Journal of Tropical Pharmacy and Chemistry

Journal homepage: https://jtpc.farmasi.unmul.ac.id

Larvicidal Effect of 96% Ethanol Extract of Lime (*Citrus aurantifolia*) Leaves with PEG 400 Diluent on *Aedes aegypti* Larvae

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Abstract

Larval control is remain the best method in reducing the high incidence of Dengue Fever, but temephos-resistance has also been reported. Lime (*Citrus aurantifolia*) leaves are thought to be a promising material to replace temephos. This studi aim to identify the larvicidal effect of lime leaves ethanolic extract on *Aedes aegypti*. Lime leaves were extracted by maceration method and PEG 400 diluent was added as a dispersing agent. Total of 400 *Aedes aegypti* larvae at stages III-IV were enrolled. At 24 hours of observation, mortality rate of *Aedes aegypti* larvae in group with extract concentration of 0.3% and 0.4% was 92% and 100%, respectively. From post-hoc Mann Whitney test, p-value of <0.05 was only found when any study group were compared to negative control group. As as conclusion is 96% ethanol extract of lime leaves in addition of PEG 400 diluent has larvicide effect on *Aedes aegypti* larvae.

Keywords: Lime leaves, PEG 400, larvicidal effect, *Aedes aegypti*

Received: 06 June 2023 **Accepted**: 13 June 2024

DOI: https://doi.org/10.25026/jtpc.v8i1.574

How to Cite:

Dewi, L. M., Mufidah, F., Sutrisna, E., Nurhayani, N., 2024. Larvicidal Effect of 96% Ethanol Extract of Lime (*Citrus aurantifolia*) Leaves with PEG 400 Diluent on *Aedes aegypti* Larvae. *J. Trop. Pharm. Chem.* **8**(1). 70-76. **DOI**: https://doi.org/10.25026/jtpc.v8i1.574

1 Introduction

World Health Organization (WHO) has included dengue fever as a global health threat in early 2020 [1]. Dengue fever is one of tropical diseases with a high incidence rate, especially in Indonesia [2]. Even in the last 50 years, there has been a trend of increasing incidence year by year. However, the morbidity rate has decreased by half in each decade, and Java Island still dominates the number of Dengue fever cases each year [3]. The global commitment for dengue fever is to reduce Dengue mortality by at least 50% by 2020, reduce Dengue morbidity by at least 25% by 2020, and estimate the true burden of disease by 2015. In order to meet these targets, WHO sets five main strategies for dengue control, they are case diagnosis and management, integrated surveillance and outbreak preparedness, continuous vector control and vaccination, as well as operational research and implementation [4].

Until now, vector control is still considered the best method in tackling dengue fever. The principles of vector control include an understanding of vector ecology and its epidemiology as well as the implementation of environmental management based on vector ecology and behavior. However, this concept has been replaced by the use of insecticides, which give the impression of being simple, easy, and effective in suppressing the existence of this vector. The use of insecticides is often not accompanied by sufficient understanding of entomology and epidemiology, and there is no adequate evaluation. Therefore, the emergence of resistance and environmental damage is being reported increasingly [5]. New solutions that can take over the role of these insecticides, but can also address the challenges of resistance and environmental damage, are urgently needed, and one of which is the use of natural-based ingredients [6]. Even WHO also recommends the use of medicinal plants as a part of traditional medicine. If it has gone through various stages of clinical trials and is proven to be safe and effective, the medicinal plants can be incorporated into the national health system [7].

Citrus aurantifolia is one of medicinal plant from the Rutaceae family. The specificity of this plant is containing limonoids which can be found in the skin of the fruit, leaves, and stems. Limonoids or also called tetranortriterpenoids, are chemical ingredient that provide a bitter taste of lime. These limonoids act by inhibiting the acetylcholinesterase enzyme. This mechanism is considered to be similar to temephos, so that *Citrus aurantifolia* is thought to be promising solution to replace temephos [8], [9]. The addition of Polyethylene glycol (PEG) 400 as dispersing agent is currently learned a lot. This is because PEG 400 is non-toxic, non-immunogenic, and has high solubility in water. These properties are utilized for PEGylation of drugs or materials, so that it is indirectly expected to increase the effectiveness of the application of a test substance in water media [10].

