

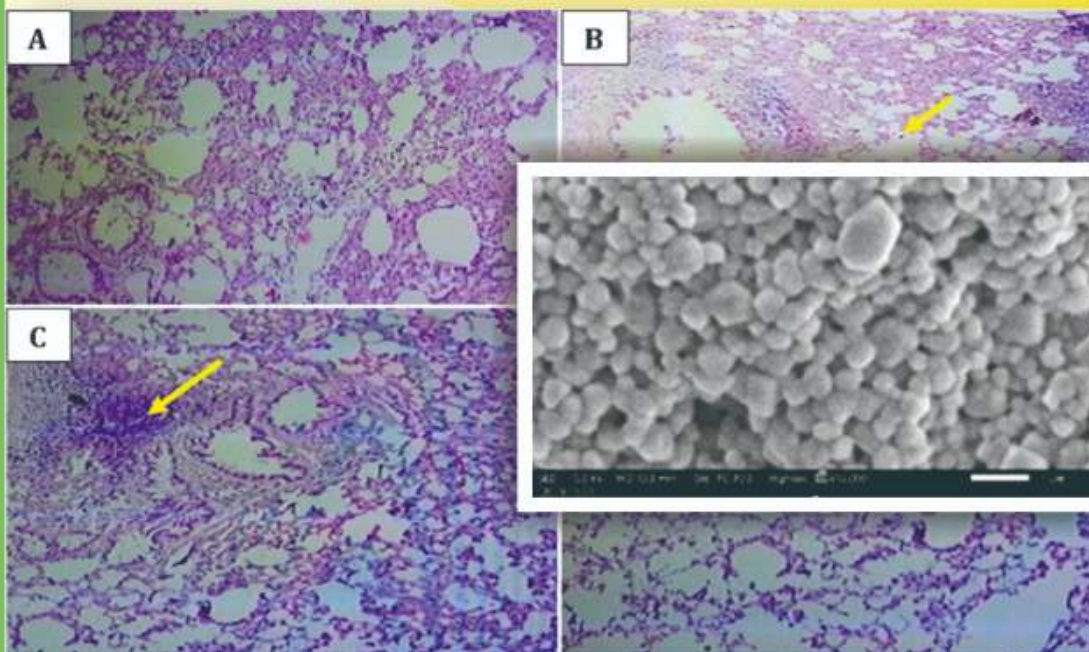
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Toxicity of *Legiayu incense* as Insecticide and Larvicide Against *Aedes aegypti* Mosquitoes Mortality

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ABSTRACT

Community-based DHF vector control has been implemented in Indonesia but has not yet obtained optimal results. Thus, in the community choosing synthetic insecticides to control disease vectors. Long term and excessive using of insecticides enable a toxic effect and resistance to mosquitoes. Burning of mosquitoes coils and incense containing synthetic dyes and fragrances have the potential to reduce environmental quality. This research was conducted to analyze the toxicity of *Legiayu incense* as an insecticide and larvicide against *Aedes aegypti* mosquito mortality. The research design is experimental with a completely randomized design. Testing was conducted by providing exposure to smoke and ash of *Legiayu incense* five times on twenty-five *Aedes aegypti* mosquitoes. Statistical analysis used one-way ANOVA, and probit test. The result showed that insecticides effective exposure for 20 minutes with a durability of 6 hours ($p=0.000$), while as larvicide effective exposure for 24 hours ($p=0.000$). Exposure *Legiayu incense* smoke obtained an LT_{50} value of $09012 \leq 5$ (super toxic category) with a time of 15 minutes 39 seconds, R^2 of 99.24%, and r of 99.62% while exposure to the ash of *Legiayu incense* obtained LT_{50} value of $0,05896 \leq 5$ (super toxic category) with time 19 hours 15 minutes 34 seconds, R^2 and r of 100%. Histopathological test results showed that *Legiayu incense* smoke did not cause tissue degeneration, necrosis, hyperplasia, and metaplasia in the lung tissue of mice (*Mus musculus*) in 12 weeks period. Thus, *Legiayu incense* is effective as insecticides and larvicides against *Aedes aegypti* mosquitoes and-potential substitute for mosquito repellent synthetic incense.

