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Efficacy and Toxicity of *Parasayu incense* ash as a Larvicide for the Eradication of *Aedes aegypti* (Diptera: Culicidae) Mosquito Larvae

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ABSTRACT: Long-term control of *Aedes aegypti* larvae with temephos has led to resistance and side effects. Therefore, natural agents have been developed to eradicate the vector that causes dengue fever. Therefore, the aim of this study is to evaluate the efficacy and toxicity of *Parasayu incense* ash as a larvicide to eradicate *Aedes aegypti* mosquito larvae. In this experimental study, a completely randomized design was used and the test was conducted by exposing 25 fourth instar larvae to *Parasayu incense* ash for two, four, six, eight and 24 hours, with five replicates for a total sample of 875 tails in the Entomology and Parasitology Laboratory for three months. Data were analyzed using one-way ANOVA and a probit test. One test showed that different exposure times to *Parasayu incense* ash significantly affected the death of *Aedes aegypti* mosquito larvae at p = 0.000 (p< 0.01). The ash toxicity test yielded a lethal time (LT_{50}) value of 57.4539, which is 5 h, 37 min, and 32 s in the extremely hazardous category. In addition, the LT_{90} value was 4.632736, which is 3 h, 21 min, and 48 s in the super toxic category. The coefficient of determination (R^2) was 0.9552 (95.52%), and the correlation coefficient (r) was 0.9773 (97.73%). Therefore, to eradicate the vector causing dengue virus infection, *Parasayu incense* ash should be used, which has been shown to be effective, efficient, and toxic to *Aedes aegypti* mosquito larvae at exposure times of 2, 4, 6, 8, and 24 hours.

KEYWORDS: *Aedes aegypti*; Ash; Dengue virus; Infectious disease vector; *Parasayu incense*; Larvicides.

1. INTRODUCTION

Dengue virus (DENV) infection is transmitted by mosquito vectors *Aedes* spp., *Aedes aegypti*, and *Aedes albopictus*, which causes dengue fever (DF), dengue hemorrhagic fever (DHF), and dengue syok syndrome (DSS) [1]. Dengue fever has become more prevalent in recent decades, with Indonesia contributing to 57% of the incidents [2]. The morbidity and mortality rates experienced significant fluctuations during COVID-19 pandemic [3,4] especially in the Province of Bali. Based on the Health Profile for Bali Province in 2021, dengue fever patients reached 12,082, with a distribution of 6,875 male and 5,207 female cases. Moreover, the incidence rate (IR) reached 278.6 per 100,000 population, with the case fatality rate (CFR) of 0.2% (23 people). Dengue hemorrhagic fever cases have tripled in Bali over the last year, while morbidity has increased 1.5 times. The reported morbidity rate has not met the national target of < 49 per 100,000 population [5,6].

The high incidence rate of DHF in Bali has been shown to be due to several factors, including ineffective mosquito nets eradication movement and 4M Plus programs such as draining, closing, burying and monitoring [7,8]. In addition, sowing of larvacide powder (temephos) in every household is less effective due to decreased community participation in dengue prevention and control efforts, lack of optimal monitoring of dengue-causing vectors and dengue cases by *jentik* monitors (*JUMANTIK*) due to the effect of the COVID-19 pandemic, and the high use of synthetic insecticides and larvicides, resulting in toxic effects on human health and the environment [9–11]. Vectors spread rapidly due to poor sanitation, climate, habitat, and the explosion of the *Aedes aegypti* mosquito population [12]. Meanwhile, the Bali community practice or tradition of burning incense (bakhour) as a kind of ceremony tends to introduce toxins into the atmosphere [13,14]. According to several studies, incense ash exposure can diminish lung function and negatively impact

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health, particularly increasing the risk of respiratory disorders [15,16]. Given that the synthetic colors and scents used to make incense are carcinogenic, the ash resulted from the burning can harm the environment [17,18].

