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The Potency of Pomegranate (Punica granatum L) Seeds Ethanol Extract as Larvacidal on *Aedes aegypti larvae*

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Abstract

In the present study, we investigated the potency of *Punica granatum* L seeds ethanol extract in against *Aedes aegypti* larvae. The research was completely randomized design with 8 treatment groups. Each group contained five times repetition using 20 third instar larvae of *Aedes aegypti* with concentrations of *Punica granatum* L seeds ethanol extract are 0, 10, 20, 40. 80, 160, 320, 640 ppm. The observation and recording of larval mortality were done for 12 hours and 24 hours. Probit analysis was used to calculate the LC_{50} and LC_{90}. . *Punica granatum* L seeds ethanol extract showed the most mortality toward the larvae of Aedes aegypti with LC_{50} = 146.9 ppm; LC_{90} = 374.7 ppm after 12 h, and LC_{50} = 84.8 ppm; LC_{90} = 177.3 ppm after 24 h. From the results of this study concluded that *Punica granatum* L seeds ethanol extract have larvicidal activity that can be developed as new bioinsecticides.

Keywords: *Punica granatum* L seeds ethanol extract, Larvicidal, Aedes aegypti

Introduction

The mosquito species -transmitted disease remains a major source of illness and death. *Aedes aegypti* L are responsible for the transmission of major diseases such as dengue haemorrhagic fever, chikungunya and yellow fever and it also act as a vector for Zika fever and it also act as a vector for Zika fever [1]. However, Dengue fever has become an important public health problem as the number of reported cases continues to increase, especially with more severe forms of the disease, dengue hemorrhagic fever, and dengue shock syndrome, or with unusual manifestations such as central nervous system involvement [2]. A. aegypti is a cosmotropical species that proliferates in water containers in the residential areas [3]. *A. aegypti* is very closely associated with the human habitat. The geographical range of *A .aegypti* is increasing in part due to rapid urbanization and increased global movement of people and cargo [4].

The common mosquito larvicides are organophosphates, temephos, methopere, and the protein extracted from the Bacillus thuringiensis and B. sphaerius. However, the high amount of chemical larvicides could lead to long

term residual effect to the environment while the use of bacterial protein requires high cost and complex purification process, significantly the increase in the resistance to larvicides of mosquito have been reported. The indiscriminate use of synthetic insecticides is creating multifarious problems like environmental pollution, insecticide resistance, and toxic hazards to humans [5]. Globally, there have been conscientious efforts to overcome these problems, and great emphasis has been placed recently on enviro-friendly and economically viable methodologies for pest control. Irrespective of the less harmful and ecofriendly methods, suggested and used in the control programmes, there is still a need to depend upon the chemical control methods in situations of epidemic outbreak and sudden increase of adult mosquitoes. Hence it is necessary find alternatives to synthetic insecticides which is more potent at low cost.

Natural products of plant origin are preferred over synthetic insecticides due to their eco-friendly nature. [6,7]. Insecticidal properties of chemical derived from plants are active against specific target species without harming other organisms and are biodegradable and potentially suitable for use in integrated management program [8]. Plant based pesticides does not have any hazardous effect on the ecosystem, and the secondary metabolites and the synthetic derivatives provide an alternative for the control of mosquitoes. Thus, innovative vector control strategies like use of phytochemicals as alternate source of insecticidal and larvicidal agents in the fight against of *A .aegypti* larvae has become inevitable [9]. Phytochemicals obtained from the huge diversity of plant species are important source for safe and biodegradable chemicals, which can be screened for mosquito repellent, larvicidal, and insecticidal activities; and tested for mammalian toxicity. Therefore, researchers are currently exploiting natural substances to be used as insecticides for controlling larval mosquitoes [8,10]. The phytochemicals of the plants serve as huge storage of compounds that have biological action. Alkaloids, flavonoid, saponins, and tannins are known to possess medicinal and pesticidal properties [11,12]. These phytochemicals present in the *Punica granatum* L seeds ethanol extract. It has been reported that *Punica granatum* L seeds ethanol extract components like phenolics, flavonoids, tannins and other constituents can be used as anthelmintic, inflammatory and microbial [13,14]. The aim of this study was to investigate the potential of *Punica granatum* L seeds ethanol extract against the larvae of *Aedes aegypti* through larvicidal bioassays.

