midgut as the site of absorption and enzyme secretion [7].

The presence of peritrophic membranes in Trichoplusia explained that peritrophic membranes are composed of insect intestinal mucin protein which is the largest protein contained by peritrophic membranes [9]. Spodoptera frugiperda midgut which was used as a control in the treatment of biopesticide using Porella chilensis algae showed a visible part of the peritrophic membrane, columnar cell, goblet cell, regenerative cell, longitudinal muscle, muscular layer, and lumen [10]. The parts contained in control midgut are microvilli, columnar cells, longitudinal muscles, peritrophic membranes, goblet cells, regenerative cells, nuclei, and lumen [11].

The histology of S. litura larvae midgut with extract concentration of 0.2% after a 48 hours treatment by using control comparison showed that some of the constituent parts were still clear, such as longitudinal muscles, muscular layers, basement membranes, and peritrophic membranes. At the extract concentration of 0.2%, regenerative cells have been formed as they play a role in the repair of damaged cells. The damage observed including the goblet cells which disintegrated, the epithelial cell nucleus epithelial which were swelling (edema) and lysis, while the control organ did not show such appearance. The lowest concentration of soursop leaf extract of 0.625%, the peritrophic membrane on the surface of the midgut wall is still visible as a solid protective layer [12]. Epithelial cells are arranged tightly adjacent to each other in the form of high columnar, and a collection of regenerative cells attached to the surface of the basement membrane.
There was no sign of damage found on midgut peritrophic membrane structure because the anonymous compounds contained in the soursop leaf extract acts as insect repellents, so they do not affect the peritrophic membrane structure of the tested larvae. The effect of *Azadirachta indica* oil dosage on *Spodoptera frugiperda* midgut tissue with the lowest concentration of 0.006% indicated that the goblet cells undergone necrosis, peritrophic membrane dislocated far from the epithelial layer, and epithelial cells became thinner and lysis [13]. The extract of *A. indica* and *M. jalapa* contained the β-sitosterol compound, whose properties are categorized as toxic with the lowest concentration used of *A. indica* and *M. jalapa* is 0.006% and 0.2%, respectively. When viewed from the midgut structure, the use of these concentrations of *A. indica* and *M. jalapa* had been observed to cause more damage compared with the use of soursop leaf extract.

Extracts of *A. indica* and *M. jalapa* contain β-sitosterol compounds, the properties of these compounds are categorized toxic with the lowest concentration used 0.006% of *A. indica* extract and 0.2% of *M. jalapa* extract when viewed from the midgut structure has been damaged compared with the extract soursop leaf.

Another property of β-sitosterol compounds is attractant (i.e., compounds that can attract insects to eat them). The effects of attracting properties increase the feeding ability of *S. litura* larvae. The phenomenon caused by the attractant property had caused death to be seen at each concentration, in which the higher the concentration the more larvae of *S. litura* had been found dead. This is supported by the morphometry of midgut *S. litura*, the higher the extract used from 0.2%, 0.4%, 0.8% and 1.6%, the more damage is experienced. The use of 0.4% *M. jalapa* leaf extract (Figure 3) showed that there were still longitudinal muscles and an intact muscular layer. However, damage was observed through the goblet cells which undergone a widespread disintegration of, basal membranes that begin to disappear, and columnar epithelial cells which undergone congestion (edema), cells undergone lysis and regenerative cells were not present at a concentration of 0.4% compared with control, while in concentration of 0.2% regenerative cells were still present (Figure 2). The midgut tissue morphology of *Anticarsia gemmatalis* larvae using Neem Seed Kernel Extract (NSKE) treatment with a dose of 500 ppm for a 2 days treatment had found that goblet cells disintegration and peritrophic membrane lysis could be observed through the treatment [14]. The result of midgut cross-section with *M. jalapa* leaf extract concentration of 0.4% show much better damage compared to the use of NSKE with 500 ppm dose.

The midgut structure of *S. litura* larvae after 48 hours of treatment with *M. jalapa* leaf extract concentration of 0.8% (Figure 4) showed a wider range of damage compared to control, 0.2% and 0.4% concentrations, in which peritrophic membranes began to disappear, many goblet cells disintegrated, all epithelial cells undergone lysis which is characterized by the swollen of cell nuclei (edema), cell degeneration zones increased, longitudinal muscles disappeared and basement membrane structure was not apparent.

