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ORIGINAL ARTICLE

Larvicidal Activity of *Melaleuca leucadendra* Leaves Extract Against *Aedes aegypti*

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KEYWORDS

larvacide activity;
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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 *Aedes aegypti*larvae and LC_{50} values after a 24-hour examination. This type of research was Experimental Design with Post-test Only Control Group Design. *M. Leucadendra* leaves was extracted through maceration process using ethanol 96%. The treatments consisted of 8 concentrations of 400mg/L (0.04%); 1000mg/L (0.1%); 1600mg/L (0.16%); 2000mg/L (0.2%); 10,000mg/L (1%); 20,000mg/L (2%); 30,000mg/L (3%); 40,000mg/L (4%) and the control group (0%). Each concentration was replicated four times and used twentyof the third larvae *A.aegypti*. The results showed that *M.leucadendra* has a lethal ability against *A.aegypti*. There was a correlation between the extract concentration and the larva mortality (p=0.000; 95%). Extract concentration 0.04-0.2% of the extract caused mortality less than 3%, and the highest mortality (47.5%) reached by concentration 4%. The LOGIT test showed that the number of LC_{50} was 3.7% (37,600mg/L) 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 in killing *Aedes aegypti* larvae, though it causes lethal effect *A.aegypti*.

INTRODUCTION

Dengue Hemorrhagic Fever (DHF) is an infectious disease caused by the dengue virus transmitted by *Aedes aegypti or Aedes albopictus*[1]. *Aedes aegypti*mosquito can reproduce rapidly and generates nearly 390 million people worldwide to be infected each year. These mosquitoespresent in tropical and subtropical areas, including the islands in Indonesia, to Australia's northern part. 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 [2]. DHF eradication can be done by controlling the *Aedes aegypti* mosquitoesthat act as carriers of the dengue virus. There are methods to manage the number of mosquitoes. Vector

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control still focuses on the use of chemical insecticides, along with the development of repeated insecticides.Chemical insecticidesmay emergea resistance and environmental pollution. Temephos is widely used for Aedes larvae control. However, its careless use promotes resistance development against temephos [3,4]. Sinaga [5] reported a 1% resistance of *Aedes aegypti*(*A.aegypti*) larvae to temephos in the city of Banjarmasin. Environmental controls are considered more appropriate and effective than chemical and biological controls [1].

Various plants in Indonesia could be used as vegetable larvicides, including lemongrass, zodiac, jasmine, tobacco, galangal, teak wood, eucalyptus[6]. The results were obtained by looking at the $LC_{50}$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 [7-10]. The natural sources of substances displaying insecticidal activity against mosquitoes are biodegradable and lower toxic towards non-target organisms [11]. *Melaleuca leucadendra (L.) L. (syn. Melaleuca viridiflora C.F. Gaertn., Myrtus leucadendra L.)* is a tree. *Melaleuca leucadendra* tree may grow as high as 40 m and find in native tropical Australia and Indonesia [12]. Cajuput, oil/eucalyptus oil, is commercially used as a medicated oil. In Indonesia, some people plant it as yard plants. *Melaleuca leucadendra* and *Melaleuca cajuputi* was 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 known as a plant that can grow in barren soils and sprouts quickly, even if it burns.The Eucalyptus plant is one of the essential oil producers widely used for various health or pharmaceutical products. Eucalyptus leaves (*Melaleuca leucadendra*) contain sineol, terpineol, terpinene, and limonene compounds that are useful as insecticides and repellents. Eucalyptus leaves have the potential to be a vegetable larvicide. However, research related to this is still limited. This study aim to evaluate the larvicidal activity of *Melaleuca leucadendra* (*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 METHODS

Leaves collection

About two kilograms of fresh leaves of *M. leucadendra* were harvestedfrom 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 hours before going under the maceration process.

Crude extract preparation

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

Larvicidal bioassay

Bioassay test of *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 [13]. Twenty numbers of third and fourth instar larvae of *A.aegypti* were introduced into the test containers with 250 ml water.*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 400mg/L (0.04%); 1000mg/L (0.1%);1600mg/L (0.16%); 2000mg/L(0.2%); 10,000mg/L (1%); 20,000mg/L (2%); 30,000mg/L(3%); 40,000mg/L (4%). The extract was added and exposed to the larvae (in 250 ml water) and observed after 2 hours, 4 hours, 8 hours, and 24 hours. 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 testingfollowed the protocol of WHO guidelines for laboratory and field testing of mosquito larvicides [13].