Materials & Methods

Mosquito Culture

Aedes aegypti colonies were maintained in our insectary in large enamel basins (45×45×40 cm) and rearing conditions were 28±2°C temperature, 65±5% relative humidity (RH) and photoperiod of 14:10 h light and dark period [12]. The egg strips were obtained from Institutet of Tropical Disease Airlangga University Surabaya to start the colony. The strips were immersed in dechlorinated tap water for hatching. Larvae were fed with a diet of finely ground brewer yeast and dog biscuits (3:1). The emerged adults were fed with rabbit blood and with 10% glucose solution. Small porcelain dishes having 50 ml of tap water lined with filter paper was kept inside the cage for oviposition.

Preparation of Ethanol Leaf Extract of *Punica granatum L*

Plant material and extract preparation leaf of *Punica granatum* L seeds were collected from Surabaya, Indonesia. Leaf were cleaned with running tap water and were chopped into pieces. They were dried under shade at

ambient temperature for 5 days and the air-dried leaf were then ground to powder for extraction. The powdered leaf (1 kg) was macerated with ethanol 96 % (5 L) for a week at 37°C. The supernatant was then collected and filtered through Whatman No. 1 filter paper in a Buchner funnel under vacuum. The filtrate was concentrated by evaporation with a vacuum rotary evaporator at 45°C. The extract was dried at reduced pressure, stored at 0-4°C and used for the experimentation.

Phytochemical Analysis

The filtrate was tested for the presence of phytochemicals such as alkaloids, flavanoids, saponins, tannins and terpenoids using standard procedures [15].

Larvicidal Bioassay *Punica granatum* L Extract.

The tests were conducted at room temperature. The *Punica granatum* L seeds ethanol extract as larvicide were tested against the third instar larvae of *A. aegypti* mosquitoes [5,16]. Five replicates of *Punica granatum* L seeds ethanol extract racts dilution with 0, 10, 20, 40, 80, 160, 320 and 640 ppm concentrations were prepared. Each replicate containing 200 ml of the described *Punica granatum* L seeds ethanol extract was placed in a 500 ml glass beaker. 20 third-instar larvae of A. aegypti were transferred into each beaker [16]. After that, the number of dead larvae in each beaker was counted after 1.5; 3; 6; 12 and 24 h. Identification of the Aedes aegypti larvae were done by tapping it with a needle in the siphon or cervical area. Each treatment was conducted in five replicates. The larvae were considered dead if, at the end of 24 hrs, they showed no sign of swimming movements even after gentle touching with a glass rod, as described in the World Health Organization's technical report series [17]. The effects of the *Punica granatum* L seeds ethanol extract were monitored through carefully counting the number of dead larvae after 24 hours of treatment, and the percentage mortality was computed.

$$\text{Percentage mortality} = \frac{\text{Number of dead larvae x 100}}{\text{Number of larvae introduced}}$$

Lethal Concentration of *Punica granatum* L Extract.

The LC_{50} and LC_{90} of *Punica granatum* L seeds ethanol extract that showed 100% mortality was determined by a similar procedure as mentioned above. 0, 10, 20, 40, 80, 160, 320 and 640 ppm concentration were tested and the observation was recorded after 1.5 ; 3; 6; 12 and 24 hrs of incubation.

