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Larvicidal activity of *Melaleuca leucadendra* leaves extract against *Aedes aegypti*

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ABSTRACT

Dengue Hemorrhagic Fever (DHF) depends on controlling *Aedes aegypti* mosquitoes and larvae. Currently, larvicide control still uses temefos larvicide, though several studies have reported resistance. Insecticides from plants can be used as an alternative. One of the plants reported to have larvicide potency was *Melaleuca leucadendra* leaves. This study aimed to look at ethanol extract of *M. leucadendra* leaves activity in killing *A. aegypti* larvae and LC_{50} values after a 24-h examination. This type of research was experimental design with post-test only control group design. *M. leucadendra* leaves was extracted through maceration process using 96% ethanol. The treatments consisted of 8 concentrations (mg L^{-1}) of 400 (0.04%); 1000 (0.1%); 1600 (0.16%); 2000 (0.2%); 10,000 (1%); 20,000 (2%); 30,000 (3%); 40,000 (4%) and the control group (0%). Each concentration was replicated four times and applied on twenty specimens of *A. aegypti* at the third larval stage. The results showed that *M.leucadendra* has a lethal ability against *A. aegypti*. There was a correlation between the extract concentration and the larval mortality (p = 0.000; 95%). The extract concentrations of 0.04-0.2% caused <3% mortality, while the highest mortality (47.5%) achieved at the conc. of 4%. The LOGIT test showed that the number of LC_{50} was 3.7% (37,600 mg L^{-1}) with 95% significance. A high concentration ($\geq$1%) of extract *M. leucadendra* caused turbid, greenish-gray color, and unpleasant smell on the water. Regarding the WHO bioassay guideline, etahnol extract of *M. leucadendra* leaves was less effective on killing *A. aegypti* larvae, though it causes lethal effect on it.

Keywords: larvacide activity, Melaleuca leucadendra leaves extract, larva Aedes aegypti.

INTRODUCTION

Dengue Hemorrhagic Fever (DHF) is an infectious disease caused by the dengue virus transmitted by *Aedes aegypti or A. albopictus* (CDC 2019). *A. aegypti* mosquito can reproduce rapidly and generates nearly 390 million people worldwide to be infected each year. These mosquitoes present in tropical and subtropical areas, including the islands in Indonesia, to northern Australia. In tropical and subtropical regions, such as Indonesia, Dengue hemorrhagic fever (DHF) is an endemic disease that occurs throughout the year, especially in every rainy season and in optimal conditions for mosquito breeding (Indonesian Health Minister 2019). DHF eradication can be done by controlling the *A. aegypti* mosquitoes that act as carriers of the dengue virus. There are methods to manage the number of mosquitoes. Vector control still focuses on the use of chemical insecticides, along with the development of repeated insecticides. Chemical insecticides may emerge a resistance and environmental pollution (Ramadhani 2013). Temephos is widely used for the *Aedes* larvae control. However, its careless use promotes resistance development against temephos (Chen *et al.* 2005). Sinaga *et al.* (2016) reported a 1% resistance of *A. aegypti* larvae to temephos in the city of Banjarmasin. Environmental controls are considered more appropriate and effective than chemical and biological controls (Arekhi & Jamshidi 2018; CDC 2019).

