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In vitro Bioactivity Evaluation of *Ziziphus mauritiana* Lam. (Bidara) Leaves Extract Against Vector Mosquitoes *Aedes* spp. and *Culex quinquefasciatus*

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ABSTRACT

Ziziphus mauritiana methanol crude extract was evaluated for its insecticidal properties against *Aedes aegypti*, *Ae. albopictus*, and *Culex quinquefasciatus* mosquito larvae. Bioassays against larvae mosquitoes were done following World Health Organization's guidelines. Late third and/or early fourth instar of mosquito larva were assayed for five different concentrations viz. 100, 150, 200, 250, and 300 mg ml^{-1} of *Z. mauritiana* crude extracts. From the results obtained, *Aedes aegypti* was the most susceptible to *Z. mauritiana* crude extracts. The percentage of mortality exhibited above 50% of 200, 250, and 300 mg ml^{-1} in 24, 48, and 72 hr exposure. Thus, it gives the lowest LC_{50} within 24 hr of exposure (121.98 mg L^{-1}), followed by *Ae. albopictus* (189.89 mg L^{-1}) and *Cx. quinquefasciatus* (246.22 mg L^{-1}). Observation of the morphology effect of the dead larvae shows *Ae. aegypti* was the most affected, followed by *Ae. albopictus* and *Cx. quinquefasciatus*. A ruptured midgut was observed in 100 and 200 mg ml^{-1} concentrations. In contrast, in higher concentrations of 300 mg ml^{-1}, the abdominal segments were indistinguishable, and the head and thorax regions were severely damaged. This study suggested that *Z. mauritiana* methanolic crude extracts were potent against *Ae. aegypti* larvae mosquitoes and have the potential to be used as an alternative larvicide in population control. However, further studies are required to establish the potential of *Z. mauritiana* larvicidal effects in the field setting.

Keywords: *Aedes* spp., bioactivity, crude extracts, *Culex quinquefasciatus*, *Ziziphus mauritiana*

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INTRODUCTION

In many tropical and sub-tropical countries, mosquitoes such as *Aedes*, *Culex*, and *Anopheles* are vectors of many infectious pathogens, such as dengue, chikungunya, Zika, lymphatic filariasis, malaria, and yellow fever (World Health Organization [WHO], 2020). Malaysia is a tropical country that provides favourable and suitable breeding sites for mosquitoes to reproduce and survive throughout the year. In addition, Malaysia's favourable temperature and humidity play a huge role in aiding the increment of the mosquito population, thus encouraging viruses and disease transmission. The cumulative number of reported dengue cases in Malaysia as of September 2021 is 18,988, with 11 deaths, although there was a drastic drop compared to 74,443 reported dengue cases and 123 deaths in 2020 (WHO, 2021). On the other hand, according to European Centre for Disease Prevention and Control (ECDC) (2021), 1,102 reported chikungunya cases as of October 2021, with an increase of 134 new cases since 11 September 2021.

Until now, there has been no effective vaccine against these mosquito-borne diseases. Thus, chemical insecticides have been used as an effective tool to combat and control the disease outbreak due to their immediate effect on suppressing the vector population. However, continuous usage and repetitive exposure to these chemical insecticides have caused mosquitoes to develop resistance due to selective pressure and compromised the success of the control interventions. In addition, chemical insecticides have also caused irreversible environmental damage, hazards to non-target species, and food web disruption. Therefore, alternative approaches, such as botanical insecticides, have become more popular in mosquito control, either for larvicidal, adulticidal, or repellent (Bakar, 2020). Toxins and secondary metabolites, a complex of compounds which act as mosquitocidal agents, are hypothesised to be responsible for distinct biological activity found in different parts of plants (Aydin et al., 2017).

For instance, *Azolla pinnata* plants have shown their potential as larvicidal and adulticidal against *Aedes* mosquitoes. The extracts can alter the morphology and behaviour of *Ae. aegypti* larvae (Zulkrnin et al., 2018). Another study by Ravi et al. (2020) has shown that a significant increase in mortality was noted as test concentration increased, indicating the existence of bioactive chemicals responsible for adulticidal activity in *Aedes* mosquitoes. In addition, phytochemicals of plant extracts would have minimal effects on resistance development when exposed to mosquitoes (Şengül Demirak & Conpolat, 2022). Therefore, this study aimed to explore the bioactivity potential of the *Ziziphus mauritiana* plant against common mosquitoes vector in Malaysia. The plant is locally known as 'Bidara' and is commonly used in traditional Islamic medicine for its health-beneficial effect (Mohd Jailani et al., 2020). *Ziziphus mauritiana*, or Bidara, is commonly used in Malay traditional medicine (Mohd Jailani, 2020). *Ziziphus*

mauritiana dried fruits are anodyne, anticancer, pectoral, refrigerant, sedative, stomachache, styptic, and tonic as it is believed to have the ability to purify the blood and aid digestion. At the same time, the root treats dyspepsia and fever. Despite that, the fruits are crunchy and have a sweet and sour taste when eaten raw. Like many other commercialized medicinal plants, *Z. mauritiana* Bidara has also come to the attention of many small and medium company industries for similar purposes. Thus, products of *Z. mauritiana*-based have been used widely in all kinds of personal care products such as hair shampoo, body care, and facial care.

