Biolarvicidal Effects of Papaya Leaves Juice Against *Aedes Aegypti* Linn Larvae

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Abstract

Generally, the control of *Aedes aegypti* mosquito has been achieved using the lethal ovitrap. Later, it is found that the papaya leaves juice inhibits the development of eggs into larvae and also larvae into pupae. This study was conducted to determine the larvicidal effect of papaya leaves juice against the larvae of *Aedes aegypti* and to analyze the phytochemicals compounds of leaves extract. Phytochemical assays were performed by UV-Vis Spectrophotometry, Thin Layer Chromatography (TLC), and High-Performance Liquid Chromatography (HPLC). The results of the analysis showed that LC_{50} and LC_{90} of the papaya leave juice to instar 1 larvae were 4.1% and 15.5%, respectively; whereas the LC_{50} and LC_{90} to instar 3 larvae were 10.6% and 18.3%, respectively. Moreover, the results of the phytochemical assays showed that papaya leaves juice have 0.25% alkaloids, 0.14% flavonoid, 0.30% saponin, ≤68 mg/L steroid, and 11.34% tannin. In conclusion, papaya leaves extract work with its compounds in serving the biolarvicidal effects against the larvae of *Aedes aegypti*.

Experimental article (J Int Dent Med Res 2019; 12(2): 780-785)

Keywords: *Aedes aegypti*, Larvicide, Lethal ovitrap, Papaya leaves juice.

Received date: 27 August 2017 **Accept date:** 26 October 2018

Introduction

Dengue Hemorrhagic Fever (DHF) is one of the vector-borne diseases and has the potential to cause the outbreaks. DHF is caused by dengue virus and transmitted by *Aedes aegypti* Linn as the primary vector and *Aedes albopictus* Linn as the secondary vector. DHF is a deadly disease, which is easily developed into an outbreak in urban areas with high population density and poor sanitation. In the world, the incidence of DHF has also increased very rapidly in recent years. It is estimated that 3.9 billion people in 128 countries are at high risk for dengue virus infection, of which 390 million people are infected with DHF each year, and 96 million of which exhibit severe disease manifestations.[1]

In Indonesia, DHF is still a major health problem. In 2014, there are 100,347 cases of DHF, IR 39.8/100,000 population, with the number of deaths reaching 907 people, Case Fatality Rate (CFR) 0.9%. The incidence of DHF in Indonesia increased significantly, from 0.05/100.000 in 1968 to 40/100,000 in 2013. The highest epidemic incidence occurred in 2010 with 86 cases of dengue per 100,000 population.[2]

Until now, temefos is still used mostly to eradicate mosquito larvae. Temefos is a chemical compound that serves as a larvicidal, which has several disadvantages such as can pollute the environment, causing insecticide resistance, causing environmental imbalance, harmful to human and other non-target organisms.[3] It is the urge to develop natural constituents such as natural plant-based larvicides to reduce the impact of the use of chemicals as larvicide such as temefos. It is because of the plant has potentially bioactive chemicals.[4,5,6] Moreover, the natural larvicides have several advantages such as easy to degrade by nature, not causing chemical resistance, and harmless to other organisms.[7,8]
One of the plants that contain the potential active ingredient as larvicide is papaya. Papaya is included in the genus Carica, from the plant family Caricaceae. Papaya leaves contain papain enzyme, which is a proteolytic enzyme. This enzyme works by denaturing the protein body by

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cutting its peptide bond; therefore, when it is applied as the larvicide, it will kill larvae by damaging the protein on the surface of its body.[2,9]

The ethanol extraction on a combination of papaya seeds and leaves and produced it in the form of granule. The extract granule was applied on the *Aedes aegypti* larvae. The results showed that Lethal Concentration $(LC)_{90}$ was obtained at a concentration of 150 ppm within 48 h. LC_{90} is the minimum dose required to kill 90% of experimental animals. Insecticides are said to be effective when they reach LC90%. However, this method of extraction using ethanol and the production of papaya extract in granule form is costly. Therefore, it is necessary to find the alternative method applying water extraction on the papaya leaves to obtain its larvicidal effect. Based on the previous study, papaya leaves have the alkaloids, flavonoids, terpenoids, saponins, steroids, and tannins.[2] In this study, the effectiveness of papaya leaves extract against larvae of Ae.aegypti which is extracted by water was investigated. To attract mosquitoes to lay eggs, researchers used the water-soaked rice straw.