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Various plants in Indonesia could be used as vegetable larvicides, including lemongrass, zodiac, jasmine, tobacco, galangal, teak wood, eucalyptus, etc. (Astriani & Widawati 2016). The results were obtained by looking at the LC_{50} which is a concentration value that can kill 50% of the total larvae tested. The use of plant-derived products, such as crude extract of natural larvicidal insecticides, could be a promising tool to control disease vectors. The natural sources of substances displaying insecticidal activity against mosquitoes are biodegradable and lower toxic towards non-target organisms (Dias *et al.* 2015). *Melaleuca leucadendra* (L.) L. (*syn. Melaleuca viridiflora* C.F. Gaertn., *Myrtus leucadendra* L.) is a tree which may grow as high as 40 m and find in native tropical Australia and Indonesia (An *et al.* 2020). Cajuput oil/eucalyptus oil, is commercially used as a medicated oil. In Indonesia, some people plant it as yard plants. *M. leucadendra* and *M. cajuputi* were a native plant in Indonesia. Eucalyptus is a familiar plant because of its benefits as a medicinal ingredient, insecticide, and fragrance. This plant can be used as a conservation plant for critical lands. Eucalyptus is a plant which can grow in barren soils and sprouts quickly, even if it burns. This plant is one of the essential oil producers widely used for various health or pharmaceutical products. Eucalyptus leaves (*M. leucadendra*) contain sineol, terpineol, terpinene, and limonene compounds useful as insecticides and repellents. These leaves have the potential to be a vegetable larvicide. However, research related to this is still limited. This study aims to evaluate the larvicidal activity of the *M. leucadendra* leaf crude extract against *A. aegypti*. This study is focused on the sustainable use of tropical local plant products to combat the larvae of dengue fever vector, *A. aegypti.*

MATERIALS AND METHOD

Leaves Collection

About 2 kg of fresh leaves of *M. leucadendra* were harvested from nature in Wonogiri city, Indonesia, in June 2019. The leaves were sorted from its branch and other parts. The leaves were dried under sunlight directly for 6 h before going under the maceration process.

Crude Extract Preparation

About 0.5 kg of dried the *M. leucadendra* leaves were grounded into a crude powder. The powder (200 g) was soaked into 300 mL 96% ethanol for 24 h in a porcelain bowl at room temperature (27± 1°C). After 24 h, the crude extract was raised on the surface and then dried in the evaporator. The dried crude extract of *M. Leucadendra* was made in the laboratory of Universitas Muhammadiyah Surakarta, Indonesia.

Larvicidal Bioassay

Bioassay test of the *M. leucadendra* leaves extract was conducted in B2P2VRP Salatiga Indonesia. This research applied an experimental design with a post-test only control group. The larvicidal activity was evaluated by following the WHO bioassay test (World Health Organization 2005). Twenty numbers of third and fourth instar larvae of *A. aegypti* were introduced into the test containers with 250 mL water. The *A.aegypti* larvae were reared in the laboratorium Institution of Research, and Vector and Reservoir B2P2VRP Salatiga, Indonesia. Larvae instar III and IV had a bigger body and easy to be observed. Small, unhealthy, or damaged larvae were removed and replaced. There were 8 variant concentrations including 400 (0.04%), 1000 (0.1%), 1600 (0.16%), 2000 (0.2%), 10,000 (1%), 20,000 (2%), 30,000 (3%) and 40,000 mg L^{-1} (4%). The extract was added and exposed to the larvae (in 250 mL water) and observed after 2, 4, 8, and 24 h. The number of larvae mortality was recorded. Each concentration was replied to four times with one control group, which was not exposed to the extract. pH and temperature of each water (after exposure to the extract) were tested. Ethics approval was gained from the Health Research Ethics Commission, Universitas Muhammadiyah Surakarta. Mosquito colonies of *A. aegypti* were obtained and maintained as previously described. This testing followed the protocol of WHO guidelines for laboratory and field testing of mosquito larvicides (World Health Organization 2005).

The data were analyzed using the non-parametric test Kruskal-Wallis to see the difference the larvae mortality among various concentrations. The Rank-spearman test was used to see the correlation between the extract concentration and larva mortalities. LC_{50} and LC_{90} of the extract were analyzed using regression equations (LOGIT test). The bioassays were conducted at a room temperature of 27 ± 1°C with five replicates for each concentration. All tests should be conducted at 25-28 °C, preferably with a 12L:12D photoperiod (World Health Organization 2005). The larva mortality was recorded and converted into percent mortality (a) and corrected mortality (b) which was calculated using Abbot's formula

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(a) Percentage of moratlity $= \frac{\text{No.of dead larvae x 100}}{\text{No of larvae introduced}}$

(b) Corrected Percentage of moratlity $= \frac{\text{1−n in T after treatment}}{\text{N in C after treatment}}$

where n is the number of larvae, T is the number of larvae survived in the treatments, and N is the number of larvae survived in control. Each concentration of the corrected percentage mortality value was considered to estimate LC_{50} and LC_{90} values using SPSS Probit analysis statistical pack. The corrected percentage mortality value of each concentration was considered to estimate LC_{50} and LC_{90} values using SPSS 25.