2 Methods

This study applies a laboratory experimental research with post-test only controlled group design. The entire process of this study was carried out in the Pharmacology Laboratory and the Parasitology Laboratory, Faculty of Medicine, Universitas Muhammadiyah Surakarta, under ethical clearance letter at number 4726/A.1/KEPK-FKUMS/I/2023. There were several research groups in this study, the positive control group, the negative control group, the treatment group with ethanol extract of lime leaves (EELL) at concentration of 0.3% and 0.4%. Each group was repeated 4 times, and each of them involving 25 larvae, as specified in the WHO guidelines for laboratory and field testing of mosquito larvicides [11]

The first stage of this study is extraction process. Lime leaves as the main ingredient, being dried under the sun for about a week, then mashed to form simplicias. They soaked in 96% ethanol with a ratio 1:7.5 for 5 days with occasional stirring every day. The filtrate is then poured into a separate container, while the residue is re-soaked in a new solvent with a ratio of 1:4 for 5 days and again stirred occasionally every day. The filtrate is then

combined with the first filtrate. We called this filtrate as the stock [12]. The extract was then prepared in two different concentrations, they are 0.3% and 0.4%.

To make the EELL composition with the specified concentration, the following formula is used in equation 1.

$$Vs \times Cs = Vn \times Cn \quad \text{(Equation 1)}$$

Description:
Vs : volume of stock taken
Cs : concentration of the stock
Vn : determined volume for each extract concentration designed
Cn : concentration of EELL designed

Based on calculations according to thus formula, the composition of each extract concentration is summarized in table 1.

Table 1. Composition of each extract concentration

Vs	Cs	Vn	Cn (EELL concentration)	Aquadest added
0.015 ml	100%	5 ml	0.3%	4.985 ml
0.02 ml	100%	5 ml	0.4%	4.98 ml

The second stage of this study is sedimentation test. At this stage, we mixed EELL with PEG 400, then left it for 24 hours and observed the precipitate formation. The composition of the mixture used is summarized in table 2.

Table 2. Composition of mixture in sedimentation test

EELL concentration	EELL volume	PEG volume	Aquadest added	Total volume
0.3%	0.03 ml	0.3 ml	9.67 ml	10 ml
0.4%	0.04 ml	0.3 ml	9.66 ml	10 ml

The third stage of this study is larval preparation. We used *Aedes aegypti*'s eggs that had been prepared on filter paper sheets. They were placed in a container which has been filled by distilled water before. We also add some fish food that has been mixed in water into the container. Wait for 2 till 3 days, so that the eggs will drip.

The next stage of this study is larvicidal test. There were 4 study groups designed. They are the positive control group with temephos, the negative control group with PEG 400, the treatment group with EELL at concentration of 0.3%, and the treatment group with EELL at concentration of 0.4%. Repetition was done for 4 times, for each study group. Totally, we prepared about 16 containers for the larvicide test, with 25 larvae in each. The composition in each container is summarized in table 3. Larval mortality was observed at 6 hours, 12 hours, 18 hours, and 24 hours. The larvae are considered to be dead when they sink or appear unresponsive when touched using a stick.

Table 3. Composition in each container for larvicidal test

Study group	Temephos	PEG 400	EELL at concentration of 0.3%	EELL at concentration of 0.4%	Aquadest	Total volume	Number of larvae
A	1 ml	0 ml	0 ml	0 ml	99 ml	100 ml	25
B	0 ml	3 ml	0 ml	0 ml	97 ml	100 ml	25
C	0 ml	3 ml	0.3 ml	0 ml	96.7 ml	100 ml	25
D	0 ml	3 ml	0 ml	0.4 ml	96.6 ml	100 ml	25

Note :
A : the positive control group
B : the negative control group
C : the treatment group with EELL at concentration of 0.3%
D : the treatment group with EELL at concentration of 0.4%
EELL : ethanol extract of lime leaves

The last stage of this study is data analysis. The number of larval-death in each study group and in each observation-period were recorded and tabulated. These data were analysed by Kruskall Wallis test and post-hoc Mann Whitney test.

3 Results and Discussion

In the extraction process, from 1000 grams of lime leaves simplicias, 79.5 grams of lime leaf ethanol extract was produced. From sedimentation test, it was obtained that no precipitate formed in each extract concentration designed, directly nor in 24 hours of observation. In other word, the addition of PEG 400 may increase the dispersion of EELL in water, so that active substance kept soluble in it. Eun-Sol (2019) through her study, states that PEG 400 has the best ability to increase material solubility compared to other dispersing agents. This is related to the formation of hydrogen bonds and nonspecific polarization of the solvent which results in increased solubility [13]. Data obtained from larvicidal test, were recorded and summarized in table 4.