Materials and methods

Plant Materials - The main ingredient of biolarvicide is papaya leaves (*Carica papaya* Linn) which were collected from Gunungpati Semarang, Indonesia. Papaya leaves were gathered in the early morning by separating the old dark green leaves from the stems. The early morning is the best time to collect the leaves since the plant has not performed the chemical process of photosynthesis. Therefore, the chemical content is still relatively the same. In this study, the leaves (lamina without petioles) were extracted. The chemical constituents of papaya leaves which were extracted using water extraction were investigated by the previous study. The result showed that larvacide contain of papaya leaves juice are alkaloid 0.25% b/v, flavonoid 0.14% b/v, saponin 0.30% b/v, steroid ≤68mg/mL, and tannin 11.34% b/v (Cahyati, 2017).

The rice straw was employed in this study to attract the mosquito to lay its eggs. The straw used was derived from Inpari-19 rice crops. The results of phytochemical analysis of hay infusion are ammonium (NH_4) 12.75mg/L and lactic acid <1.20ppm.[2]

Preparations of Biolarvicide Extract and Hay Infusion **-** The biolarvicide extract was prepared by blending 1 kg fresh papaya leaves with 1 litre water. Then, the mixture was left for 1 hour at room temperature in a sealed plastic container. Subsequently, it was filtered to separate the papaya leaves with its aqueous phase. After the filtration process, the papaya leaves juice was then poured into the plastic container, and it was stored in the refrigerator cabinet at a temperature of 10-15 ºC.

The hay infusion was made by cutting 125 g of dried rice straw into small pieces (± 3 cm^2) to attract the mosquito to lay its eggs. Then, it was soaked in 15 litres water for 7 days. After submerging, it was filtered and 100% hay infusion was obtained. The soaking water was then mixed with water until reaching the desired concentration. Usually, the concentration used as an attractant is 10%. 10% hay infusion could be obtained by mixing 15 litres of 100% hay infusion with 135 litres water.

Analytical Conditions **-** In this study, instar 1 larvae and instar 3 larvae were placed in a plastic bowl, where each bowl contained aqueous extract of papaya leaves with different concentrations. Each bowl was filled with 25 larvae. The death of larvae was then observed periodically at 5, 10, 15, 30, 45, 60, 120, 180, 1440 mins. pH and temperature of the medium in each bowl were measured. The ambient temperature was also determined to ensure that the larval death was not caused by the environmental conditions.

Aedes aegypti Larvae Hatching- The eggs of *Aedes aegypti* was obtained from the area of Yogyakarta Province, Indonesia, using ovitrap. Paper strips were placed slightly above the water level on the ovitrap. Installation of ovitrap was done for 5 days. Ovistrip with eggs attached was then taken to the laboratory; where temperature, humidity, lighting were set according to the optimal conditions for the development of mosquitoes. Ovitstrip was placed in the basin, and it was soaked in water. After 3-6 days, the eggs hatched into larvae. The larvae then were moved to the bowls placed in mosquito coils and were fed by dog food as larvae food. After 7-10 days, the larvae initiated to turn into mosquitoes. After 13 days, the unhatched larvae were discarded. The mosquitoes were fed by the blood of Tikus Putih (*Rattus norvegicus*) and sugar water every day. A basin of water was provided for a place to lay the eggs for mosquitos inside the cage. After the mosquitoes lay its eggs (F1), and then it hatched into larvae, this larva was used as the subject of this study.

Instar 1 larvae and instar 3 larvae were used in this study, since it is easy to die when it is applied by the larvicide, especially instar 1 larvae. Also, the low dose of larvicide can be applied considering the instar 1 larvae as the most vulnerable phase; therefore, it is more beneficial. In addition to instar 1 larvae 1, the researchers used instar 3 larvae. The reason for the selection of instar 3 larvae is because the larvae eat and have a strong enough condition compared to instar 1 and instar 2 larvae in this phase. Researchers did not choose instar 4 larvae since it is the phase of preparation to be pupa. Pupa phase is the fasting phase where the larvae do not eat; therefore, the use of larvicidal is not effective as a stomach poison.