RESULTS

Bioassay water temperature was measured and showed as an initial temperature 25°C, and the final temperature average was 22°C ± 1. The optimum average water temperature for larval growth was 20 °C – 30 °C (Costa *et al.* 2010). These temperatures supported the presence of the *Aedes aegypti* larvae during laboratory study. It was found that the water temperature did not affect the growth and development of larvae during the test. The pH measurement showed no significant difference in bioassay water pH before and after extract addition (pH ranged 4.7-5.3). This pH range still supports larvae life. The *A. aegypti* can develop in waters from pH 4-11, and larvae develop optimally at pH 7 (Clark *et al.* 2004).

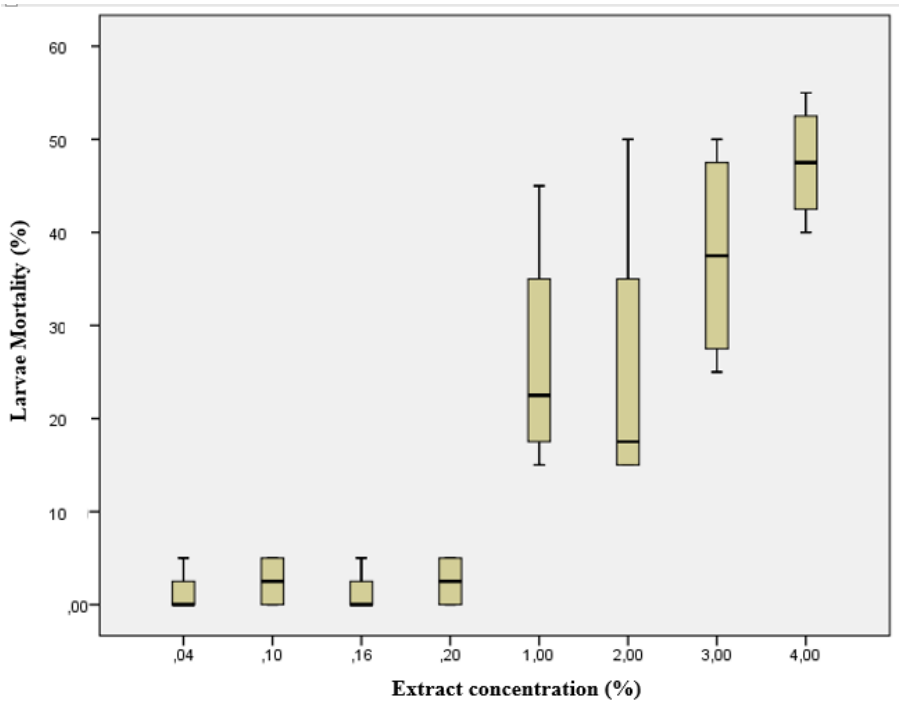

Fig. 1. Larva mortality based on *M. leucadendra* larvicidal concentration (in %).

Fig. 1 shows that the number of larvae mortality raised by the elevated concentration of the extract after 24 h. At the concentrations (mg L^{-1}) of 0.04% (400), 0.1% (1000), 0.16% (1600), 0.2% (2000), the observed larvae mortality was less than 3%. The higher mortalities (>25%) were observed at the concentrations of 1% (10.000), 2% (20,0000), 3% (30,000), 4% (40,0000). The highest mortality (47.5%) was observed at 4%. It shows that the average mortality of *A. aegypti* larvae has increased at concentrations $\geq$ 1%.