Due to these problems, aromatic pandan leaves (*Pandanus amaryllifolius* Roxb.), lemongrass leaves (*Andropogon nardus*), and wood powder packaged in *Parasayu incense* compositions were devised. *Parasayu incense* is designed not only for use as a ceremonial means but burning ash can be used as a larvacide to control the larval population that causes dengue virus infection [14,19] . Therefore, this study aims to examine the efficacy and toxicity of *Parasayu incense* ash as a natural larvicide for controlling and eradicating *Aedes aegypti* (Diptera: Culicidae) mosquito larvae in the Bali Province. Furthermore, it seeks to report the harmful effects of *Parasayu incense* ash exposure on body morphological damage in larvae, which is crucial in determining the target organs of larvicidal activity. The results are expected to contribute essentially to the management and eradication of *Aedes aegypti* (Diptera: Culicidae) mosquito larvae using a plant-based solution that is safe and ecologically benign.

2. RESULTS

2.1 Efficacy test as larvicide

The efficacy test of exposure to *Parasayu incense* ash as a larvicide for 2, 4, 6, 8, and 24 h on the mortality of *Aedes aegypti* mosquito larvae produced an F-value of 484,728 with a probability value of p = 0.000 which is less than $p<0.01$, indicating that the average mortality of the mosquito larvae was significantly different between treatment groups with a 99% confidence level. The post hoc Fisher's LSD test results indicated that treatment K(+) was not statistically significant compared to P4 and P5, and treatment P2 was not statistically significant compared to P3. Table 1 shows the exposure to *Parasayu incense* ash on the mortality of *Aedes aegypti* mosquito larvae.

Table 1. Exposure to *Parasayu incense* ash on mortality of *Aedes aegypti* mosquito larvae

Treatment Group	Mean ± S.E	Shapiro Wilk *(Sig.)*	Levene Statistic	*p*-value
K(-)	00.00 ± 0.000a	0.000		
K(+)	23.80 ± 0.374b	0.314		
P1	15.40 ± 0.510c	0.814		
P2	21.20 ± 0.374d	0.314	0.079	0.000***
P3	21.60 ± 0.510d	0.814		
P4	22.80 ± 0.374e	0.314		
P5	24.20 ± 0.374f	0.314		

Abbreviations: *** = Very significant difference ($p<0.01$); Numbers by different letters = Very significant difference ($p<0.01$).

2.2 Toxicity test of *Parasayu incense* ash

The toxicity test of *Parasayu incense* ash on the death of *Aedes aegypti* mosquito larvae yielded a probability value of $p = 0.0041$ which is less than $p < 0.01$. This indicates that the time of exposure to *Parasayu incense* ash (X) influenced the mortality of the larvae (Y). The 50 percent Lethal Time (LT_{50}) test yielded the equation y = ax+b, specifically 5 = 1.3921x + 2.5509, leading to an LT_{50} value of 57.4539. Therefore, the 5 h, 37 min and 32 s exposure to *Parasayu incense* ash caused 50% death of *Aedes aegypti* mosquito larvae. Meanwhile, the lethal time test of 90% (LT_{90}) obtained the equation 9 = 1. 3921x + 25509 with an LT_{90} value of 4.632736. Therefore, the 3 h, 21 min, and 48 s exposure to *Parasayu incense* ash caused 90% death of *Aedes aegypti* mosquito larvae. The regression coefficient value for the period of exposure was 1.3921, indicating that when the mosquito larvae are exposed to *Parasayu incense* ash for an additional 1 h, the mortality of *Aedes aegypti* (Y) is expected to increase by 1.3921. Furthermore, the coefficient of determination (R^2) was 0.9552, indicating that the period of exposure affected the death rate of the mosquito larvae by 95.52%, while other variables only had an effect which amounted to 4.48%. The correlation coefficient (r) was 0.9773 (97.73%), this suggests a high association between the period of exposure and the mortality rate of the mosquito larvae [20]. Table 2 and Figure 1 show the results of the probit test and the association between the period of exposure to *Parasayu incense* ash and the mortality of the *Aedes aegypti* mosquito.