In this study, the larvae of *Aedes aegypti* mosquito used were instar 1 and instar 3 which were obtained from the breeding center in the parasitology laboratory of Gadjah Mada University, Yogyakarta. The purpose of the use of larvae from the laboratory was to obtain the larvae with a homogeneous condition; therefore, the certain reason of death can be observed that the same thing causes it.

Larvicidal Activity **-** The larvae of *Aedes aegypti* used in this study were instar 1 and instar 3 larvae. This study was conducted through two stages of the test to determine the effective dose of papaya leaves juice as a biolarvicide. For the study on instar 1 larvae, the first stage used 6 treatments and 1 control, i.e. concentrations at 32%, 16%, 8%, 4%, 2%, and 1%. While for instar 3 larvae used 6 treatments and 1 control as well, i.e. concentrations at 25%, 20%, 15%, 10%, 5%, and 1%. The mixture was made by diluting papaya leaves juice with the hay infusion. The control used was the hay infusion without biolarvicide addition. It serves as a regular environment which does not cause the larval mortality. This step was done by 4 replications.
In the first stage of testing, 700 instars 1 larvae (7 treatments x 4 replicates x 25 heads per cup) and 700 instars 3 larvae (7 treatment x 4 replicates x 25 heads per cup) were used. For the next stage of research, 800 instars 1 larvae (8 treatments x 4 replicates x 25 heads per cup) and 800 instars 3 larvae (8 treatments x 4 replicates x 25 heads per cup).

Based on the results of this first stage of research, the LC_{80} and LC_{20} were determined; and then, the dose for the second stage of research was determined using the increment formula. The results from the research stage 2 were applied to determine LC_{50} and LC_{90} using probit test.

The death of the larvae was determined by providing a touch stimulus within 30 minutes. When there was no motion as a response; then, the larvae were transferred to clear water and were given a touch stimulus. If there was still no motion response, the larvae were confirmed to be dead.

Results

The state of the environment was controlled to control the confounding variables. Factors that are expected to affect larval life are water pH, water temperature, and humidity. In this research, from initial until final research, pH of the papaya leaves juice in all treatments tended to increase in acidity and the pH was in the range of 6.1-7.0. In fact, the optimum pH for the breeding of *Aedes aegypti* mosquitoes is varied from 5.8-8.8. It shows that the difference in the number of dead larvae in the 8 types of concentration was not caused by the pH of the solution. Moreover, the temperature measurement shows that the temperature in each treatment group and the control group were the same where the initial temperature and the final temperature was at 25ºC. It showed that the attractants' temperature did not cause the difference in the number of dead larvae at 8 different concentrations. According to the results of the air humidity measurement at the time of the research at the initial and the final stage, it showed the same level of air humidity which was equal to 60%. It shows that air humidity did not cause the difference in the number of dead larvae in the seven types of concentration.

The doses used for the instar 1 larvae were 2.8%, 4.2%, 6.5%, 9.9%, 15.1%, and 23.1%; with diluents using 10% hay infusion. The control used 10% hay infusion without the addition of papaya leaves juice. The results obtained during 24 hours of observation are shown as follows:

Based on the data shown in Table 1, it can be seen that the death of instar 1 larvae has started since the first 5 minutes. At doses of 15.1% and 23.1%, it experienced more than 50% death in 180 mins (3rd hour). After 24 hours (1,440 mins), the dose at 4.2% showed that the larvae which experienced death were more than 50%. In the treatment of water and 10% hay infusion, there was no larval mortality found after 24 hours.

A probit test was used to see the dose required to cause 50% mortality (LC_{50}) and 90% mortality (LC_{90}) to examine the effectiveness of a material as a larvicide. The result of probit test shows that the LC_{50} value of papaya leaves juice was 4.1%. It means that the concentration of papaya leaves juice that killed 50% of the tested larvae was at 4.1%. The LC_{90} value of papaya leave juice was 15.5%. It means that the concentration of papaya leaves juice that killed 90% of tested larvae was 15.5%.

In accordance with the provisions of WHO to analyze whether papaya leaves extract can be used as a larvicide, a series of tests was conducted to determine the effectiveness of papaya leaves extract as the larvicide against *Aedes aegypti* instar 3 larvae. The reason for selection of instar 3 larvae as the target test is because the size of larvae is large and has a stable body condition comparing with instar 1 and 2 larvae. Also, it has a high appetite; therefore, it is easy to absorb the larvicide. Researchers did not use instar 4 larvae because it was nearly transformed into the pupa. In pupa phase, the larvae will stop their eating habit. It is feared the appetite drops or the larvae turn into pupa at the time during research. This test was intended to find out how the killing effectiveness of papaya leaves extract against instar 3 larvae.