Table 1. The result of correlation and different test between extract solution and larvae mortality

Extract Solution (%)	0.04	0.1	0.16	0.2	1	2	3	4
Larvae Mortality (%)	1.25	2.5	1.25	2.5	26.25	25	27.5	47.5
Differentiate test	(p = 0.12; 95%)							
Coorelation test	(p = 0.000; 95%)							
LOGIT test	LC_{50}= 37,600 mg L^{-1}; LC_{90}= 65,920 mg L^{-1}							

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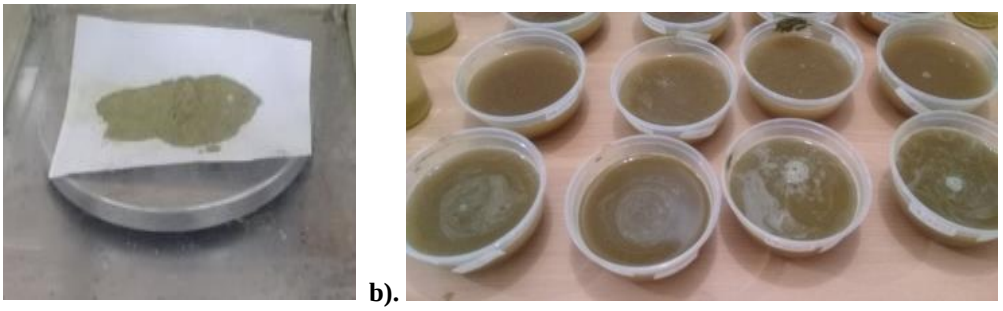
As shown in Table 1, there was a significant difference among various concentrations towards larvae mortality. The higher concentration likely led to the higher mortality of larvae. The LC_{50} of the *M. leucadendra* extract was 37,600 mg L^{-1}, while the predicted LC_{90} was 65,920 mg L^{-1}.

Extract of *M. leucadendra* can cause lethal on the *A. aegypti* larva at the very high concentration (>1%, 10,000 mg L^{-1}) yet gives low mortality rate (<50%). The statistic showed a correlation between extract concentration and larva mortality. However, an insignificant difference was observed among the extract concentration toward the larvae mortalities ($p > 0.05$). The LOGIT test showed that the number of LC_{50} of the *M. leucadendra* extract is 3.7% (3760 mg L^{-1}).



a) b).

Fig. 2. Crude extract of *M. leucadendra*; b) Appearance of crude extract solution (≥10,000 mg L^{-1}).

The water added by the extract (concentration >1%) showed turbid, brownish color, and cloudy appearance. It made the observation difficult. It also leaved a lot of sediment in the bottom. At the concentration of 0.04%; 0.1%; 0.16%; 0.2%, the water were clear and no smell was detected. However, at 1%; 2%; 3%; 4% the water were really turbid and the smell was unpleasant.

DISCUSSION

Based on the result, *M. leucadendra* ethanol extract was observed to be lethal on the *A. aegypti* larvae. However, this extract was less effective since it requires a high concentration to kill half the larvae population (LC_{50} = 3760 mg L^{-1}). At this concentration, it causes high turbidity and an unpleasant smell. This extract caused death to the *A. aegypti* larvae. However, it is not suitable for clean water sources. In the present study, the lowest concentration was 400 mg L^{-1} (0.04%), which is acceptable to be applied, although yielded less than 2% mortality. The extract could not cause 95% mortality, in spite of exposure to high concentration. Larvicides are considered effective if they cause >95% mortality in the targeted population (World Health Organization, 2005).

