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Potential of Natural Larvicide Microemulsion Based on Pomegranate Peel Extract (*Punica granatum* L) against Dengue Haemorrhagic Fever Vectors

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ABSTRACT

Background: Dengue fever, caused by the dengue virus which is transmitted by the *Aedes aegypti* mosquito, is a significant global health problem. Controlling the *Aedes aegypti* vector is key in efforts to reduce the spread of this disease. However, the use of chemical larvicides in vector control has raised concerns regarding environmental impacts and mosquito resistance. Therefore, research related to the development of natural larvicides based on pomegranate peel extract contain flavonoids, terpenoids, tannins, steroids, and saponins, in microemulsion form has an urgency to provide a safer and more sustainable alternative. **Objective**: To determine the larvicidal activity of microemulsion ethanol extract of pomegranate peel (*Punica granatum*) against *Aedes Aegypti* larvae. **Methods**: This research uses an experimental study design, post-test only controlled group design. The samples used were *Aedes aegypti* larvae instar III-IV. There were 12 treatment groups consisting of positive control, negative control, and treatment groups with microemulsion ethanol extract and ethyl acetate fraction of pomegranate peel extract, each with five different concentrations. **Results**: The probit analysis results indicate that the LC_{50} (Lethal Concentration for 50% mortality) for the ethanol extract microemulsion of pomegranate peel is 51,607 ppm, while the LC_{50} for the ethyl acetate fraction microemulsion of pomegranate peel is 52,401 ppm against *Aedes aegypti* larvae. **Conclusion:** the probit analysis shows that the ethanol extract microemulsion of pomegranate peel is slightly more effective in killing *Aedes aegypti* larvae compared to the ethyl acetate fraction microemulsion. These results emphasize the potential of pomegranate peel extracts as natural larvicides, contributing to sustainable and environmentally friendly mosquito control measures.

KEYWORDS:
Aedes aegypti, Dengue, Larvicide, Microemulsion, Punica granatum

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INTRODUCTION

Research into larvicidal activity against *Aedes aegypti* has explored various natural and synthetic compounds due to the increasing resistance of mosquitoes to conventional insecticides. One promising area of study involves the use of plant-based extract, such as those derived from *Punica granatum* peel. *Punica granatum,* commonly known as pomegranate, has been extensively studied for its diverse medicinal properties, including its potential as a larvicidal agent. The peel of *P.granatum*, often considered agricultural waste, has shown significant insecticidal efficacy against mosquito larvae, particularly *Culex pipiens*. Studies have demonstrated that pomegranate peel extract can cause high mortality rates in mosquito larvae, with

median lethal concentrations (LC_{50}) decreasing over time, indicating increased effectiveness with prolonged exposure[1]. The extract not only induces mortality but also causes morphological malformations and biochemical disruptions in the larvae, such as reduced enzyme activities and altered protein, carbohydrate, and lipid contents[1]. Additionally, methanol-derived leaf extracts of *P. granatum* have been effective against the larvae of *Anopheles stephensi* and *Culex quinquefasciatus,* with identified compounds like phenol, 2-methyl-5-(1-methylethyl) contributing to its larvicidal activity[2]. The predation rate of tadpoles on mosquito larvae increased post-exposure to the extract, suggesting its compatibility with biological control methods[2]. Furthermore, the synthesis of magnesium oxide nanoparticles (MgO-NPs) using *P. granatum* peel extract has shown promising antimicrobial and larvicidal activities. These green-synthesized nanoparticles effectively inhibited the growth of various pathogenic microbes and demonstrated high larvicidal activity against *Culex quinquefasciatus* larvae, with significant mortality rates and prevention of adult hatching[3]. The phytochemical profile of *P. granatum*, rich in polyphenols, terpenoids, and other bioactive compounds, underpins its broad-spectrum biological activities, including its larvicidal properties[4]. The antibacterial and antimicrobial properties of pomegranate extracts, attributed to compounds like punicalagin, further support its potential in integrated pest management strategies[5].