In Malaysia, however, there are limited to no studies conducted on their insecticidal properties. A thorough search of recent documented studies on *Z. mauritiana* has succeeded in finding a similar study conducted by researchers in Indonesia, which investigated the effectiveness of *Z. mauritiana* extracts against *Ae. aegypti* larva (Amaliyah et al., 2021; Askur et al., 2021). It has initiated the author(s) attention to give insight into *Z. mauritiana* larvicidal effects against common mosquitoes vector in Malaysia, such as *Ae. aegypti*, *Ae. albopictus*, and *Cx. quinquefasciatus*. While *Ae. aegypti* is commonly known as the primary dengue vector in Malaysia, *Ae. albopictus* is currently the most invasive mosquito in the world and serves as a potential vector of the dengue virus in rural and suburban areas (Benedict et al., 2007; Higa, 2011). In contrast to the *Aedes* spp. clean and clear water habitat, *Cx. quinquefasciatus* preferences of breeding in highly polluted water and being capable of surviving in high larval densities have allowed exploitation of the human-made polluted environment. As a result, it created a high risk of human transmission of zoonotic pathogen diseases (Harvey-Samuel et al., 2021). In the present study, the bioactivity of *Z. mauritiana* was evaluated *in vitro* against *Ae. aegypti*, *Ae. albopictus*, and *Cx. quiquefasciatus* for its larvicidal and morphological effects on dead larvae. The comparisons were discussed.

METHODOLOGY

Collection and Preparation of *Ziziphus mauritiana*

The leaves specimen of *Z. mauritiana* were collected at the Islamic Centre, Universiti Sains Malaysia (USM), Pulau Pinang, Malaysia (N 5° 24' 40.428''E 100° 20' 7.548''). The botanist staff of the School of Biological Sciences (SBS), USM, Pulau Pinang, identified the specimens. All collected leaves were brought to the SBS, USM laboratory for sample processing. The leaves were washed, cleaned thoroughly, and dried at room temperature (29.5–31.5°C) for three to four days. The cleaned and dried *Z. mauritiana* leaves were grounded using a household KitchenAid® blender (USA) and stored in zip-lock plastic bags until further use.

Maceration Extractions

In this study, crude extracts of the plant leaves were prepared with the maceration extractions method. The ratio applied was 1

g (grounded leaves) to 10 ml (solvents). The desired amounts of *Z. mauritiana* grounded leaves and methanol were measured and placed in tight containers for 72 hr. The mixture was stirred and shaken periodically to ensure complete extraction. At the end of the extraction process, the micelles were filtered with filter paper Whatman No.1. and concentrated with a rotary evaporator at a temperature of 50 to 60℃ and pressure of 1,000 mbar for eight hours. The extracted residue was carefully kept in the refrigerator at 4℃ in an amber glass vial for subsequent assay.

Mosquito Preparation

Mosquitoes used in this study were obtained from Vector Control Research Unit (VCRU), SBS, USM Pulau Pinang. The mosquito colony was cultured and reared in the laboratory with a temperature of 28.0 ± 2.0°C and relative humidity (RH %) of 80.5 ± 3.0 %. Throughout the maintenance period, mosquito larvae were provided with prepared portioned ratio powdered food (2 : 1 : 1 : 1): cat's biscuit, dried cow liver (grounded), powdered milk, yeast, and eight tablets of the vitamin B complex. The colony was continuously maintained during the study period.

Larval Bioassay

The bioassay larval was conducted according to the standard guidelines of the WHO (2005). Five concentrations of *Z. mauritiana* methanol extracts were prepared: 100, 150, 200, 250, and 300 mg ml^{-1}. Bioassay larvae were in five replicates of 20 instar larvae for each concentration. Larvae were aged between late instar three and early instar four. The number of dead larvae was recorded after 24 and 48 hr of exposure. The control solutions were prepared by mixing 1.0 ml methanol in 199.0 ml distilled water. During the observation, food was not supplied to the larvae.

Morphological Effects of Dead Larvae

All dead larvae mosquitoes were collected and transferred to a Petri dish to observe morphological effects. The observation was conducted under an Olympus® stereo microscope (Model: SZX16-CCD, Japan), and photos were captured.

Statistical Analysis

For any mortality in the control which is less than 20%, Abbott's formula was used (Abbot, 1925) as below:

$$Mortality\% = \frac{Test\ mortality\% - Control\ mortality\%}{100 - Control\ mortality\%} \times 100\%$$

The lethal concentrations 50% (LC_{50}) and 90% (LC_{90}) were calculated using probit analysis (Finney, 1971), respectively. All data obtained were analyzed with Statistical Product and Service Solutions (SPSS) software (version 27) with a confidence interval of 95%. Determination of the most effective concentration of the extract based on the analysis of variance (ANOVA) followed by least significant differences (LSD) tests.