In other studies, the *M. leucadendra* leaf essential oil was reported to be effective against *A. aegypti* by LC_{50} = 7.4 µg mL^{-1} after 24 h and 1.4 µg mL^{-1} after 48 h. This oil is rich in α-eudesmol (17.6%), guaiol (10.9%), linalool (5.1%), (E)-caryophyllene (7.0%), and bulnesol (3.6%) (An *et al.* 2020). About 104 compounds were identified in this oil. Oxygenated sesquiterpenoids and sesquiterpene hydrocarbons were the dominant chemical classes (An *et al.* 2020). The components contained in *M. leucadendra*, such as cineol, terpineol, terpinene, and limonene, can be used to kill many insect species. Astriani and Widawati also reported that the *M. leucadendra* leave essential oil had LC_{50} 78.64 mg L^{-1} with components such as α-terpinena, terpinolena, dan γ-terpinena (Astriani & Widawati 2016). Melaleuca is belonged to Myrtaceae family that thrives in barren areas. Leyva *et al.* (2016) suggested that *M. quinquenervia* preferential oil has a lethal effect on *A. aegypti* (LC_{50} = 0.0047%). In a literature review research, 50 ml/L essential oil of Cajeput (*M. leucadendra*) resulted in 3.3% mortality, Niaouli (*M. quinquenervia* Madagascar) 30% mortality, after 24 h (Amer & Mehlhorn 2006). Indeed, *M. leucadendra* was less toxic compared to other plants such as *Cinnamomum camphora, Amrys balsamifera, Citrus lemon, Paper nigrum,* and others. Essential oil through the hydrodistillation process is supposed to be more effective for *M. leucadendra* extraction than maceration process. In this study, the *M. leucadendra* ethanol extraction using a conventional maceration process produced a less toxic effect (LC_{50} =3700 mg L^{-1} or 3.7%). The less effectiveness of *M. leucadendra* against *A. aegypti* larvae was likely due to the extraction process. The maceration process was reported to have the lowest result of phenols and flavonoids of *A. clavatus* flower compared to other extraction processes (soxhlet, heating, and reflux extraction) (Aliboudhar & Tigrine-Kordjani 2014). They reported that soxhlet extraction yielded the highest number of phenol and flavonoids results. Conventional soxhlet extraction (CSE) was better to extract phenolic compounds than other methods (Aliboudhar & Tigrine-Kordjani 2014). In

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CSE, the plant material is not in contact with the solvent, and the solvent was heated separately (extraction by vapor). Drying under sunlight directly and a heated evaporation process likely reduced the extract toxicity. Nevertheless, as hydrodistillation is often conducted at a temperature above the boiling point of water, hence, some natural pigments, volatile components, and heat-labile bioactive compounds may be lost (da Silva Ramos et al. 2017). Most biolarvicide extracted by hydrodistillation result in the LC_{50} to mosquitoes by less than 100 ppm (mg L^{-1}). Although, in this study the heat may be produced during maseration process, the *M. leucadendra* leaves essential oil extract using hydrostilistation with higher heat indeed results in a higher toxicity as a larvicide than those with maseration process.

The maceration process is one of the bioactive natural product extraction methods. It uses water, aqueous and non-aqueous solvents and conducts at room temperature. It is a simple extraction method with the disadvantage of long extraction time, large organic solvent consumption, and low extraction efficiency. It suits for the extraction of thermolabile components. The extraction efficiency of luteolin, orient side, and total flavonoids were the lowest in this method. However, reflux extraction is the most commonly applied technique for preparative separation. Pressurized liquid extraction and microwave-assisted extraction, ultrasound-assisted extraction, supercritical fluid extraction are regarded to be green extraction due to their high extraction yields, the stability of the target extracts, selectivity, and process safety merits (Zhang *et al.* 2018). Although the maceration is appropriate for some thermolabile components, it seems not suitable for *M. leucadendra* larvicide production. Some of the bioactive components may lose during solvent exposure for long hours, and other coarse components make the solution too concentrated (Zhang *et al.* 2018).