Table 2. LT_{50} and LT_{90} exposure to *Parasayu incense* ash on mortality of *Aedes aegypti* mosquito larvae

Group	Time (min)	Time Log	Probit value	LT_{50} value	LT_{50} (h)	LT_{50}	LT_{90} value	LT_{90} (h)	LT_{90}
P1	120	2.079	5.31	1.1779	00:28:16	x= 57.4539 (05:37:32)	3.1017	01:14:27	x =4.632736 (03:21:48)
P2	240	2.380	6.04	1.0289	00:24:42		2.7095	01:05:02	
P3	360	2.556	6.08	0.9581	00:23:00		2.5228	01:00:33	
P4	480	2.681	6.34	0.9134	00:21:55		2.4053	00:57:44	
P5	1440	3.158	6.88	0.7754	00:18:37		2.0419	00:49:00	

Abbreviations: LT_{50} = Lethal Time 50%; LT_{90} = Lethal Time 90%.

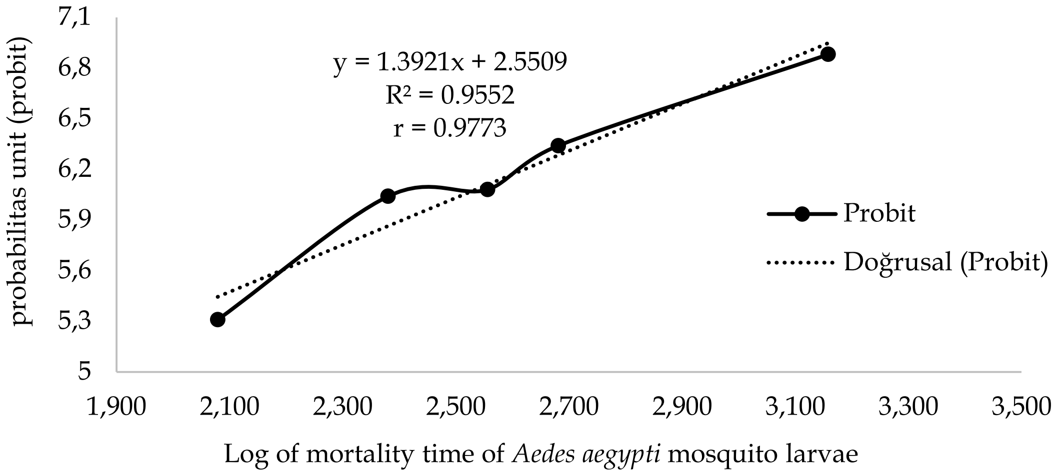

Figure 1. The relationship between time of exposure to *Parasayu incense* ash and mortality of *Aedes aegypti* mosquito.

2.3 Damage to the larval body

Test results and microscopic observations confirmed that seven-day-old larvae (instar IV) appeared dead, did not move, and had black-brown body parts. After treatment with *Parasayu* fumigated ash at various times, the head, spines, siphon, abdomen, exoskeleton, and tarsus skin of the larvae were detached (destroyed). Figure 2 shows the damage to the body of *Aedes aegypti* mosquito larvae.

2.4 Laboratory assays of *Parasayu incence*

The results of laboratory tests conducted in three different locations, namely the laboratory room, outside the laboratory, and in residential areas, determined that the use of *Parasayu incense* was safe and met environmental quality standards. All test parameters contained up to seven indicators (Particulate Matter ($PM_{2,5}$); Sulfur Dioxide (SO_2); Nitrogen Dioxide (NO_2); Carbon Monoxide (CO); Ozon (O_3); Hydrogen Sulfide (H_2S); and Total Suspended Particulate (TSP) following the national quality standards of Indonesia, allowing them to be used as a means of ceremony or as natural larvicides while being environmentally friendly. Table 3 summarizes the laboratory findings.