Statistical Analysis

The statistical tools that were used in this study are the following: the Arithmetic Mean to get the average number of dead mosquito larvae, and Probit Analysis to calculate LC_{50} and LC_{90} values to determine Lethal Concentrations of the *Punica granatum* L seeds ethanol extract on *Aedes aegypti* mosquito larvae after 12 and 24 hours of treatment

Results

The preliminary phytochemical analysis of *Punica granatum* L seeds ethanol extract (Table 1) showed the presence of alkaloids, saponins, flavonoids, triterpenoids and tannins of phytochemicals. Any of these phytochemicals, either singly or in a combination with each other could be responsible for the larvacidal activity of the *Punica granatum* L seeds ethanol extract.

Table-1: Phytochemical analysis of *Punica granatum* L seeds ethanol extract

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Punica granatum L seeds ethanol extract	
Phytochemicals	**Level**
Alkaloids	++
Flavanoids	+++
Saponins	+
Tannins	++
Terpenoids	+++

+: low, ++: immediate, +++: high

Different concentrations (0, 10, 20, 40, 80, 160, 320 and 640 ppm) of *Punica granatum* L seeds ethanol extract solutions were bioassayed against the third instar larvae of *Aedes aegypti*. The results were shown after 1.5, 3, 6, 12 and 24 h of treatment (Table 2). In control treatments, no larvicidal effect was observed; the larvae remained alive, and they moulted into fourth instar larvae. Whereas, when the different *Punica granatum* L seeds ethanol extract concentrations were tested, different mortality rates were recorded with respect to exposure time. .

At 40 ppm *Punica granatum* L seeds ethanol extract, the larvae remained immobile after 3 h of treatment. When 80 ppm *Punica granatum* L seeds ethanol extract was tested, 1.4 % and 46.3 % larval mortality was recorded after 1.5 h and 24 h of treatment respectively. When 160 ppm *Punica granatum* L seeds ethanol extract solution was tested, 3.4 % and 87.2 % mortality was recorded after 1.5 h and 24 h of treatment respectively. At 320 ppm *Punica granatum* L seeds ethanol extract, complete mortality (100 %) was recorded after 24 h of exposure, while at 640 ppm, 100 % mortality was recorded after 12 h. The maximum result (100 %) was recorded with 320 ppm *Punica granatum* L seeds ethanol extract concentration after 24 h exposure (Table 2).

Table- 2: Effect of different concentrations of *Punica granatum* L seeds ethanol extract solution and exposure time on larvicidal bioassay of third instar larvae of *Aedes aegypti*

Ocimum sanctum Extract (ppm)	Larval Mortality Rate (%) After				
	1.5 h	3 h	6 h	12 h	24 h
0 (Control)	0	0	0	0	0
10	0	0	0	0	0
20	0	0	0	1.5 ± 0.3	4.6 ± 0.9
40	0	2.5 ± 0.4	4.6 ± 0.7	7.3 ± 1.2	21.6 ± 2.4
80	1.5 ± 0.4	3.7 ± 0.7	11.8 ± 2.2	22.8 ± 2.4	46.5 ± 3.8
160	3.6 ± 0.5	9.2 ± 0.6	2053 ± 4.1	58.6 ± 5.7	87.3 ± 6.2
320	5.8 ± 0.6	14.6 ± 1.5	32.6 ± 3.7	85.7 ± 4.6	100 ± 0
640	8.1± 0.5	24.4 ± 2.8	40.7 ± 6.2	100 ± 0	100 ± 0

*The values are mean ± SEM of five replecate

After 12 h, lethal concentration 50 (50 % larvicidal activity) of *Punica granatum* L seeds ethanol extract was 146.9 ppm while lethal concentration 90 (90 % larvicidal activity) was 37474 ppm. However after 24 h, lethal concentration (50 % larvicidal activity) of *Punica granatum* L seeds ethanol extract was 84.8 ppm while lethal concentration (90 % larvicidal activity) was 177.3 ppm (Table 3) . The exposure time is very important for larvicidal activity of the *Punica granatum* L seeds ethanol extract solution.