In the other study, other plants extracted with maceration process showed better results. Ravi *et al.* reported larvicidal effects of the *Azolla pinnata* extracts using methanol that showed LC_{50} and LC_{95} values of 867 and 1293 mg L^{-1} at 24 h against *A. albopictus* (Ravi *et al.* 2018). Besides, Krzyzaniak *et al.* (2017) reported that *Tagetes patula* extracted by ethyl acetate was reported to have LC_{50} on 50 mg L^{-1} on *A. aegypti* after 24 h. Ethyl acetate was reported to result in a higher concentration of patuletin in *Tagetes patula* fraction (Krzyzaniak *et al.* 2017). However, methanol extract of *Clione celata* (red boring sponge) resulted in the highest larvicidal activity at 500 mg L^{-1} against the *C. quinquefasciatus* larvae (LC_{50} = 95.63 mg L^{-1}; Reegan *et al.* 2015). Sharma *et al.* (2016) reported the larvicidal activity of the *Achyranthes aspera* leaf extracts. It exhibits LC_{50} of 82.5 mg L^{-1} against *A. aegypti*. Ethanol extract of *Inula racemosa* have potential for use in the control of the *A. albopictus* larvae with LC_{50} of 25.23 µg Ml^{-1} (He *et al.* 2014). Lakshmi Naidu *et al.* reported that plants produce a broad range of bioactive chemical compounds consisting of secondary metabolites such as flavonoids, tannins, terpenoids, and alkaloids which would significantly produce biological activities and chemical defenses against insects (Naidu *et al.* 2006). *Azolla pinnata* causes lethal on *A. aegypti* at late third- stage larvae by LC_{50} of 1262 mg L^{-1} (Husna Zulkrnin *et al.* 2018). Dias *et al.* (2015) reported that the Brazilian Legal amazon flora ability could be a potential larvicide by LC_{50} ranging from 230 to 292 after 24 h-exposure. Most of those plants' ethanol extraction gives LC_{50} higher than 20 mg L^{-1} except for *Inula racemosa* (LC_{50} = 25.23 µg/mL). There were not many plant extractions with LC_{90} lower than 20 mg L^{-1} as requested from the WHO bioassay guideline. WHO recommends that only the aqueous and alcoholic extracts of plants that cause the death of 90% of the animal population when tested at concentrations equal to or lower than 20 mg L^{-1} (after 24-h exposure) deserve attention in studies and should be further tested in the field (World Health Organization 2005). In the present study, since LC_{90} of the *M. leucadendra* ethanol extract was not reached and statistically predicted to be higher than 20 mg L^{-1} (LC_{90} = 65,920 mg L^{-1}). Therefore the extract is not able to be tested in the field.

Plant products produced positive outcomes as an alternative for synthetic chemical agents for insect biocontrol programs. Bioactive agents in plants, such as alkaloids, steroids, terpenes, and phenolic constituents were investigated earlier for biocontrol potency, exhibiting positive results (Mathew *et al.* 2009; Pavela 2015). Moreover, the ability to control mosquito larvae and their application efficacies vary with species, plant parts, age, the solvent used, and collection site of plants (Isman 2015; Stevenson *et al.* 2017). Botanical pesticides emerge as a potential source for mosquito control tools since they contain a rich source of bioactive compounds that are biodegradable and potential for controlling mosquitoes. However, regarding the WHO bioassay recommendation, only plant extractions that result in LC_{90} = 20 mg L^{-1} are considered to be tested further. Moreover, the larvicide experiment should observe not only its ability to kill larvae, but also observed and reported the water condition after the extract addition. It is due to the health and safety of the people who used the water, especially for hygiene and sanitation use. The *M. leucadendra* leaves extract, at concentrations of 0.04%, 0.1%,

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0.16% and 0.2% resulted in a clear water condition and no smell detected. However at the concentrations of 1%, 2%, 3% and 4%, the water was turbid, and the smell was unpleasant. Even though the *M.leucadendra* leaves extraction results in a higher number of LC_{90} than its recommend, it could be useful for the other researcher. Most researchers only state that a plant extract's killing ability, but they mostly have no explanation about the water condition after the extract added and the lethal concentration required by WHO bioassay guideline. Further research is suggested to test another formula or extraction of *M. leucadendra* leaves into a more lethal larvicide to better understand.