Table 3. Laboratory assay of *Parasayu incense*

Indicators	Unit	Analysis Methods	Result			Quality standards	Information
			A1	A2	A3		
Particulate Matter ($PM_{2,5}$)	μg/Nm³	Gravimetri	0.13	0.7	0.6	66	***
Nitrogen Dioxide (NO_2)	μg/Nm³	SNI 7119-2-2017	0.42	0.36	0.27	400	***
Sulfur Dioxide (SO_2)	μg/Nm³	SNI 7119-7-2017	3,67	3,89	3,22	900	***
Carbon Monoxide (CO)	μg/Nm³	Direct Reading	258	198	193	30.000	***
Ozon (O_3)	μg/Nm³	SNI 7119-8-2017	1.88	2.71	1,46	235	***
Hydrogen Sulfide (H_2S)	ppm	Methylene Blue	0,006	0,002	0,003	0,02	***
Total Suspended Particulate	μg/Nm³	Gravimetri	3.40	3.77	3.49	230	***

Abbreviations: *** = Safe and meets quality requirements.

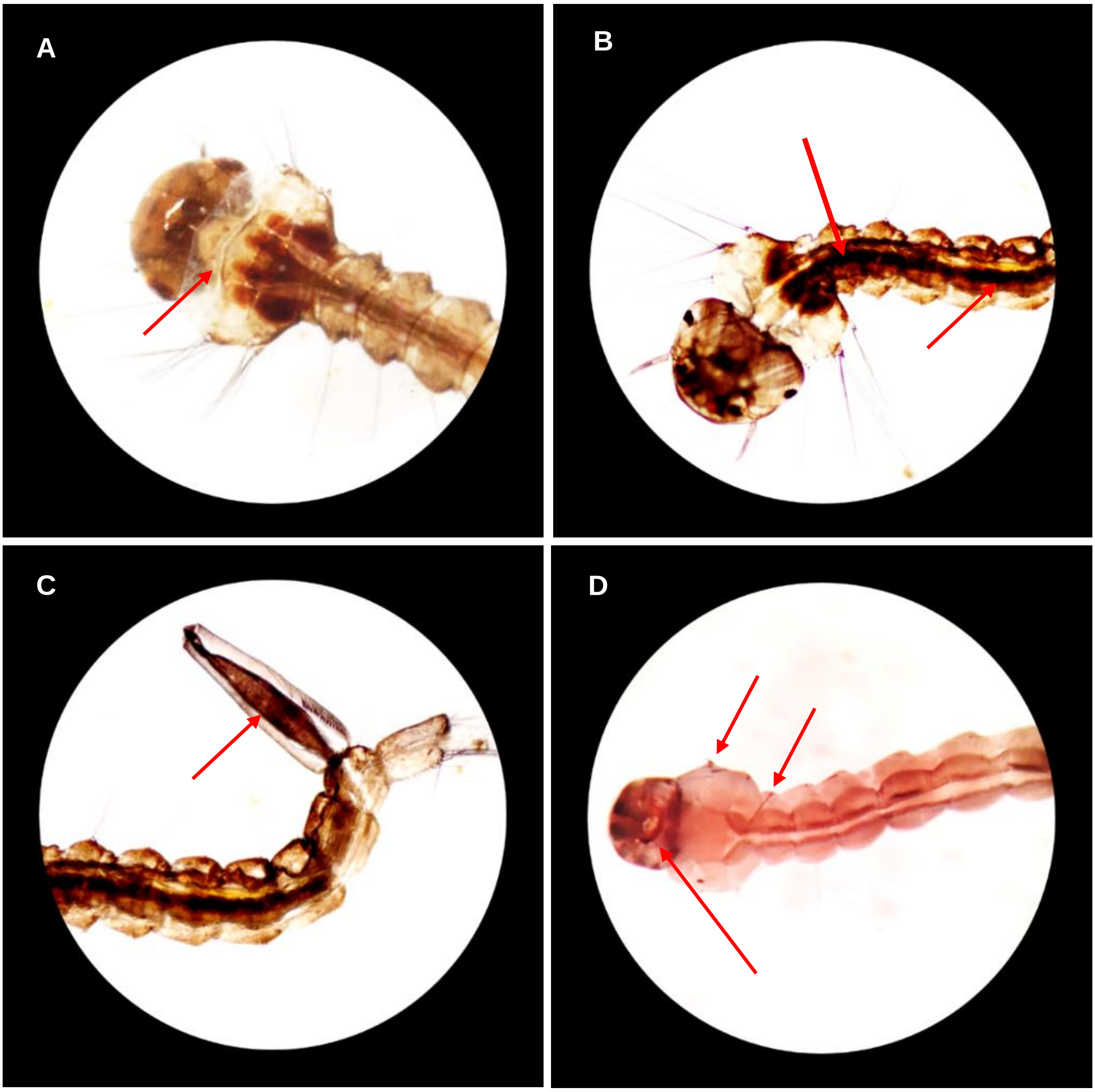

Figure 2. Visualization of body damage to *Aedes aegypti* mosquito larvae. No staining and 40X magnification. **A** = Head and thorax are difficult to identify; larval neck looks elongated. **B** = The abdomen (digestive tract) is blackish brown and filled with *Parasayu incense* ash, which is swallowed by the larvae. **C** = Anal papillae, ventral brush, comb scales, caudal hairs, and spiracles detached from the body and appear transparent, and Shipon experiences thickening and widening because it is filled with *Parasayu incense* ash. **D** = Lateral hair on the abdomen detached from the body, antennae, palps, scutum, eyes, and labellum on the head fall out and appear blackish brown.

3. DISCUSSION

Our results indicated that the exposure of *Aedes aegypti* mosquito larvae to *Parasayu incense* ash for 2, 4, 6, 8, and 24 h increased mortality. The 2 h exposure (P1) caused 62% of deaths in the test larvae, hence, the shortest duration of exposure proved efficient as a larvicide against *Aedes aegypti* mosquito larvae. An insecticide is deemed effective when it can kill 50% of the total test animals. Moreover, the use of larvicides is recognized to have a significant effect according