Key words: *Aedes aegypti*, Insecticide, Larvicide, *Legiayu incense*, Toxicity.

INTRODUCTION

Aedes aegypti mosquito is dengue virus vector transmitted on the human body (Ministry of Health, 2018). The number of Dengue Hemorrhagic Fever (DHF) cases in Indonesia from 2006-2015, 57% of the cases are from Indonesia (Mayasari *et al.*, 2020). A very significant increase in incidence rate (IR). DHF is widespread in the Indonesian Province of Bali. In Bali Province in 2019, a total of 5.956 cases of dengue occurred, with the IR was $137.3 > 49$ per 100.000 people. It is considerably higher than the national (Bali Health Department, 2020).

High cases of DHF in Bali may be due to the application of the 3M Plus program are not going well and low of community participation. Furthermore, the increased use of synthetic

insecticides, which is causing a decline in environmental standards and mosquitoes experiencing resistance (Garcia *et al.*, 2018; Marcombe *et al.*, 2017). The substance of synthetic insecticides has positive attributes as an insect repellent and has adverse health effects due to high acetylcholine accumulation levels (Wong *et al.*, 2020) with the symptoms include vomiting, muscle spasms, bradycardia, paralysis, and death (Hung *et al.*, 2020; Jerry *et al.*, 2012). Environmental quality degradation is caused by the excessive use of synthetic insecticides. Besides, the use of synthetic dye incense and fragrances is increasing significantly in Bali. This synthetic fragrance contains carcinogenic substances which pollute the air (Amador-Muñoz *et al.*, 2020; Geng *et al.*, 2017)

and produce environmental damage (Guo *et al.*, 2020). The high side effects and impacts of the use of incense containing synthetic fragrances resulted in people switching to utilizing natural ingredients because it is considered safer for health, cheaper, and environmentally friendly (Susilowati *et al.*, 2018; Zulfikar *et al.*, 2019).

Based on this, developing environmentally friendly incense organic raw materials such as *Legundi* leaf (*Vitex trifolia* L.), lemongrass leaves (*Andropogon nardus*), and wood grain packaged in *Legiayu incense*, are necessary. The pharmacologically content of oleum citronellal, geraniol, tannin, flavonoids, saponins, champine, pinine and polyphenols that serve as insecticides and larvicide (Bao *et al.*, 2018; Chan *et al.*, 2016). These compound has benefits as contact, stomach and respiratory toxins that result in death (Susilowati *et al.*, 2018). *Legiayu incense* has potential a substitute for mosquito an organic repellents–synthetic fragrance-based incense. The objective of this research was to analyze the toxicity of *Legiayu incense* as insecticide and larvicide against *Aedes aegypti* mosquito mortality. The results of this research are initial efforts to prevent, and suppress of *Aedes aegypti* mosquitoes by developing herbal products based on local wisdom which is effective, safe, are not toxins and no environmental pollution.

MATERIAL AND METHODS

The material research were *Aedes aegypti* imago mosquitoes aged 3 weeks and larvae aged 6 days (Instar IV) and 8 weeks old strain Balb/c (25 tails) mice weighing 24-35 gr. The tools used were 2 large buckets, 50 mL plastic glass, test tube, 4-piece drop pipette, digital scale, measuring flask, cutter, medium size scissors, petri dish, filter research, Peet Grady Chamber size 45×45×45 cm³, sterile handscone, cotton, hygrometer®, sterile gauze, tissue, room thermometer, glass aspirator®, gas lighter, stopwatch®, gadgets and stationery. While the materials used in this research were *Aedes aegypti* mosquito eggs as many as 1.250 grains, temephos 1%®, mosquito repellent coils contain *transfluthrin*® 1 box, *Legiayu incense*, water, fish pellets brand takari®, and sugar 50 g.