Microemulsion delivery systems have been explored as a potential method to produce natural biolarvicides for mosquito control. Research on the use of microemulsion systems in biolarvicides to control *Aedes aegypti* larvae is still limited. Further research is needed to determine the effectiveness of the microemulsion system in biolarvicide to control *Aedes aegypti* larvae. This research aims to develop a natural larvicide based on *pomegranate* peel extract (*Punica granatum* L) in the form of a microemulsion and to assess its effectiveness in controlling *Aedes aegypti* larvae.

METHODS

This study was conducted in Pharmacology Laboratory and Parasitology Laboratory of the Faculty of Medicine at Universitas Muhammadiyah Surakarta (UMS).

Preparation of extracts and fractions: Pomegranate peels were collected from Surakarta and surrounding areas. The dried peels are ground into a fine powder and subjected to maceration using ethanol 96%. The extract is then concentrated using a rotary evaporator to obtain the ethanol extract. A portion of the ethanol extract is further fractionated using ethyl acetate as the solvent. This fraction is also concentrated using a rotary evaporator.

Formulation of microemulsion as follow:

Table 1. Microemulsion Formulations for Ethanol Extract of Pomegranate Peel

Component	E1	E2	E3	E4
Ethanolic Pomegranate peel extract	0,5gr	0,25gr	0,125gr	0,0625gr
Palm oil	50 gr	50gr	50gr	50gr
PEG 400	100gr	100gr	100gr	100gr
Tween 80	200gr	200gr	200gr	200gr
Aquadest	ad 1L	ad 1L	ad 1L	ad 1L

Table 2. Microemulsion Formulations for Ethyl Acetate Fraction of Pomegranate Peel

Component	E1	E2	E3	E4
Ethyl acetate fraction Pomegranate peel	0,5gr	0,25gr	0,125gr	0,0625gr
Palm oil	50 gr	50gr	50gr	50gr
PEG 400	100gr	100gr	100gr	100gr
Tween 80	200gr	200gr	200gr	200gr
Aquadest	ad 1L	ad 1L	ad 1L	ad 1L

The ethanol extract and ethyl acetate fraction are individually incorporated into the microemulsion systems by mixing with the selected components using high-speed homogenizer. The microemulsions are characterized for droplet size to ensure the proper formation of microemulsion.

Larvacidal Assay: Different concentrations of the microemulsions of ethanol extract and ethyl acetate fraction are prepared in containers. Group of 25 *Aedes aegypti* larvae are exposed to each concentration of the microemulsions in separate containers. A negative control and positive control (using Themephos) are also maintained. The larvae are observed for mortality at 24 hours.

Data collection and Analysis: The number of dead larvae in each group is recorded at each observation period. The mortality data are analysed using probit analysis to determine the LC_{50} values for both ethanol extract and ethyl acetate fraction microemulsions.

RESULT AND DISCUSSION

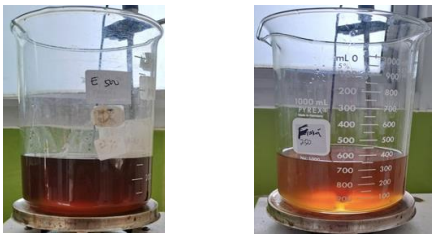
The study was conducted in accordance with current ethical considerations for human animal subjects from Faculty of medicine Universitas Muhammadiyah Surakarta, No. 5283/a.2/KEPK-FKUMS/VIII/2024.

The yield obtained from the ethanol extract of pomegranate peel was 30.28%, while the yield from the ethyl acetate fraction of pomegranate peel was 32.22%. These yield percentages are indicative of the efficiency of the extraction process and the concentration of bioactive compounds within the pomegranate peel.

The 30.28% yield from the ethanol extract suggests a significant extraction of bioactive components, as ethanol is a polar solvent known to effectively extract a wide range of phytochemicals, including phenolics, flavonoids, and other antioxidants. This relatively high yield is consistent with the presence of numerous compounds that contribute to the extract's overall efficacy, particularly in terms of antioxidant and larvicidal activities.