to the World Health Organization Pesticide Evaluation Scheme (WHOPES) when they cause 10–95% death of test larvae for 24 h, while the Ministry of Health for the Republic of Indonesia set a target of 90–100% for 24 h. Based on these requirements, treatments P1, P2, and P3 representing exposure to *Parasayu incense* ash for 2, 4, and 6 h respectively met the WHOPES target [21]. Meanwhile, positive controls namely giving *Abate*® 1%, as well as P4, and P5 representing exposure to

Parasayu incense ash for 8 and 24 h respectively fit within the category established by the Republic of Indonesia's Ministry of Health [22]. Thus, exposure to *Parasayu incense* ash for 2, 4, 6, 8, and 24 h proved efficacious and toxic as a larvicide against *Aedes aegypti* mosquito larvae mortality.

Observations performed throughout the investigation showed that *Aedes aegypti* mosquito larvae exposed to *Parasayu incense* ash were harmed and apparently destroyed. The majority of the dead larvae settle on the bottom of the tube, but some float on the water's surface. These results are consistent with previous studies, which reported that dead larvae were destroyed and marked by organ rupture, as well as deposits on the bottom or floats on the top [9,23]. The mortality of *Aedes aegypti* mosquito larvae increased significantly in each treatment group. This might be due to the bioactive constituents in *Parasayu incense*, specifically silica compounds, which are capable of inhibiting larvae growth. Silica compounds have been reported to operate as a stomach, respiratory, and contact poison and to interfere with larval spiracles that penetrate the exoskeleton through the tarsus [14,19]. They inhibit metabolism and cell synthesis activities, such as electron transport in the mitochondria, leading to a reduction in cell function of creating energy, which is utilized as a food source for larvae [23–25].