The results of *S. litura* midgut observation with *M. jalapa* leaf extract of 1.6% concentration (Figure 5) showed that midgut tissue constituents such as goblet cells, epithelial cells, and regenerative cells were not found. The normal midgut tissue constituents were not present due to cellular damage, such as goblet cell disintegration which was observed throughout the midgut tissue causing irregular goblet cells shape, all epithelial cells have undergone lysis, muscular layer became thin, the longitudinal muscle disappeared, and the basal membrane structure could not be observed. This was the most severe damage compared to control and the concentrations of 0.2%, 0.4%, and 0.8%.

### 3.2 Discussion

The effect of *Sclerotium rolfsii* lectin (SRL) with the highest concentration of 0.06% on midgut tissue showed that peritrophic membrane still appeared intact, there were regenerative cells, the longitudinal muscle was still visible, and basal membrane still appeared intact. The damage obtained was still in the moderate category, in which epithelial cells have undergone swelling (edema), many goblet cells undergone lysis and many epithelial cells undergone lysis. When observed the effect of *M. jalapa* leaf extract of 1.6% concentration, the damage obtained was much better than the SRL [15].
Spodoptera frugiperda midgut which was treated with a concentration of 100 μg/g biopesticide from Porella chilensis algae. The result of observation showed that midgut tissue constituents such as epithelial cells, peritrophic membrane, goblet cells, and regenerative cells were not found [10]. The visible damage including the lysis of peritrophic membrane, the disintegration of goblet cells, and disappearance of basal membranes. The observed effects were similar to the effect caused by treatment with M. jalapa leaf extract at a concentration of 1.6% (Figure 5).

Figure 4. Histological Structure of Spodoptera litura larva midgut; (A) Control, (B) M. jalapa extract concentration of 0.8%. Magnification 10x10. Staining: Harris Hematoxylin-Eosin (HE). Description: membrane peritrophic (PM), regenerative cell (RC), longitudinal muscle (LM), basal membrane (BM), epithelial cell (EC), goblet cell (GC), muscular layer (ML), microvilli (MV), Cell Nuclei Edema (CNE), disintegration (D), cell lysis (CL) and lumen (L) in the middle.

Figure 5. Histological Structure of Spodoptera litura larva midgut; (A) Control, (B) M. jalapa extract concentration of 1.6%. magnification 10x10. Staining: Harris Hematoxylin-Eosin (HE). Description: membrane peritrophic (PM), regenerative cell (RC), longitudinal muscle (LM), basal membrane (BM), epithelial cell (EC), goblet cell (GC), muscular layer (ML), microvilli (MV), disintegration (D), cell lysis (CL) and lumen (L) in the middle.

The absence of regenerative cells in the midgut tissue constituents at the concentrations of 0.4%, 0.8%, and 1.6% can be inferred that the compounds of β-sitosterol, tannin, saponin, terpenoid and flavonoids of M. jalapa leaf extract have damaged the histology structure of the midgut gastrointestinal tract. Thus, its constituent tissue cannot regenerate and the damage would cause death in larvae. The properties of M. jalapa compounds are attractant (i.e. compounds that can attract insects to eat them). The effects of attracting properties had caused the increase of feeding power in S. litura larvae. The phenomenon of attractive properties had caused larval death to be seen at each concentration, in which the higher the concentration the more S. litura larva would be found dead. This was supported by S. litura midgut morphometry, in which the higher the extract used from 0.2%,
0.4%, 0.8% and 1.6%, the more damage was experienced. Therefore, *M. jalapa* has the potential to be used as a botanical insecticide to control pests in plants effectively and environmentally friendly. Botanical insecticides are categorized as toxic if it is able to kill *S. litura* larvae within 48 hours. The smaller the concentration needed to kill the larvae, the more toxic the compound for the tested animal.