Table 4. Larval mortality in each study group

Study group	Repetition	Number of larval mortalities at each of observation time				Average of the larva mortalities in 24 hours	Percentage of the larva mortalities in 24 hours
		6	12	18	24		
A	I	25	0	0	0	25±0	100%
	II	25	0	0	0		
	III	25	0	0	0		
	IV	25	0	0	0		
B	I	0	0	0	0	0±0	0%
	II	0	0	0	0		
	III	0	0	0	0		
	IV	0	0	0	0		
C	I	9	7	6	3	23.75±1.875	95%
	II	2	5	7	6		
	III	7	12	3	3		
	IV	5	7	5	8		
D	I	20	3	1	1	25±0	100%
	II	12	5	5	3		
	III	12	4	7	2		
	IV	15	2	1	7		

Note :
A : the positive control group
B : the negative control group
C : the treatment group with EELL at concentration of 0.3%
D : the treatment group with EELL at concentration of 0.4%
EELL : ethanol extract of lime leaves

The results of the larvicidal test in the positive control group, at each repetition, the larvae mortality reached 100% in the first 6 hours of observation. Here, we can say that the use of temephos as a positive control is appropriate. As we know, larvicide was chosen as one of the additional efforts in the 3M Plus Movement launched by the Indonesian Ministry of Health in 2016 [14]. Temephos is a kind of organophosphate larvicide that inhibits the cholinesterase enzyme, causing acetylcholine deposits in the larvae's body. This resulting a muscular spasms or seizure of the larvae, which will cause the larvae to tire and eventually die [15]. This is why it can be said that the use of larvicides, including temephos, is considered being simple, easy, and effective in controlling the dengue vector [5]. Although on the other hand, knowledge and attitudes about dengue prevention greatly influence the accuracy of the practice of temephos usage. Inadequate knowledge and attitude about dengue prevention can lead to inappropriate use of temephos, which may impact on resistance [16]. Drastic differences were found in the second study group, the negative control group, where the percentage of larval mortality at 24 hours was 0%. There was no larva were found to be dead in this study group. It means that PEG 400 has no larvicidal effect, especially on *Aedes aegypti* larva. Therefore, the usage of PEG 400 as negative control in this study was appropriate. It is also proved that the addition of PEG 400 will not confound the results of the EELL used in this study.

In the treatment groups, 0.3% and 0.4% of EELL, the percentage of larval mortality was 95% and 100%, respectively. The larvae had started to die in the first 6 hours of observation, and the longer they were, the number increased. The longer exposure of the larvae to the EELL, the greater amount of active ingredients that enter the body of the larvae. This shows that EELL has a larvicidal effect, especially against *Aedes aegypti* larvae. This larvicidal effect is due to the presence of a number of active ingredients, such as essential oils, flavonoids, alkaloids, terpenoids, phenolics, and limonoids [17], [18], [19]. These materials have various mechanisms as larvicides, including neural toxic, digestive toxic, respiratory toxic, and growth toxic. Especially for limonoida which is specific to the Rutaceae family, apart from being neuro-toxic as well as temephos, limonoida is also an analogue of juvenile hormones in insects which regulates larval growth, and is able to suppress the appetite of larvae [18], [19], [20] .

Data analysis in table 4, through the Kruskal-Wallis test, obtained a p-value of <0.05. This statistical test is intended to find out whether there are groups of data that have

significant differences. Because of the p-value obtained is <0.05, this indicates that from all of the groups that were observed, there were at least two groups that had significantly different. As a continuation, a statistical test was carried out using post-hoc Mann-Whitney test to find out which groups had significantly different results. P-value obtained from post-hoc Mann Whitney test were presented in the table 5.

Table 5. The Result of Post-Hoc Mann Whitney Test

Study group	A	B	C	D
A	--	0.008*	0.317	1.000
B	0.008*	--	0.011*	0.008*
C	0.317	0.011*	--	0.317
D	1.000	0.008*	0.317	--

Note :
* : significantly different (p-value < 0.05)
A : the positive control group
B : the negative control group
C : the treatment group with EELL at concentration of 0.3%
D : the treatment group with EELL at concentration of 0.4%
EELL : ethanol extract of lime leaves

From the data Table 5, we can see that significantly different data were only found when each of study group were compared to the negative control group. Because in the previous discussion, it was known that PEG 400 as a negative control did not have a larvicidal effect, so here we can say that the positive control and EELL at concentrations of 0.3% and 0.4% were proven to have a larvicidal effect. We also found that when each of EELL at concentrations of 0.3% and 0.4% were compared to the positive control group, the data obtained were not significantly different. It means that EELL at concentrations of 0.3% and 0.4% as potent as temephos as larvicide. Similar result was found when EELL at concentrations of 0.3% and 0.4% was compared. It is also can be said that both concentration of EELL statistically have similar potential each other.