The slightly higher yield of 32.22% from the ethyl acetate fraction indicates that ethyl acetate, a semi-polar solvent, is also highly efficient in extracting bioactive compounds from pomegranate peel. The ethyl acetate fraction is likely to contain a variety of non-polar and semi-polar compounds, including terpenoids, which can enhance the larvicidal properties of the extract. The higher yield might reflect the extraction of specific compounds that are more soluble in ethyl acetate than in ethanol. The formulation

results of the microemulsion of ethanolic extract and ethyl acetate fraction of *P. granatum* peel can be seen in Figure 1.



A B

Figure 1. Microemulsion of pomegranate (*Punica granatum*) peel extract and its ethyl acetate fraction**.** (A) Microemulsion of pomegranate peel extract at a concentration of 500 ppm, showing uniformly dispersed micro-sized particles. (B) Microemulsion of the ethyl acetate fraction of pomegranate peel extract at a concentration of 250 ppm, demonstrating stable phase distribution with well-dispersed particles

The results of larval mortality percentages after 6 hours, 12 hours, 18 hours, and 24 hours of treatment with various concentrations of microemulsions of ethanol extract of pomegranate peel and ethyl acetate fraction of pomegranate peel are presented in Tables 3 and 4.

Table 3. Percentage Mortality of *Aedes aegypti* Larvae after 24 Hours of Exposure Various Concentrations of Ethanolic *Punica granatum* Peel Extract Microemulsion

Concentration of microemulsion ethanolic extract *Punica granatum* peel (ppm)	Number of dead larvae at 4 repetitions in hour (n)				Larvae per treatment (n)	Larva mortality at 24 hours (%)
	6	12	18	24		
500	15	25	0	0	25	100
	20	25	0	0	25	100
	22	25	0	0	25	100
250	21	25	0	0	25	100
	22	25	0	0	25	100
	25	0	0	0	25	100
	22	25	0	0	25	100
125	22	25	0	0	25	100
	22	25	0	0	25	100
	20	25	0	0	25	100
	20	25	0	0	25	100
	20	25	0	0	25	100
62,5	20	23	24	24	25	96
	21	21	23	24	25	96
	20	20	22	23	25	92
	20	21	22	24	25	96
Control (+)	25	25	25	25	25	100
Control (-)	0	0	0	0	25	0

Table 4. Percentage Mortality of *Aedes aegypti* Larvae after 24 Hours of Exposure Various Concentration of Ethyl acetate fraction *P. granatum* Peel Microemulsion

Concentration of microemulsion ethyl acetate fraction *Punica granatum* peel (ppm)	Number of dead larvae at 4 repetitions in hour (n)				Larvae per treatment (n)	Larva mortality at 24 hours (%)
	6	12	18	24		
500	23	25	0	0	25	100
	21	25	0	0	25	100
	24	25	0	0	25	100
250	25	0	0	0	25	100
	22	25	0	0	25	100
	22	25	0	0	25	100
	23	25	0	0	25	100
125	21	25	0	0	25	100
	25	0	0	0	25	100
	23	25	0	0	25	100
	19	25	0	0	25	100
	25	0	0	0	25	100
62,5	19	22	23	23	25	92
	16	20	21	23	25	92
	19	20	21	22	25	88
	20	21	21	22	25	88
Control (+)	25	25	25	25	25	100
Control (-)	0	0	0	0	25	0

In the treatment with microemulsions of ethanol extract of pomegranate peel, repeated four times, the percentage of *Aedes aegypti* larval mortality reached 100% at concentrations of 500 ppm, 250 ppm, and 125 ppm. Meanwhile, at the concentration of 62.5 ppm, the percentage of larval mortality from the four repetitions was 92% and 96%.