Table-3: LC_{50} and LC_{90} of *Punica granatum* L seeds ethanol extract

Time	*Punica granatum* L seeds ethanol extract	
	LC_{50} (ppm)	LC_{90} (ppm)
After 12 h	146.7	374.7
After 24 h	84.8	177.3

Discussion

Mosquitoes in the larval stage are attractive targets for pesticides because mosquitoes breed in water, which makes it easy to deal with them in this habitat. The use of conventional pesticides in the water sources, however, introduces many risks to people and the environment. *Aedes aegypti*, the primary carrier for viruses that cause dengue and dengue hemorrhagic fever and yellow fever, are widespread over large areas of the tropics and subtropics [18]. There is no vaccine to prevent dengue infection, nor are there drugs to combat the disease in infected persons, so vector control is the most opted solution available so far for reducing the morbidity. The prevention of mosquito breeding through the use of larvicides is the most effective way to fight with this mosquito importunation. Synthetic insecticides have been used as larvicide in several countries for the last 30 years [19]. However, the non-selectiveness of insecticides and

harmful effects on other organisms is the major hindrance with the use of these chemical insecticides [20].

The recently increased interest in developing plant-based insecticides as an alternative to chemical insecticides should be taken into consideration due to the toxicity problems, together with the increased incidence of insect resistance. In this study was undertaken to access the toxicant potential of the *Punica granatum* L seeds ethanol extract against mosquito larvae of *A. Aegypti*.

The *Punica granatum* L seeds ethanol extract exhibited a concentration dependent activities against *Aedes aegypti* larvae since the percentage mortality were observed to increase with increasing concentrations of the *Punica granatum* L seeds ethanol extract. The increase of percentage mortality of the treated *Aedes aegypti* larvae is supported by the presence of phytochemicals in the *Punica granatum* L seeds ethanol extract which have insecticidal activities. The least percentage mortality was noted in the control group (0 ppm concentration) which is extremely lower compared to those in the experimental groups. It reveals that all tested concentrations of the *Punica granatum* L seeds ethanol extract (20, 40, 80, 160, 320 and 640) caused mortality of Aedes aegypti larvae in comparison to those in the control group.

Result also indicates that 160 ppm concentrations of *Punica granatum* L seeds ethanol extract have the high percentage of mortality after 24 h. The Punica granatum L seeds ethanol extract high larvicidal activity is supported by the presence of phytochemicals such as alkaloids, saponins, flavonoids, triterpenoids and tannins which are known to have insecticidal and pesticidal properties [3,12,13]. This is supported by the presence of phytochemicals in Punica granatum L seeds contain a wide variety of active compounds such as gallotannins, gallic acid, ellagic acid, punicalins and punicalagins are known to possess medicinal and pesticidal properties [12,21].

It is further noted that the percentage mortality increased with increasing concentrations of the *Punica granatum* L seeds ethanol extract. Moreover, the mortality of mosquito larvae was also increased in relation to the time of exposure. The larvicidal activity of the highest concentration (320 ppm) of the *Punica granatum* L seeds ethanol extract on A. aegypti mosquito larvae within 24 hours of exposure. Moreover, the results also exhibited that there is a significant difference on the mortality of A. aegypti mosquito larvae between the control group and various concentration of the *Punica granatum* L seeds ethanol extract. This result denotes that higher concentration of the *Punica granatum* L seeds ethanol extract would lead to greater number of mortality in the A. aegypti mosquito larvae.

The lethal concentrations (LC50 and LC90) values of these *Punica granatum* L seeds ethanol extract on *Aedes aegypti* mosquito larvae after 24 hours of exposure are 84.8 ppm and 177.3 ppm. It shows that *Punica granatum* L seeds ethanol extract is the most effective in terms of insecticidal activity.

Conclusion

From the results of this study concluded that *Punica granatum* L seeds ethanol extract have larvicidal activity that can be developed as new bioinsecticides.

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