This result is in line with previous study by [18], in which they also used lime leaf extract with different solvent, N-hexane, and the larvae used were *Culex quinquefasciatus* larvae. In this study, it was found that the highest larval mortality rate, which was 93.33%, was obtained from lime leaves extract with a concentration of 4000ppm or equivalent to 0.4%. At the same extract concentration, the mortality rate of the larvae in our study was higher, when the extraction using ethanol was accompanied by the addition of PEG 400. Even at a lower EELL concentration, at 0.3%, a higher mortality rate was obtained, it is about 95%. This may reinforce that the addition of PEG 400 may increase the effectiveness of the extract indirectly. This finding is in line with research by [21], who also added PEG 400 as diluent, and produced an effective larvicidal effect [21]. PEG 400 which has no larvicidal effect is able to increase the solubility of active ingredient of the extract in water media which is the growth medium for the larvae, so that the chances for the larvae to get contact with the active ingredient will increase, furthermore more active ingredient will enter the larvae's body and subsequently trigger the death of the larvae [22].

4 Conclusions

96% ethanol extract of lime leaves (EELL) in addition of PEG 400 diluent has larvicide effect on *Aedes aegypti* larvae. 96% ethanol extract of lime leaves (EELL) has similar potential to temephos as larvicide.

5 Declarations

5.1 Acknowledgements

The authors would like to thank to Universitas Muhammadiyah Surakarta for permission, facilities, and funding for this research

5.2 Author contribution

LMD designed the study. FM carried out the laboratory work and analyzed the data. FMD and FM wrote the manuscript. All authors read and approved the final version of the manuscript.

5.3 Etic

Ethical clearance letter at number 4726/A.1/KEPK-FKUMS/I/2023, issued by Ethics Committee of Faculty of Medicine, Universitas Muhammadiyah Surakarta.

5.4 Conflict of Interest

All of authors state that there are no competing interests

6 References

[1] World Health Organization, "Dengue and severe dengue," 2021. Accessed: Jun. 03, 2023. [Online]. Available: https://www.who.int/news-room/fact-sheets/detail/dengue-and-severe-dengue

[2] M. Akram *et al.*, *Neglected Tropical Diseases and Phytochemicals in Drug Discovery*. 2021.

[3] H. Harapan, A. Michie, M. Mudatsir, R. T. Sasmono, and A. Imrie, "Epidemiology of dengue hemorrhagic fever in Indonesia: Analysis of five decades data from the National Disease Surveillance," *BMC Res Notes*, vol. 12, no. 1, Jun. 2019, doi: 10.1186/s13104-019-4379-9.

[4] Kementerian Kesehatan Republik Indonesia, *Strategi Nasional Penanggulangan Dengue 2021-2025*. Kementerian Kesehatan RI, 2021.

[5] A. L. Wilson *et al.*, "The importance of vector control for the control and elimination of vector-borne diseases," *PLoS Neglected Tropical Diseases*, vol. 14, no. 1. Public Library of Science, pp. 1–31, Jan. 01, 2020. doi: 10.1371/journal.pntd.0007831.

[6] N. L. Achee *et al.*, "Alternative strategies for mosquito-borne arbovirus control," *PLoS Neglected Tropical Diseases*, vol. 13, no. 1. Public Library of Science, 2019. doi: 10.1371/journal.pntd.0006822.

[7] L. M. Dewi and M. Q. Nafi'ah, "Effectiveness of 96% Ethanol Extract of Clove Leaves (Syzygium aromaticum (L.) Merr & Perry) as an Antibacterial Agent Against Staphylococcus epidermidis And Providencia stuartii," *MAGNA MEDICA Berkala Ilmiah Kedokteran dan Kesehatan*, vol. 9, no. 2, p. 139, Aug. 2022, doi: 10.26714/magnamed.9.2.2022.139-146.

[8] Y. S. Shi *et al.*, "Limonoids from Citrus: Chemistry, anti-tumor potential, and other bioactivities," *Journal of Functional Foods*, vol. 75. Elsevier Ltd, Dec. 01, 2020. doi: 10.1016/j.jff.2020.104213.