Research design

This experimental research follows to Completely Randomized Design (CRD), completed from January to June 2020, in Entomology

Laboratory, Department of Biology, Hindu University of Indonesia, UPTD. Balai Hyperkes & Work Safety and National Agency of Drug & Food Bali Province.

Testing legiayu incense as insecticide

The treatment in this research gave exposure *Legiayu incense* to 25 heads of adult population of *Aedes aegypti* mosquitoes with a different time of 10, 20, 30 and 40 minutes also 0 minutes (as control) negative control (no treatment) and positive control (with repellent burn), Replication are five times with a total sample of 750 *Aedes aegypti* mosquitoes.

Testing legiayu incense as larvicides

The testing as larvicide was conducted by pouring 100 mg ash from the burning of *Legiayu incense* to test tube that has been filled with 25 heads of larvae of the 6-day-old *Aedes aegypti* mosquito (instar IV) replicatioans are five times with an incubation period of 12 and 24 hours, 2 (negative control and positive control (temephos 1%®), the total sample was 500 *Aedes aegypti* mosquito larvae.

Toxicology and histopathology testing

Testing of smoke and ash toxicity of *Legiayu incense* was conducted through the calculation of the number of *Aedes aegypti* mosquito mortality after being treated with Lethal Time (LT₅₀) parameters or exposure time resulting in larvae and imago experiencing death of 50% of the total population (OECD, 2009). Histopathology testing was conducted to determine the histological picture of rat lung tissue (*Mus musculus*) after being given exposure to *Legiayu* smoke incense for 12 weeks. The test was conducted in the Food and Drug Administration of Bali Province. *Legiayu incense* smoke was tested for toxicity by exposing BALB/c strain mice to incense smoke for 40 minutes every day for a period of 12 weeks. Mice that had been exposed were subsequently physically sacrificed by having their necks dislocated. If the animal is deceased, do surgery to extract the lungs and proceed with histological preparations using Hematoxylin Eosin (HE) staining. These results are used to analyze the impact of *Legiayu incense* smoke exposure as well as analyze the damage caused (OECD, 2018). The testing the safety of smoke and ash of *Legiayu incense* in the UPTD. Balai Hiperkes & work safety Bali Province.

Table I. The mortality rate of *Aedes aegypti* mosquitoes exposed to different times

Treatment	Mean ± SE	Shapiro Wilk	Levene Statistic	ANOVA
K(-)	00.00 ±0.000a	0.000		
K(+)	15.40 ±2.522b	0.739		
P1	11.40 ±1.860b	0.617	0.031	0.000**
P2	17.80 ±1.530c	0.814		
P3	23.40 ±0.510d	0.814		
P4	24.20 ±0.837d	0.314		

Abbreviations: SE= Standard error; ** = Very significantly different ($p < 0.01$); Numbers with letters = Very significant differences ($p < 0.01$).

Population and samples

The *Aedes aegypti* mosquitoes were collected from the Entomology Laboratory at the East Java Health Office in Surabaya. The samples used *Aedes aegypti* with a criteria three-weeks for testing as insecticides and larvae aged six days for testing as larvicides. The number of treatments and repeats in this research is based on World Health Organization Pesticides Evaluation Scheme procedures (WHOPES, 2018). The Health Science Ethics Commission (KEPK) Denpasar Health Polytechnic approved the use of *Aedes aegypti* mosquitoes and rats (*Mus musculus*) strain Balb/C as a research study with Ethical Approval number LB.02.03/EA/KEPK/0113/2020.

Statistical analysis

The mortality of *Aedes aegypti* larvae and imago was analyzed by a one-way ANOVA test, followed by a Fisher's least significant difference (LSD) post hoc test with a significance level of ($p < 0.01$). The toxicity test uses the Unit Probability (Probit) test to obtain a Lethal Time value of 50% (LT₅₀). Data is presented in the form of tables, graphs, pictures and narration. This data was compiled using the Statistical Product and Service Solutions (SPSS) Inc. 25.0.