The ash of *Parasayu incense* contains bioactive substances such as polyphenols, saponins, and alkaloids which are destroyed during the combustion process. Although it has been degraded into a simpler form, the chemical constituents have a larvicidal action. After several time variations of exposure, *Parasayu incense* ash functioned as a larvicide against *Aedes aegypti* mosquito larvae. The bioactive chemicals found in the ash have been investigated and showed a mode of action that includes silica compounds, polyphenols, saponins, and alkaloids that inhibit larval growth. They interfere with the reaction and stimulus for eating, preventing the larvae from distinguishing between the position and location of their food. Also, taste receptors in the mouth region are inhibited, causing the larvae to lose excitability of the surrounding nutrients, culminating in death [22], [26-27]. Saponins and alkaloids have been reported to function as stomach poisons in larvae by blocking the cholinesterase enzyme system. They are anti-cholinesterase chemicals involved in larval growth and development. The cholinesterase enzyme in larvae can be phosphorylated and rendered inactive by anti-cholinesterase, this leads to the inhibition of acetylcholine breakdown, which causes its buildup in the synaptic cleft, thereby culminating in muscle spasms and paralysis as well death [28-29]. Meanwhile, polyphenols can function as digestive toxins by interfering with cell metabolic processes [31] such as electron transport in the mitochondria which are organelles within cells that turn nutrients into energy. When the process in the mitochondria is impeded, the generation of food and energy in the cell is hampered, consequently, the larvae are unable to create food, and the cell dies [32].

Flavonoids operate as respiratory toxins, inhibiting the spiracles and pores on the surface of the larva's body, causing the breathing process to be impeded, which leads to the withering of the nervous system, as well as muscular spasms, and death [33]. Although flavonoids are desisccant, ingestion tends to cause disruptions in a lack of oxygen intake into the respiratory system, consequently, the larvae experience excessive heat, their bodies will turn brown, and the intestines become black and rigid due to the poison's potency. According to a study by Chan, flavonoid and tannin chemicals have had toxic impacts that can agglomerate the proteins of the *Aedes aegypti* mosquito [34]. They can denature proteins, leading to a decrease in the permeability of the larval cell wall, loss of nutrition stimulation, and larvae death [35]. Based on this mechanism, *Parasayu incense* ash can be used as a larvicide and applied into drains, containers, and larval breeding places to eradicate *Aedes aegypti* mosquito larvae, especially in endemic regions of Bali Province.

A safety check of *Parasayu incense* products in the laboratory revealed that *Parasayu incense* is suitable for use as a means of ceremony and the burning ash is safely used as a larvacide and categorized as environmentally friendly. Laboratory test results indicate *Parasayu incence* ash deserves to be used as a larvacide for the eradication of the *Aedes aegypti* mosquito (Diptera: Culicidae). All inspection indicators meet the standard environmental criteria of Government Regulations 22 of 2021 [36]; the World Health Organization Pesticide Evaluation Scheme [21,37,38]; and Regulation of the Minister of Health, 50 of 2017 concerning standards for environmental health quality standards and health requirements for vectors and animals carrying diseases and their control [39].

4. CONCLUSION

Exposure to *Parasayu incense* ash for 2, 4, 6, 8 and 24 hours proved to be effective larvicide against mortality of *Aedes aegypti* mosquito larvae. Therefore, this incense can be developed as a natural larvicide to reduce the vector responsible for dengue virus infection. In the future, chronic toxicity experiments will be required to evaluate the effects and consequences of long-term exposure to *Parasayu* frankincense ash.

5. MATERIALS AND METHODS

5.1 Research design

This research was an experimental study conducted in the laboratory using a completely randomized design. *Aedes aegypti* mosquito eggs collected from entomology laboratory, East Java Health Office, Surabaya. The efficacy and toxicity tests were performed by dividing 875 *Aedes aegypti* mosquito larvae into seven treatment groups, with each consisting of 5 sample units and each unit containing 25 mosquito larvae (n = 25) with the following details: 1) negative control group (K-) without exposure, 2) positive control group (K+) exposed to 1% *abate*®, and 3) the treatment group exposed to *Parasayu incense* ash for 2 h (P1), 4 h (P2), 6 h (P3), 8 h (P4), and 24 h (P5), with five repetitions each. This test was based on insecticide testing guidelines from the Republic of Indonesia's Ministry of Health [22] and the Food and Drug Supervisory Agency [40] and World Health Organization Pesticides Evaluation Scheme [38] procedures for non-clinical toxicity studies *in vivo*. The exposure results, namely the number of dead larvae was reported on an assessment sheet, which was then statistically analyzed.