In the treatment with microemulsions of ethyl acetate fraction of pomegranate peel, repeated four times, the percentage of *Aedes aegypti* larval mortality reached 100% at concentrations of 500 ppm, 250 ppm, and 125 ppm. Meanwhile, at the concentration of 62.5 ppm, the percentage of larval

mortality from the four repetitions was 88% and 92%. For the positive control (temephos), the percentage of *Aedes aegypti* larval mortality was 100% over the 24-hour observation period. In contrast, for the negative control, all larvae remained alive during the 24-hour observation period.

From the probit analysis, the LC_{50} value for the microemulsion of pomegranate peel ethanol extract was determined to be 51.607 ppm, while the LC_{50} value for the microemulsion of the ethyl acetate fraction of pomegranate peel was 52.401 ppm. According to the probit analysis results, the 24-hour LC_{50} value of the pomegranate peel ethanol extract microemulsion against *Aedes aegypti* larval mortality was obtained at a concentration of 51.607 ppm. This indicates that at this concentration, the pomegranate peel ethanol extract microemulsion can kill 50% of *Aedes aegypti* larvae within 24 hours.

The ethyl acetate fraction microemulsion of pomegranate peel at a concentration of 52.401 ppm can kill 50% of *Aedes aegypti* larvae within 24 hours. Therefore, it can be concluded that both the pomegranate peel ethanol extract microemulsion and the ethyl acetate fraction microemulsion of pomegranate peel are effective against *Aedes aegypti* larvae.

The ethanolic peel extract of *Punica granatum* enhances larvicidal properties through a multifaceted approach involving its rich phytochemical composition and bioactive compounds. The extract contains significant amounts of 5-hydroxymethylfurfural, D-sucrose, sorbitol, palmitic acid β-monoglyceride, 2-furancarboxaldehyde, and β-D-glucopyranose, which contribute to its potent biological activities[6]. The larvicidal efficacy against *Culex pipiens* larvae is evident from the reduction in mortality rates at various concentrations and time intervals, with median lethal concentrations (LC_{50}) decreasing significantly over 96 hours.[1] This extract not only causes mortality but also induces morphological malformations and biochemical disruptions in the larvae, such as reduced enzyme activities and altered protein, carbohydrate, and lipid profiles[1]. Additionally, the presence of flavonoids, terpenoids, tannins, steroids, and saponins in the extract further enhances its bioactivity, contributing to its broad-spectrum antibacterial and potentially larvicidal effects[7]. The extract's neuroprotective properties, demonstrated through its ability to mitigate oxidative stress and modulate the Nrf2-HO-1 pathway, suggest a mechanism that could also be relevant in disrupting larval development and survival.[8] Moreover, the extract's efficacy against various microbial strains, including multi-resistant bacteria, underscores its potential as a robust bioactive agent. The combination of these phytochemicals and their synergistic effects likely underpins the enhanced larvicidal properties of the ethanolic peel extract of

Punica granatum, making it a promising candidate for developing natural insecticides and contributing to integrated pest management strategies.

The ethyl acetate fraction of *Punica granatum* (pomegranate) peel extract enhances larvicidal properties through a combination of its potent phytochemical constituents and its multifaceted biological activities. The extract's efficacy against *Culex pipiens* larvae is evident from its significant reduction in larval mortality rates, with median lethal concentrations (LC_{50}) decreasing over time, indicating a strong and sustained larvicidal effect.[1] The ethyl acetate fraction is particularly rich in bioactive compounds such as tannins, flavonoids, phenols, alkaloids, saponins, and terpenoids, which contribute to its potent antibacterial and insecticidal properties.[9] These compounds disrupt the biological processes of the larvae, leading to morphological malformations and significant reductions in enzyme activities, total proteins, carbohydrates, and lipids[1]. Additionally, the ethyl acetate extract has demonstrated strong antibacterial activity against multidrug-resistant bacterial pathogens, suggesting that its bioactive compounds can effectively target and disrupt microbial cell functions[10]. This antibacterial property is crucial as it can prevent secondary infections in larvae, further enhancing the larvicidal efficacy. The extract's ability to inhibit α-amylase activity also suggests a potential mechanism for disrupting the digestive processes of the larvae, thereby impairing their ability to metabolize nutrients and survive.[11] Furthermore, the presence of compounds like 5-hydroxymethylfurfural and D-sucrose in the ethanolic extract of *P. granatum* enhances its overall antimicrobial profile, making it more effective against a broad spectrum of microorganisms, including those that may affect larval health.[6] Collectively, these multifaceted actions of the ethyl acetate fraction from *P. granatum* peel extract underscore its enhanced larvicidal properties, making it a promising natural agent for controlling mosquito populations and potentially reducing the spread of mosquito-borne diseases.