RESULT AND DISCUSSION

Legiayu incense smoke as an insecticide

The Shapiro-wilk normality test revealed that the probability value between treatment groups was normally distributed ($p > 0.01$). The Levene test results revealed that the variance value was homogeneous ($p = 0.031$), which was greater than $p > 0.01$. The one-way ANOVA test results (Table I) revealed that the probability value ($p = 0.000$) was smaller than $p < 0.01$. Thus, exposure to *Legiayu incense* smoke among the treatment (10, 20, 30, and 40 minutes & control) had a very significant effect on

the mortality of *Aedes aegypti* mosquitoes. The LSD test showed that the treatments of negative control (K-), positive control (K+), and exposure treatment for 20 minutes (P2) are very significantly different ($p < 0.01$). While exposure to *Legiayu incense* smoke for 30 minutes (P3) compared to exposure for 40 minutes (P4) was not significant ($p > 0.01$) (Table I).

Based on the unit probability test, exposure of *Legiayu incense* smoke to the mortality of imago *Aedes aegypti* mosquitoes received a significance value ($p = 0.000$) smaller than value ($p < 0.01$). Thus, there is an effect of exposure time to *Legiayu incense* (X) smoke on the mortality of *Aedes aegypti* mosquitoes (Y). The result of the Lethal Time 50% test on the mortality of the imago *Aedes aegypti* mosquito given exposure to *Legiayu incense* smoke at different times obtained LT₅₀ value of $0.9012 \leq 5$ (super toxic category). Thus, exposure to *Legiayu incense* smoke was able to result in 50% mortality of imago *Aedes aegypti* mosquitoes within 15 minutes 39 seconds. The results of the analysis of the relationship between exposure time to *Legiayu incense* smoke (Figure 1) shows the value of variable regression coefficient of smoke exposure time range (X) of 0.0753 means that if there is an increase of 1 minute after exposure to *Legiayu incense* smoke, the mortality of imago *Aedes aegypti* mosquitoes (Y) will increase by 7.53%, coefficient of determination (R²) of 0.9924 (99.24%) thus, the mortality of *Aedes aegypti* mosquitoes was influenced by the length of time exposure to *Legiayu incense* smoke. While the correlation coefficient value (r) is 0.9962 (99.62%) the relationship between the time of smoke exposure and the mortality rate of imago is very strong (Darwin *et al.*, 2021).

Based on the smoke testing *Legiayu incense* as an insecticide there is an increase in the mortality of *Aedes aegypti* mosquitoes, after exposure to *Legiayu incense* smoke.

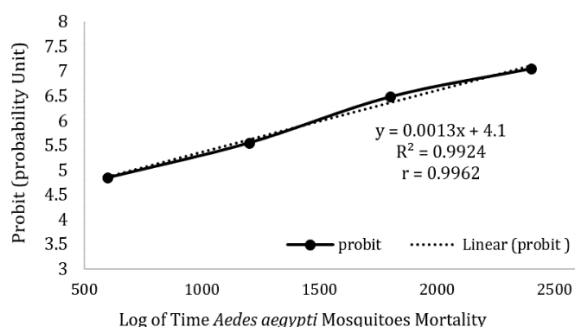


Figure 1. Relationship of exposure time to *Aedes aegypti* mosquitoes mortality

It is caused by compounds in *Legiayu incense* such as essential oils, chalcone, dyhydrocalcon, flavonoids, 3-geranylflavones, xanthenes, saponins, tannins, polyphenols and alkaloids that inhibiting the excitatory power of eating insects, inhibitors of the respiratory system and inhibiting moulting hormones (Saklani *et al.*, 2017; Siew *et al.*, 2019). *Legiayu incense* have an effect as insecticides such as flavonoids, saponins, citoronellal, and polyphenols. The mode of action of these flavonoid compounds is inhibiting effect on the respiratory poison. If the insect inhales *Legiayu incense* smoke will breathing disorders and excessive dehydration and result in death (Saputra and Pakkan, 2018). Saponin and citronellal compounds are able to decrease the elasticity of the surface of the insect's body, lower the turgor voltage of the insect's body, as a result of skin trauma and die of dryness (Ekawati, 2017; Susilowati *et al.*, 2018). Polyphenol compounds can provide an insecticidal effect by binding to factor adhesions, extra cellular proteins and soluble proteins resulting in increased damage to cuticles and cells that have an impact on the decrease in the ability in producing colinesterase enzymes for the growth and transport of food throughout the body, resulting in insect death (Aji, 2017). Based on this, the use of raw materials of lemongrass leaves and legundi effective as an insecticide (Fitriansyah *et al.*, 2018). The impact of use of these ingredients provide healthy, environmentally, minimizing of side effects and pollution. Also, the use of smoking methods is an effective apply of insecticide (EPA, 2014).