CONCLUSION

The extract of *M. leucadendra* can cause lethal on the *Aedes aegypti* larva at very high concentration (>1%, 10 mg/mL) yet gives low mortality percentage (<50%). This study showed a correlation between the number of *M. Leucadendra* ethanol extract concentration and larva mortality. However, it showed an insignificant difference among the extract concentration toward the larvae mortalities ($p>0.05$). The LOGIT test showed that the number of LC_{50} was 3.7% (37,600 mg/mL) with 95% probability. A high concentration of extract *M. leucadendra* caused turbid, greenish-gray color, and unpleasant smell on the water. Ethanol extract of *M. leucadendra* leaves was less effective in killing *A. aegypti* larvae. Further research is suggested to test another formula or extraction of *M. leucadendra* leaves into a more lethal larvicide for better understanding.

CONFLICT OF INTERESTS

The authors declare that they do not have any conflict of interests

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فعالیت لاروکشی عصاره برگ *Melaleuca leucadendra* در برابر پشه *Aedes aegypti*

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چکیده

درمان بیماری تب دنگی (DHF)، به کنترل پشه‌های تب زرد (*Aedes aegypti*) و لاروهای آن بستگی دارد. امروزه، برای کنترل لارو، از سم لاروکش تمه فوس استفاده می‌شود، اگرچه چندین مطالعه، مقاومت پشه‌ها و لاروها در برابر این سم را نشان داده‌اند. حشره‌کش‌های گیاهی را می‌توان به‌عنوان جایگزین سموم شیمیایی استفاده کرد. یکی از گیاهانی که خاصیت لاروکشی دارد، برگ‌های گیاه کاجوپوت است. هدف این مطالعه بررسی فعالیت عصاره اتانول برگ‌های کاجوپوت در کشتن لارو پشه تب زرد و مقادیر LC50 بعد از ۲۴ ساعت آزمایش است. طرح آزمایش این تحقیق، طرح پس‌آزمون با یک گروه شاهد است. عصاره‌ی برگ‌های کاجوپوت از طریق فرایند خیساندن در اتانول ۹۶ درصد استخراج شد. تیمارها شامل ۸ غلظت ۴۰۰ میلی‌گرم در لیتر (۰,۰۴ درصد)، ۱۰۰۰ میلی‌گرم در لیتر (۰,۱ درصد)؛ ۱۶۰۰ میلی‌گرم در لیتر (۰,۱۶ درصد) ۲۰۰۰ میلی‌گرم در لیتر (۰,۲ درصد)، ۱۰۰۰۰ میلی‌گرم در لیتر (۱ درصد)، ۲۰۰۰۰ میلی‌گرم در لیتر (۲ درصد)، ۳۰۰۰۰ میلی‌گرم لیتر (۳ درصد)، ۴۰۰۰۰ میلی‌گرم لیتر (۴ درصد) و گروه شاهد (۰ درصد) بود. هر غلظت چهار بار تکرار داشت و بیست‌وسه لارو پشه استفاده شد. نتایج نشان داد که عصاره برگ کاجوپوت قابلیت کشندگی لارو پشه تب زرد را دارد. بین غلظت عصاره و مرگ‌ومیر لارو ($p = 0.000$، ۹۵ درصد) همبستگی وجود داشت. غلظت عصاره ۰,۰۴-۰,۲ عصاره منجر به مرگ‌ومیر کم‌تر از ۳ درصد شد و بالاترین مرگ‌ومیر (۴۷,۵ درصد) در غلظت ۴ درصد حاصل شد. تست LOGIT نشان داد که تعداد LC50 برابر با ۳,۷ درصد (۳۷۶۰۰ میلی‌گرم بر لیتر) بامعنی داری ۹۵ درصد بود. غلظت بالای (۱ درصد≥) عصاره برگ کاجوپوت باعث ورم، رنگ خاکستری مایل به سبز و بوی نامطبوع آب شد. با توجه به دستورالعمل سازمان بهداشت جهانی، عصاره اتانول برگ‌های کاجوپوت، اثربخشی کم‌تری در کشتن لاروهای پشه تب زرد داشت، اگرچه اثر کشندگی بر روی *A. aegypti* داشت.

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