The treatment of *Aedes aegypti* larvae with natural biolarvicides leads to significant morphological change as shown in Figure 2. These alterations are crucial for understanding the efficacy of biolarvicides as alternatives to chemical insecticides.

A B C

Figure 2: Figure showing morphological changes in *Aedes aegypti* larvae after treatment with the microemulsion of pomegranate peel extract and fraction. A). *Aedes aegypti* larvae in the control group, showing normal morphology indicating a healthy and living state. B). *Aedes aegypti* larvae after treatment with microemulsion of ethanol extract of pomegranate peel displaying physical deformities, indicating death. C). *Aedes aegypti* larvae after treatment with microemulsion of ethyl acetate fraction of pomegranate peel, showing physical deformities, indicating death.

Morphological changes in *Aedes aegypti* larvae after treatment with microemulsions of pomegranate (*Punica granatum*) peel extract and fractions can be understood through several studies that demonstrate the larvicidal effects of various natural extracts. Pomegranate peel is known to contain bioactive compounds such as flavonoids, saponins, and tannins, which have the potential to inhibit larval growth and cause significant morphological changes[12]. However, the research by Novitri and Kurniati focused more on the antibacterial activity of ethanol extract of pomegranate peel and lemongrass stems against *Escherichia coli*, and therefore does not directly support claims regarding larvicidal effects[13].

Meanwhile, other studies have shown that treatments with natural extracts, such as those from tobacco leaves and lemongrass, can lead to morphological changes in *Aedes aegypti* larvae. Tobacco leaf extract contains alkaloids and saponins that can damage the larvae's nervous system, leading to death and morphological alterations.[14] Research by Wulandari *et al.* (2022) also demonstrated that lemongrass infusion can slow larval movement and cause visible morphological changes, such as larvae floating on the water surface[15].

Other studies reported that some extracts were effective in killing *Aedes aegypti* larvae, they are lime leaf extract 2.5%, 5% and 10% with PEG 5%, basil leaves in addition of TWEEN 80 and PEG 400, suren leaves and lime extracts 0.5%, 1% and 1.5%, tobacco leaves[16-19].

Further studies have reported that exposure to botanical insecticides can cause damage to the midgut epithelium of larvae, marked by dilation of the apical surface and damage to the brush border[20]. This indicates that treatment with natural extracts not only affects larval mortality but can also lead to significant structural changes in the larvae's internal organs.

Overall, the morphological changes in *Aedes aegypti* larvae after treatment with microemulsions of pomegranate peel extract and fractions may include larval death, changes in behavior (such as reduced movement), and damage to internal structures. Further research is needed to understand the specific mechanisms involved in these morphological changes and to explore the potential of pomegranate peel extract as an effective larvicide.

The limitation of this study was variability in larval susceptibility. Besides, there need to do further study to determine the potential environment impacts of large-scale application.

CONCLUSION

The study demonstrates that microemulsions of pomegranate peel *Punica granatum* extracts exhibit larvicidal activity against *Aedes aegypti* larvae. The ethanol extract microemulsion was found to be slightly more effective, with an LC_{50} of 51.607 ppm,

compared to the ethyl acetate fraction microemulsion, which had an LC_{50} of 52.401 ppm. These findings highlight the potential of pomegranate peel extracts as natural larvicides, offering a safer and more environmentally sustainable alternative to chemical larvicides in controlling mosquito populations and mitigating the spread of dengue fever. For further study, researcher can use formulation optimization for the better result.

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