Legiayu incense smoke as an larvicide

The Shapiro-Wilk normality analysis showed that the probability value between treatment groups was normally distributed

($p > 0.01$). The Levene test results showed that the variance value was homogeneous ($p = 0.080$) was greater than $p > 0.01$. The one-way ANOVA test analysis (Table III) showed that the probability value ($p = 0.000$) was less than $p < 0.01$. Thus, exposure to *Legiayu incense* ash had a very significant impact on *Aedes aegypti* mosquito larvae mortality. The LSD test showed that the treatment of the positive control (K+), treatment of 12 h (P1), and treatment of 24 h (P2) were not significantly different ($p > 0.01$) (Table II).

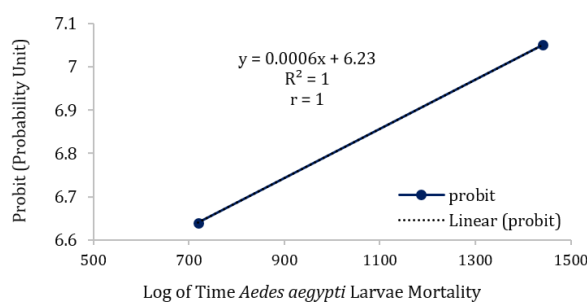


Figure 2. Relationship of ash exposure time to mortality of mosquito larvae.

Based on the unit probability test, among treatment of exposure of *Legiayu incense* ash to the mortality of *Aedes aegypti* mosquito larvae obtained a significance value ($p = 0.000$) smaller than the value ($p < 0.01$). Thus, this indicated that exposure time of *Legiayu incense* (X) ash affect on the mortality of larvae *Aedes aegypti* mosquitoes (Y). The Lethal Time 50% analysis results on the mortality of *Aedes aegypti* mosquito larvae exposure to *Legiayu incense* ash at different times obtained LT_{50} a value of $0,05896 \leq 5$ (super toxic category). Thus, exposure to *Legiayu incense* ash was able to result in 50% mortality of *Aedes aegypti* mosquito larvae within 19 h 15 min 34 s. The analysis of the relationship between exposure time to *Legiayu incense* ash (Figure 2) showed the regression coefficient of ash exposure time range (X) of 0.0342 means that if there is an increase of 1 hours after exposure to *Legiayu incense* ash, the mortality rate of *Aedes aegypti* mosquito larvae (Y) would increase by 3.42%, with a coefficient of determination (R^2) of one, implying that the time of exposure to *Legiayu incense* ash has a 100% effect on the mortality rate of *Aedes aegypti* mosquito larvae. While the correlation coefficient (r) is 1 (100%) the relationship between the time of exposure to ash and the mortality rate of larvae is perfect.

Table II. The mortality rate of larvae *Aedes aegypti* mosquitoes exposed to different times

Group	Mean±SE	Shapiro Wilk	Levene Statistic	ANOVA
K(-)	00.00±0.000a	0.000		
K(+)	23.20±1.700b	0.421		
P1	23.80±1.700b	0.421	0.080	0.000**
P2	24.40±0.800b	0.046		

Abbreviations: SE= Standard error; ** = Very significantly different ($p < 0.01$); Numbers with letters = Very significant differences ($p < 0.01$).