5.2 Execution time

The Denpasar Health Polytechnic ethics committee granted ethical approval under registration number LB/02.03/EA/KEPK/0131/2021. This research was approved and granted a research permit by the Bali Province Investment and One-Stop Service with registration number 070/1500/ IZIN-C/DISPMPT. The study lasted three months from February–April 2021 and was carried out at the Entomology and Parasitology Laboratory, Department of Environmental Health, Denpasar Health Polytechnic.

5.3 Materials

The materials used include *Parasayu incense*, fish pellets, 1% *abate*®, and *Aedes aegypti* mosquito eggs.

5.4 Inclusion and exclusion criteria

Aedes aegypti mosquito larvae aged seven days (Instar IV), mosquito larvae moving actively and swiftly, and a consistent number of 25 individuals were the inclusion criteria in this study. In comparison, the exclusion criteria include samples that were not *Aedes aegypti* mosquitoes, aged > 7 days, and slow movement or death, and a number of < 25 tails.

5.5 *Parasayu incense* production

The composition of *Parasayu incense* consists of aromatic pandan leaves (*Pandanus amaryllifolius* Roxb.) collected from Kumbasari market traders, as much as 3 kg. Lemongrass leaves (*Andropogon nardus*) up to 5 kg collected from the aromatic lemongrass farmer depot in Baturiti, Tabanan, and wood powder up to 3 kg obtained from the wood cutting place in Serkel Adijati, Klungkung. The production of *Parasayu incense* is carried out in sole proprietorship (*UD*) Prapen Bali, Klungkung. All ingredients were thoroughly washed and then milled separately until smooth. The whole material was drained by aeration. Furthermore, the manufacture of incense wet dough was done by putting all the ingredients into the dough making tube. At this stage, a ratio of 1.2.1 was used, namely 2 kg of fragrant pandan leaves, 4 kg of fragrant lemongrass leaves, and 2 kg of wood powder. To the dough, was added 0.3 kg of wood adhesive and 750 mL of water. The dough that had been mixed well was then inserted into the molding machine. In bamboo bitting, as many as 680 stems were prepared for incense printing with a diameter of 0.4 cm and a length of 32 cm. The dried incense that had been printed was then dried by aerating for 2 days in a closed room.

5.6 Laboratory assay of *Parasayu incense*

Parasayu incense safety testing was carried out at the Technical Implementation Unit of the Hyperkes Hall and work safety with laboratory test request number 0131/I/Hyperkes/III/2021. A Sampling point was carried out at A1 = Post Monitor PTLSA (S. 080 43' 336*E. 1150 13' 397*); A2 = Area of IPL (S. 060 43' 3610E.155013'174*) and A3 = Residential Area (S. 080 43' 361* E. 1150 13' 203*). Environmental Conditions A1 = Post Monitor PTLSA (S. 080 43' 336*E. 115013'397*), Temperature = 27 °C, Air Pressure = 7601 mmHg; A2 = IPL Area (S. 060 43' 3610 E.1550 13' 174*), Temperature = 24°C, Air Pressure = 761 mmHg; A3 = Residential Area (S. 080 43' 361* E. 1150 13' 203*). Temperature = 32 °C, Air Pressure = 763 mmHg. Testing was based on the standard environmental criteria of Government Regulations 22 of 2021 [36]; the World Health Organization Pesticide Evaluation Scheme [21,37,38]; and Regulation of the Minister of Health, 50 of 2017, concerning standards for environmental health quality standards and health requirements for vectors and animals carrying diseases and their control [39].

5.7 Statistic analysis

The statistical analysis was conducted using one-way ANOVA followed by Fisher's LSD test with a significance level of 99% (p <0.01). The toxicity test used Lethal Time 50% (LT_{50}) and 90% (LT_{90}), calculated using the unit probability test with linear regression. The data were processed using the SPSS (*Statistical Package for the Social Sciences*) Inc. program version 25.0.

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