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 [13].The larva mortality was recorded and converted into percent mortality (a), and corrected mortality (b) was calculated using Abbot's formula

(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 thenumber of larvae, T is the number of larvae survived in treated, and Nis thenumberoflarvaesurvivedin control. Each concentration's corrected percentage mortality valuewas considered to estimate LC_{50} andLC_{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 showedan initial temperature 25^0C, and the final temperature average was 22^0C$\pm$1. The optimum average water temperature for larval growth is 20^0C - 30^0C[14].These temperatures supported the presence of *Aedes aegypti* larvas during laboratory study. It showed 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 larva life.The A.*aegypti* can develop in waters from pH 4 to pH 11, and larvae develop optimally at pH 7 [15].

Figure 1 showed that the number of larvae mortality raised as the concentration of the extract increased after 24 hours. At concentration 0.04% (400mg/L); 0.1% (1000mg/L); 0.16% (1600mg/L); 0.2% (2000 mg/L), the observed larvae mortality was less than 3%. The higher mortality (>25%) was performed by concentration 1% (10.000mg/L); 2% (20,0000mg/L); 3% (30,000mg/L); 4% (40,0000 mg/L). The highest mortality observed on 4% with 47.5% mortality. It shows that the average mortality of *Aedes aegypti* larvae has increased at concentration $\geq$ 1%.

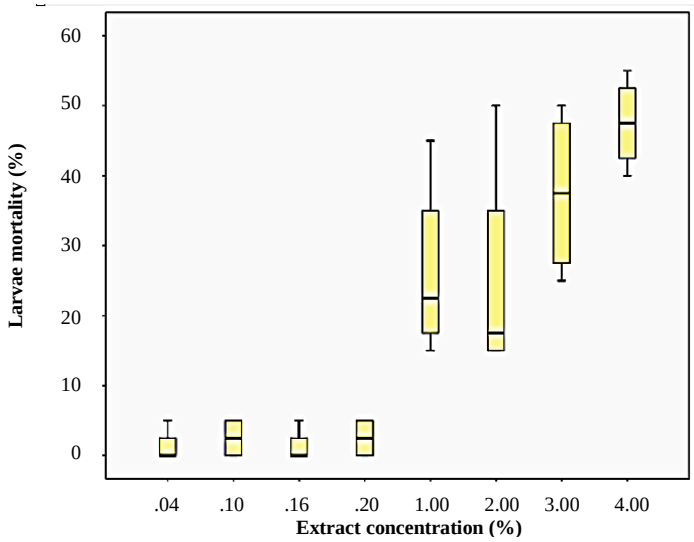


Figure 1. Larva mortality based on *M. leucadendra* larvicide concentration (in %)

As shown in Table 1, there was a significant difference among various concentration towards larvae mortality number. The higher concentration was likely causing the higher mortality of larvae. The LC_{50} of *M. leucadendra* extract was 37,600 mg/L, and the predicted LC_{90} was 65,920 mg/L.

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; LC_{90}= 65,920 mg/L							

Extract of *M. leucadendra* can cause lethal on larva *Aedes aegypti* on very high concentration (>1%, 10,000mg/mL) yet gives low mortality percentage (<50%). The statistic showed a correlation between 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} of *M. leucadendra* extract is 3.7% (37,600mg/L).

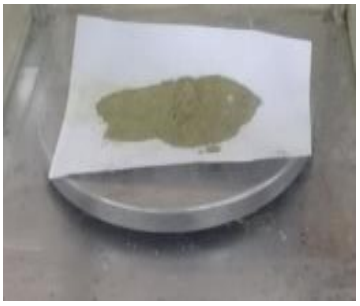
a).  b). 

Figure 2. a) Crude extract of *M.leucadendra*; b) Appearance of crude extract solution (≥10,000mg/L)

The water added by the extract (concentration >1%) showed turbid, brownish color, and cloudy appearance (Figure 2). It made the observation difficult. It also leaves a lot of sediment in the bottom. At concentration 0.04%; 0.1%; 0.16%; 0.2%, the water were clear and the smell was not detected, however at concentration 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*A.aegypti* larvae. However, this extract was less effective since it requires a high concentration to kill half the larvae population (LC_{50}=37,600mg/L). At this concentration,it causeshigh turbidity and an unpleasant smell. This extract caused death to the *A.aegypti* larvae; however, it is not suitable for clean water sources.This study's lowest concentration was 400mg/L (0.04%),which acceptable concentration to be applied however yielded less than 2% mortality. The extract could not cause 95% of mortality though the high concentration exposed. Larvicides are considered effective if they cause >95% mortality in the targeted population [13].