Table III. Laboratory results of smoke and ash safety *Legiayu incense*

Parameter	Unit	Test Results			Quality Standard	Test Result
		A 298	A 299	A 300		
PM _{2.5}	µg/Nm ³	0.5	0.7	0.9	66	Safe
Nitrogen Dioxide (NO ₂)	µg/Nm ³	0.2	0.4	0.2	400	Safe
Sulfur Dioxide (SO ₂)	µg/Nm ³	3.48	3.69	1.78	900	Safe
Carbon Monoxide (CO)	µg/Nm ³	0,1	0.1	0.4	30.000	Safe
Oxidant (O ₃)	µg/Nm ³	0.3	0.7	0.8	235	Safe
Hydrogen Sulfide (H ₂ S)	ppm	0.002	0.002	0.002	0.02	Safe
Total Dust (TSP)	µg/Nm ³	1.78	1.96	1.27	230	Safe

The increasing number of *Aedes aegypti* mosquito mortality after exposure caused by compounds content of *Legiayu incense*. By 45% silica compounds enable to inhibit the development of *Aedes aegypti* larvae. Silica compounds is stomach poison, respiratory poison, and contact poison. Silica compounds work by inhibiting the metabolism and synthesis of cells, one of which is transport electrons contained in mitochondria. This results in a decrease in cell performance in producing energy used as a food source of larvae (Vasanth-Srinivasan *et al.*, 2018). *Legiayu incense* ash contains compounds of silica, polyphenols, saponins, alkaloids as bioactive substances that slow the development of larvae. The bioactive substance interferes with the response and excitatory of eating insects. Receptors in the mouth area will be inhibited and the larvae lose excitation to the surrounding nutrients (Sholeha *et al.*, 2018). Saponins and alkaloids compounds as stomach poisoning by inhibiting the working system of enzymes cholinesterase in larvae (Rochmat *et al.*, 2017). Polyphenol compounds as digestive toxins work by inhibiting the work of cell metabolism processes (Astriani and Widawati, 2017). Flavonoid and tannin compounds act as a respiratory toxin in larvae, inhibiting respiration and nerve wither (Susilowati *et al.*, 2018). Flavonoid and tannin are able to provide toxic effects that are able to clump the protein of *Aedes*

aegypti larvae. Flavonoid compounds are able to regulate proteins that result in the permeability of the cell walls of larvae decreased which has an impact on the loss of excitatory stimulus of eating and larvae experience death (Bao *et al.*, 2018; Siew *et al.*, 2019).

Legiayu incense product toxicity test

Environmental toxicology testing of *Legiayu incense* smoke and ash at seven parameters and three different locations revealed that the smoke and ash met environmental quality standards (Table III).

The environmental toxicity tests for the safety of *Legiayu incense* smoke and ash based on 7 parameters with 3 different locations (the laboratory (A298), in the outside area of the laboratory (A299) and in residential areas (A300), showed that PM_{2.5}, NO₂, SO₂, CO, O₃, H₂S, and TSP have meet the minimum standard values and meet the criteria of environmental quality standards and are safe to use indoors and outdoors. In accordance with Government Regulation of the Republic of Indonesia Number 22 of 2021, Bali Governor Regulation number 16 of 2016, and Indonesian National Standard 06-3566-1994 (Indonesian National Standards, 1995). *Legiayu incense* products are feasible and safe to use as insecticides and larvacides to minimize the population of *Aedes aegypti* mosquito.