[9] Thankhoe A. Rants'o, Lizette L. Koekemoer, and Robyn L. van Zyl, "In vitro and in silico analysis of the Anopheles anticholinesterase activity of terpenoids," *Parasitol Int*, vol. 93, Apr. 2023, doi: https://doi.org/10.1016/j.parint.2022.10271.

[10] Z. Peng, C. Ji, Y. Zhou, T. Zhao, and R. M. Leblanc, "Polyethylene glycol (PEG) derived carbon dots: Preparation and applications," *Appl Mater Today*, vol. 20, p. 100677, Sep. 2020, doi: 10.1016/j.apmt.2020.100677.

[11] World Health Organization, *Guidelines for Laboratory and Fields Testing of Mosquito Larvicides*. 2005. Accessed: Jun. 04, 2023. [Online]. Available: https://www.who.int/publications/i/item/WHO-CDS-WHOPES-GCDPP-2005.13

[12] E. Sutrisna, *Herbal Medicine : Suatu Tinjauan Farmakologis*. Muhammadiyah University Press, 2016.

[13] E.-S. Ha *et al.*, "Solvent effect and solubility modeling of rebamipide in twelve solvents at different temperatures," *J Mol Liq*, vol. 288, p. 111041, Aug. 2019, doi: 10.1016/j.molliq.2019.111041.

[14] Kementrian KEsehatan Republik Indonesia, "Kemenkes Keluarkan Surat Edaran Pemberantasan Sarang Nyamuk dengan 3M Plus dan Gerakan 1 Rumah 1 Jumantik."

[15] J. P. Martínez-Mercado, A. Sierra-Santoyo, F. A. Verdín-Betancourt, A. E. Rojas-García, and B. Quintanilla-Vega, "Temephos, an organophosphate larvicide for residential use: a review of its toxicity," *Crit Rev Toxicol*, vol. 52, no. 2, pp. 113–124, Feb. 2022, doi: 10.1080/10408444.2022.2065967.

[16] W. Hary Cahyati and N. Siyam, "Perilaku Masyarakat dalam Penggunaan Temephos," *Higeia Journal of Public Health Research And Development*, 2019, doi: 10.15294/higeia/v3i1/28665.

[17] P. D. Muniandy, S. F. Riswari, and K. Ruchiatan, "Larvicidal Activity of Citrus aurantifolia Decoction against Aedes aegypti Larvae," *Althea Medical Journal*, vol. 7, no. 1, pp. 35–39, Mar. 2020, doi: 10.15850/amj.v7n1.1814.

[18] M. Hanif, N. D. R. Lastuti, and R. Kurnijasanti, "Effect of Larvicidal Extract N-Hexane Lime Leaves (Citrus hystrix) on Larva Instar III Mosquito (Culex quinquefasciatus)," *World's Veterinary Journal*, vol. 11, no. 3, pp. 416–421, 2021, doi: 10.54203/scil.2021.wvj53.

[19] N. N. Indriyani, J. Al Anshori, N. Permadi, S. Nurjanah, and E. Julaeha, "Bioactive Components and Their Activities from Different Parts of Citrus aurantifolia (Christm.) Swingle for Food Development," *Foods*, vol. 12, no. 10, p. 2036, May 2023, doi: 10.3390/foods12102036.

[20] C. J. Kumara, R. S. Bestari, and L. M. Dewi, "Efektivitas Flavonoid, Tanin, Saponin dan Alkaloid terhadap Mortalitas Larva Aedes aegypty," in *The 13 th University Research Colloqium 2021*, 2021. Accessed: Jun. 06, 2023. [Online]. Available: http://repository.urecol.org/index.php/proceeding/article/view/1311

[21] L. M. Dewi, H. Z. Ariffah, R. Aisyah, and N. Nurhayani, "Bio-larvicidal Potential of Betel Leaves (Piper betle L) Ethanolic Extract in Addition of PEG 400 Diluent on Aedes aegypti Larvae," *Biology, Medicine, & Natural Product Chemistry*, vol. 12, no. 2, pp. 451–455, Aug.

2023, doi: 10.14421/biomedich.2023.122.451-455.
[22] M. A. Desheesh, D. M. El-Masry, M. M. Fargand, and H. M. Youssef, "Larvicidal Activity of Nano-Encapsulated Lambda – Cyhalothrin Against Susceptible Mosquito Larvae (Culex Pipiens) in Comparison with Conventional form," *Alexandria Science Exchange Journal*, vol. 40, no. October-December, pp. 568–573, Dec. 2019, doi: 10.21608/asejaiqjsae.2019.58477.