A probit test was used to see the dose required to cause 50% mortality (LC_{50}) and 90% mortality (LC_{90}) to see the effectiveness of a material as a larvicide. The result of probit analysis showed that the LC_{50} value of papaya leaves juice was 10.6%. It means that the concentration of biolarvicide that killed 50% of the tested larvae was 10.6%. The LC_{90} value of papaya leaves juice was 18.3%. It represents that the concentration of biolarvicide that killed 90% larvae was 18.3%.

Dose of Papaya Leaf Juice	Time (second)								
	5'	10'	15'	30'	45'	60'	120'	180'	1.440'
2.8%	2	2	3	3	3	6	6	7	40
4.2%	0	1	2	2	6	8	8	9	55
6.5%	0	0	1	3	14	16	16	16	60
9.9%	1	2	4	4	4	4	4	7	76
15.1%	0	12	12	19	26	38	44	52	88
23.1%	0	7	22	52	68	80	90	94	100
10% Hay Infusion	0	0	0	0	0	0	0	0	0
Water	0	0	0	0	0	0	0	0	0

Table 1. Percentage of dead instar 1 larvae based on time period.

Dose of Papaya Leaf Juice	Time (second)								
	5'	10'	15'	30'	45'	60'	120'	180'	1.440'
4.9%	0	4	4	4	5	5	5	5	7
6.9%	1	1	3	3	6	6	6	6	10
9.6%	0	2	2	3	7	7	7	11	35
13.5%	0	0	0	13	18	31	37	39	81
18.8%	0	0	11	18	23	23	40	45	91
26.4%	0	5	5	12	32	19	46	60	97
10% Hay Infusion	0	0	0	0	0	0	0	0	1
Water	0	0	0	0	0	0	0	0	0

Table 2. Percentage of dead instar 3 larvae based on time period.

Discussion

Phytochemical test of papaya leaves juice using UV-Vis spectrophotometer shows that the extracts contained 0.25% alkaloids, 0.14% flavonoids, 0.30% saponin, and 11.34% tannins. Moreover, the phytochemical test using thin layer chromatography (TLC) method resulted that it had steroid ≤68 mg/mL, whereas terpenoid was not detected. This result is in accordance with research conducted by S Dewi Astuty 2017 which states that papaya leaves contain alkaloids, triterpenoids, steroids, flavonoids, saponins, and tannins. Other studies have also shown that the papaya leaves extract contains alkaloids, phenols, flavonoids, and amino acids.[10,11]

The chemical constituents contained in the extract of papaya leaves such as flavonoids, tannins, saponins, steroids, and alkaloids serve as a botanical insecticide, and it has the entomotoxicity (toxic to insects). Chemical compounds eradicate the eggs that can not hatch itself. Also, it can inhibit the process of organ completion from eggs to instar 1 larvae, so that its development becomes imperfect, the larvae can not survive and eventually die. Papaya leaves extract also contains juvenile hormones substances in which the element affects the titer of juvenile hormone inside the *Ae. aegypti* causing an abnormal development and can also affect the egg hatching. [2,12]

The process that interferes the development or inhibits the hatchability of *Aedes aegypti* is thought to be the introduction of active insecticidal substances into the egg. This process takes place on the surface of the shell on the polygonal points through the diffusion process. It can be said that this process occurs on the entire surface of the egg. The insecticide in water which is potential active substance starts to enter the egg since the environment outside the egg is in the state of higher concentration (hypertonic) than the water potential inside the egg (hypotonic). With the introduction of potentially effective insecticides into eggs, it interferes the metabolic process, which can eventually interfere with the development of eggs or other harmful effects.[13,14]

Flavonoids and alkaloids have juvenile hormone activity that affects the development of insects from eggs into larvae. It is in accordance with research conducted by (Aarti et al., 2018; Sara, 2016) which states that flavonoid compounds can cause the eggs of *Aedes aegypti* can not hatch into larvae. [15,16] Alkaloids contained in the extract of papaya leaves can stimulate the endocrine glands to produce the ecdysone hormone. Increased ecdysone hormone leads to failure of metamorphosis.[17,18]