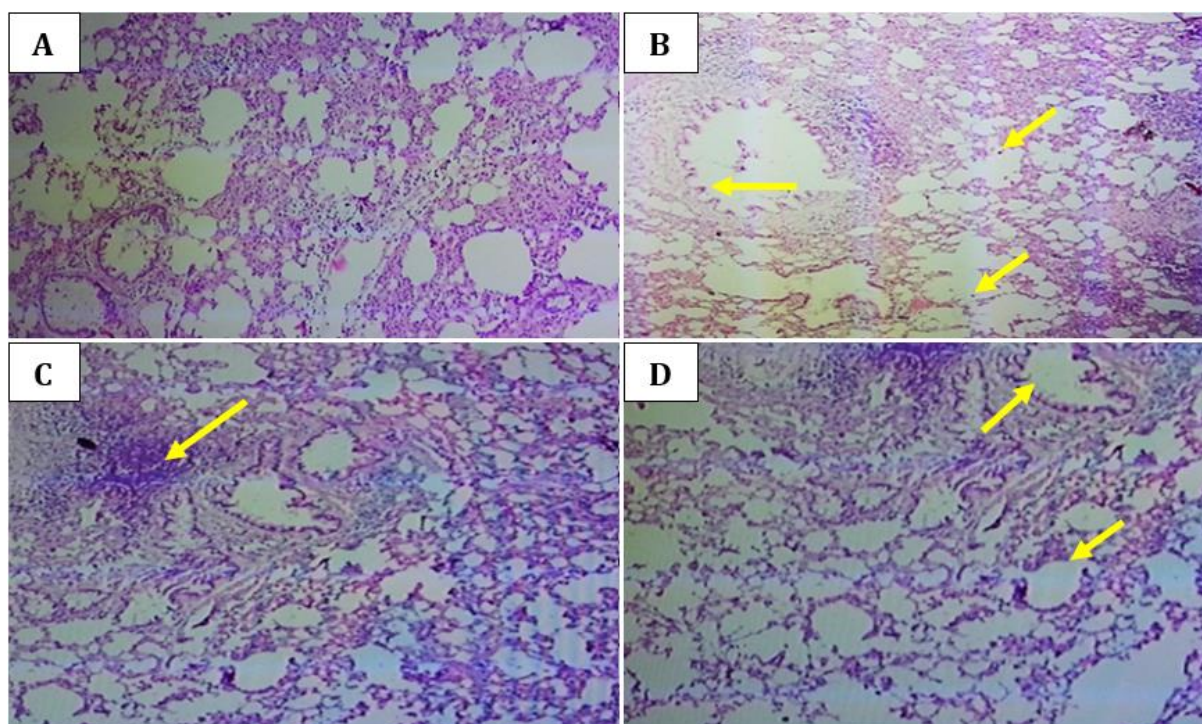


Figure 3. Mice lung tissue given exposure to *Legiayu incense* smoke for 12 weeks.
 Note: A = Before the trial, the tissue with in lungs is normal (Control); B = after 4 weeks of treatment (Lymphoid cells found in the alveoli); C = after 8 weeks of treatment (Found lymphoid foci in the alveoli); D = after 12 weeks of treatment (The alveolar cavity remains unchanged).

Lung tissue histology overview mice (*Mus musculus*) strain Balb/C

The sub-acute toxicity tests (Figure 3) at the Food and Drug Administration showed *Legiayu incense* smoke did not result in histological changes in the mice lung tissue (*Mus musculus*) exposed for 40 minutes each day within a period of 12 weeks. The sub-acute toxicity tests at the Food and Drug Administration (BPOM) showed *Legiayu incense* smoke did not result in histological changes in the mice lung tissue (*Mus musculus*) for exposure 40 min each day 12 weeks periods. The picture of preparations colored using *Haematoksilin-Eosin®* appears to be non-inflammatory in the lung tissue of test animals, judging by the absence of polymorfonuclear cells (Granulocytes), edema and mononuclear cell infiltration in pulmonary tissue morphology. The observed results showed no changes in tissue morphology (degeneration), absence of cells experiencing necrosis, hyperplasia and metaplasia this has met the standard criteria OECD test number 413 Subchronic Inhalation Toxicity: 90-day Study, SNI 06-3566-1994 and Food and Drug Administration regulations number

7 of 2014 (Food and Drug Administration Republic of Indonesia, 2014).

CONCLUSION

Legiayu incense products are potential insecticides and larvicides against *Aedes aegypti* mosquitoes and potential as a substitute for mosquito repellent and synthetic incense. The effects of sub-chronic toxicity should be done to determine its long-term safety, for further research.

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