The effect of *M. jalapa* extract used as biopesticide along with *Beauveria bassiana* fungi in *Crocidolomia binotalis* larva midgut showed that terpenoid on *M. jalapa* leaves has a role in inhibiting feeding activity as a physiological disorder condition [16]. The physiological disorder conditions will affect the immune system by lowering hemoxin and lectin, thus facilitating other pathogens to invade the host body. The peritrophic membrane starts from the foregut section to the midgut, and ends in the hindgut. These membranes are transparent and have different thicknesses along the digestive tract and separate food from intestinal epithelial cells. It is not connected to the epithelium and there is a space between the two layers (ectoperitrophic space) which is filled with secretions of cells, granular cells, stem cell secretion products synthesized by epithelial cells, and also the solution taken from the food which is filtered by peritrophic membranes [13].

It is known that only the peritrophic membrane of the midgut part which is not coated by chitin. Thus, the midgut can experience severe damage compared to the foregut and the hindgut. Moreover, the midgut serves as the site for food absorption, thus it is the ideal site to observe the damage of its constituent tissues. The result of *S. litura* larva midgut cross-section through leaf dipping method using wild spinach leaves which have been given *M. jalapa* leaf extract with the highest concentration of 1.6% showed the most severe damage compared to the concentration of 0.2%, 0.4%, and 0.8%. The highest number of larval mortality occurred at a concentration of 1.6%, which was indicated by the occurrence of larval death since the first two hours of treatment. Death increased gradually until 48 hours of treatment. Morphological changes in proleg legs which are used for walking became smaller and dysfunctional. The proleg did not stick strongly on the leaves’ surface when it was moved, while normal larva did not fall from leaves surface when moved.

This confirms that the presence of peritrophic membranes also serves as midgut protection against strong damage caused by toxic food particles. The occurred damage increased along with the increase of the extract concentration used. The higher the concentration of the extract, the more active ingredients it contains. Hence, the resulting damage would be more effective as shown in Figures 3, 4, and 5. *S. litura* is a group of lepidopteran orders that metamorphosed perfectly (holometabola). The occurred changes were not only due to internal factors but also due to unexpected external factors such as the pathogenic substance of toxic *M. jalapa* leaf extract. In addition, the combination of leaf *M. jalapa* extract and entomopathogenic fungi *Metarhizium anisopliae*, can cause the highest mortality level of *S. exigua* 87% with the combination interval of 6 hours [4].

One of the compounds in *M. jalapa* leaf is alkaloids which serve as an antimicrobial by disrupting peptidoglycan components in bacterial cells. Thus, the bacterial cell walls cannot be formed completely and it will lead to cell death. Saponins act as an anti-inflammatory by inhibiting prostaglandin dehydrogenase pathways. Tannins are astringent compounds with a bitter taste from its polyphenol groups which can bind and precipitate proteins [17]. Tannin works through the disruption of cell membrane permeability by causing the wrinkling of the cell membrane. The disruption of cell membrane permeability impedes the exchanges of substances needed for cell survival. Thus, cell growth will be inhibited and finally die. Several types of active secondary metabolites which serves as pesticide include saponins, alkaloids, and tannins. These chemicals can act as inhibitors of insect growth, antifeedant, and repellents for insects.

The absorption of *M. jalapa* leaf extract into the body of *S. litura* can be promoted by first soaking the leaves of wild spinach in *M. jalapa* leaves extract for 15 minutes. Then, let the leaves to be eaten by *S. litura*. When the leaves go through its digestive tract, particularly when it reaches the midgut part, an alkaline intestine enzymatic reaction will cause the malfunction of the intestine tract and body cavity, midgut tissue structure undergo histological damage, and the larvae become physiologically inactive. Hence, its body become pale and oily. After 1 -2 days, the larvae will be die and releasing polyhedral fluid which will infect healthy larvae.
4. Conclusion

The histological structure of *S. litura* larva midgut undergone more damage on midgut tissue constituent when the concentration of *M. jalapa* leaf extract was higher. The observed microphotography at a concentration of 0.2%; 0.4%; 0.8% showed some cells undergone swelling, disintegration, and lysis. Meanwhile, the worst damage was found at the concentration of 1.6% which showed epithelial cells in the bottom undergone wide disintegration, peritrophic membrane disappears and loosen, all epithelial cells lysis, muscular layer became thinner, the structure of basement membrane was not visible, the form of goblet cell become irregular and longitudinal muscles disappear.

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