A study conducted by Julien (2017) shows that the newly hatched larvae (instar 1 larvae) have body conditions that tend to be susceptible, the organs of the body are not ready to function fully.[19] Therefore, it can not survive when exposed to alkaloid-containing plant insecticides. In the newly hatched larvae, the head is still wrinkled, triangular and not wider than the thorax, the comb to feed on the labrum is still tucked into the preoral cavity. The larvae will soon suck water to contract their thorax and abdomen, pushing hemolymph into the head that will dilate more than thorax, the expansion mainly occurring in the frontal. Furthermore, the head will grow wider and darker. Comb for the eating function will grow and ready to work. The condition of the larvae proves that alkaloids inhibit the process of organ completion in instar 1 larvae make its development becomes imperfect, the larvae can not survive and eventually die.

Saponin is an entomotoxicity that can inhibit the development of eggs into larvae by destroying the egg membrane. By this eradication, the other active compounds will enter the egg and cause developmental disorders in the eggs of mosquito that resulted in the failure of the eggs to hatch into larvae. Saponin will bind to the aglycons of the flavonoid acting as ecdysone blockers which also play a role in inhibiting the development of eggs into larvae.[20]

The content of steroids in papaya leaves extract protective functions, such as phytoecdyson which has a structure similar to insect molting hormone; therefore, the steroid content can inhibit larval molting process if ingested. The presence of steroids will affect the thickening of the chitin cell wall on the surface of insect body; then, the insects become abnormal. Steroids lead to increased rate of cell extension in larval deaths by steroid treatment.[12]

Chemical constituents in the extract of papaya leaves also serve as a larvicidal. Research conducted by Oche et al., (2016) shows the result that papaya infused contains the alkaloid, tannin, flavonol, and papain which have the larvicidal effect on *Culex sp*.[11] The chemical content of alkaloids and steroids proven to kill *Aedes albopictus* larvae. Papaya leaf extracts containing carcinic alkaloids, papain enzymes, tocophenols, flavonoids, saponins, and tannins also have the larvicidal effects on mortality of *Anopheles sp* larvae. Other studies have also shown that the chemical content of alkaloids, flavonoids, saponins, tannins, sterols, and triterpenes have larvicidal effects on *Aedes aegypti.*[13] These studies prove that chemicals such as alkaloids, tannins, saponins, steroids, and flavonoids in the extract of papaya leaves serve as larvicide.

The effects of larvicide on papaya leaves extract obtained from alkaloids, flavonoids, saponins, tannins, and steroids. These components can act as juvenile hormones, where the hormone can affect the abnormal development time and can also affect the eggs hatching of the mosquito. The active substance also has the potential to diffuse into the egg, because the egg is in a hypertonic environment rather than hypotonic state inside the egg. With the introduction of potentially powerful insecticides into eggs, it interferes with the metabolic process, which can eventually interfere with the development of eggs or other adverse effects, and even death. The results showed that the concentration of papaya leaves extract that killed 50% tested larvae (LC_{50}) was 4.1% and the concentration of papaya leaves extract that killed 90% test larvae (LC_{90}) was 15.5%. The higher dose required to kill instar 3 larvae. Based on the probit analysis, it showed that for instar 3 larvae, the papaya leaves extract concentration that killed 50% test larvae (LC_{50}) was 10.6% and papaya leaves extract concentration that killed 90% test larvae (LC_{90}) was 18.3%.

Conclusions

The results of papaya leave juice analysis shows that it contains alkaloids, flavonoids, saponins, tannins, and steroids. The chemical constituents act as a botanical insecticide and larvicide. It also has an entomotoxicity activity where it reaches the LC_{50} and LC_{90} against instar 1 larvae at 4.083 and 15.332, respectively; whereas LC_{50} and LC_{90} against instar 3 larvae were at 10.682 and 18.623, respectively. Since in the form of liquid the chemical molecules are easy to be degraded, it is expected to formulate the biolarvicide in granular preparation to make it more durable in the future.

Acknowledgements

The authors would like to thank the Head of Laboratory and Technician of Integrated Research and Testing Laboratory of UGM and Technician of Parasitology Laboratory of Faculty of Medicine UGM.

Declaration of Interest

The authors report no conflict of interest.

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