In other studies, *M. leucadendra* leaf's essential oil was reported to be effective against *A. aegypti* had LC_{50} = 7.4 µg/mL after 24 hours and 1.4 µg/mL after 48 hours.*M. leucadendra* leaf essential oil is rich in α-eudesmol (17.6%), guaiol (10.9%), linalool (5.1%), (E)-caryophyllene (7.0%), and bulnesol (3.6%) [12].About 104 compounds were identified in the *M. leucadendra* essential oils. Oxygenated sesquiterpenoids and Sesquiterpene hydrocarbons were the dominant chemical classes [8].The components contained in *M. leucadendra*, such as cineol, terpineol, terpinene, and limonene, can be used to kill many insect species. Astrianiand Widawati [6] also reported that *Melaleuca leucadendra* leaves' essential oil had LC_{50} 78.64 mg/L with component α-terpinena, terpinolena, dan γ-terpinena [6]. Melaleuca is considered as Myrtaceae family that thrives in barren areas. A study byLeyva[14] suggested that *Melalueca quinquenervia* preferential oil has a lethal effect on *Aedes aegypti* (LC_{50} = 0.0047%). In a literature review research, 50 ml/L essential oil of Cajeput (*Melaleuca leucadendra*) resulted in mortality 3.3%, Niaouli (*Melaleuca quinquenervia* Madagascar) mortality 30%, after 24 h [16]. 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 processis supposed to be more effective for *M.leucadendra* extraction than maceration process.

In this study, *M.leucadendra* ethanol extraction using a conventional maceration process produce a less toxic effect (LC_{50}=37,600 mg/L 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 compare to other extraction processes (soxhlet, heating, and reflux extraction) [17]. 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 [17]. In Conventional Soxhlet Extraction (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 were likely reducing the extract toxicity. Nevertheless, hydrodistillation is often conducted at a temperature above the boiling point of water, some natural pigments, volatile components, and heat-labile bioactive compounds may be lost [18]. Most biolarvicide extracts use essential oil extraction (hydrodistillation) and produced the LC_{50} to mosquitoes less than 100 ppm (mg/L). Although, in this study the heat may exposed during maseration process, *M.leucadendra* leaves essential oil extracted with hydrostilistation with higher heat indeed resulted 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, orientside, 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 [19].Although the macerationis appropriate for some thermolabile components, itseems 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 [19].

In the other study, other plants extracted with maceration process showed better results. Ravi [20]reported larvicidal effects of the *Azolla pinnata* extracts using methanol that showed LC_{50} and LC_{95} values of 867 mg/L and 1293 mg/L at 24 hours against *A. Albopictus*[20]. Besides, Krzyzaniak [21] reported that *Tagetes patula* extracted with ethyl acetate was reported to have LC_{50} on 50 mg/L on *A.aegypti* after 24 hours. Ethyl acetate was reported to result ina higher concentration of patuletin in *Tagetes patula* fraction[21]. However, methanol extract of *Clione celata* (red boring sponge) resulted in the highest larvicidal activity at 500 mg/L against *C. quinquefasciatus* larvae (LC_{50} 95.63 mg/L) [22]. Sharma [23] reported the larvicidal activity of *Achyranthes aspera* leaf extracts. It has LC_{50} 82.5 mg/L against *Aedes aegypti*. Ethanol extract of *Inula racemosa* have potential for use in the control of *A. albopictus* larvae with LC_{50} 25.23 µg/mL [24]. Lakshmi Naidu [25] reported that plants produce a broad range of bioactive chemical compounds consisting of secondary metabolites such as flavonoids, tannins, terpenoids, and alkaloids would significantly produce biological activities and chemical defenses against insects [24]. *Azolla pinnata* cause lethal on *A.aegypti* late third- stagelarvae 1262 mg/L (LC_{50}). Zulkrnin [26] and Dias [11] reported Brazilian Legal amazon flora's ability could be a potential larvicide with LC_{50} ranging from 230 to 292 mg/L after 24 h of exposure. Most of those plants' ethanol extraction gives LC_{50} higher than 20mg/L except *Inula racemosa* (LC_{50} 25.23 µg/mL). There were not many plant extractions with LC_{90} lower than 20 mg/L 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 20mg/L (after 24-hour exposure) deserve attention in studies and should be further tested in the field [13]. This study, since LC_{90} of *M.leucadendra* ethanol extract was not reached and statistically predicted to be higher than 20mg/L (LC_{90}65,920 mg/L). 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 are various, such as alkaloids, steroids, terpenes, and phenolic constituents were investigated earlier for biocontrol potency, and it has positive results [27]. 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 [28,29]. 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 LC90 20mg/L 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. *M.leucadendra* leaves extract, at concentration 0.04%; 0.1%; 0.16%; 0.2%, resulted in a clear water condition and no smell detected. However at concentration 1%; 2%; 3%; 4%, the water was turbid, and the smell was unpleasant. Eventhough *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 [30].

CONCLUSIONS

The extract of *M. leucadendra* can cause lethal on larva *Aedes aegypti*at very high concentration (>1%, 10mg/mL) yet gives low mortality percentage (<50%). This study showed a correlation betweenthe number *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% (37,600mg/mL)with 95% significancy. 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 betterunderstanding.

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Conflict of interests

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

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