RESULTS

Larvicidal Assays

Table 1 shows the mean mortality of mosquito larvae against *Z. mauritiana* leaves extracts at different concentrations and time exposure (hrs) during larvae assay. From the results obtained, mean mortality of different concentrations and time exposure among three mosquito species were increased with high concentrations and time exposure. Of these, *Ae. aegypti* larvae population was the most susceptible compared to *Ae. albopictus* and *Cx. quinquefasciatus*. It is notable that, at a concentration of 200 mg ml^{-1}, the mean mortality of *Ae. aegypti* population was the constant highest at 23.25 ± 0.48, 23.75 ± 0.25, and 24.25 ± 0.25 throughout the time exposure of 24, 48, and 72 hr, respectively, compared to *Ae. albopictus* and *Cx. quinquefasciatus*. The highest mean mortality was 25.00 ± 0.00 (100 % mortality), demonstrated by *Ae. aegypti* exposed to 300 mg ml^{-1} methanolic *Z. mauritiana* leaves extract for 48 and 72 hr. The *Ae. aegypti* larvae marked more than 50% mortality from exposure of 150 mg ml^{-1} and above for 24, 48, and 72 hr. The

Table 1
Mean mortality of Ziziphus mauritiana *extracts against mosquito larvae*

Mosquito species	Concentrations (mg ml^{-1})	Mean mortality ± S.E		
		24[a] hr	48 hr	72 hr
* [a]*Aedes aegypti*	100	5.00 ± 2.55	7.00 ± 2.94	9.50 ± 2.90
	150	17.3 ± 1.25	21.50 ± 0.87	22.50 ± 0.87
	200	23.25 ± 0.48	23.75 ± 0.25	24.25 ± 0.25
	250	23.25 ± 0.48	24.75 ± 0.25	25.00 ± 0.00
	300	24.75 ± 0.25	25.00 ± 0.00	25.00 ± 0.00
	Control	0	0	0
* *Aedes albopictus*	100	11.50 ± 1.85	13.25 ± 2.39	13.25 ± 2.39
	150	11.50 ± 1.55	13.50 ± 1.85	13.75 ± 1.89
	200	14.75 ± 3.09	18.00 ± 1.29	19.25 ± 1.44
	250	17.00 ± 3.67	19.50 ± 2.96	21.00 ± 2.12
	300	23.75 ± 0.63	25.00 ± 0.00	25.00 ± 0.00
	Control	0	0	0
* [a]*Culex quinquefasciatus*	100	1.00 ± 0.41	2.75 ± 0.25	3.75 ± 0.63
	150	2.75 ± 0.85	14.25 ± 1.89	15.75 ± 1.44
	200	13.25 ± 1.44	21.00 ± 0.00	22.50 ± 0.29
	250	16.25 ± 2.10	23.00 ± 0.41	23.50 ± 0.29
	300	13.50 ± 1.19	24.50 ± 0.29	24.75 ± 0.25
	Control	0	0	0

Note. Number of studies = 27; Number of effects = 216; Total $N = 5400$
*Significant difference in mean mortality of tested mosquito species, $p < 0.05$ ($p = 0.00$)
[a] Significant difference in mean mortality of 24 hr, $p < 0.05$ ($p = 0.015$)

mean mortality was 17.3 ± 1.25, 21.50 ± 0.87, and 22.50 ± 0.87, respectively. At the end of 72 hr observation, each concentration assayed of 100, 150, 200, 250, and 300 mg ml^{-1} *Z. mauritiana* methanolic extracts gives mean mortality of 9.50 ± 2.90, 22.50 ± 0.87, 24.25 ± 0.25, 25.00 ± 0.00, and 25.00 ± 0.00, respectively. In brief, *Ae. aegypti* larvae give the highest mean mortality throughout the time exposure period compared to *Ae. albopictus* and *Cx. quinquefasciatus*. Nevertheless, both mean mortality of *Ae. albopictus* and *Cx. quinquefasciatus* showed a gradual increment for each concentration with increasing exposure hours. However, extracts of *Z. mauritiana* were more effective against *Ae. albopictus* larvae compared to *Cx. quinquefasciatus*. Cx recorded no 100% mortality. *quinquefasciatus* at the highest concentrations in any time exposure of 24, 48, and 72 hr. Statistical analysis of one-way ANOVA analysis showed a significant difference ($p < 0.05$) in the mean mortality of all tested mosquito species and tested concentrations of *Z. mauritiana* leaves extract ($p = 0.00$). However, the paired samples test analysis revealed the significant difference ($p < 0.05$) in the mean mortality was only between *Ae. aegypti* and *Cx. quinquefasciatus* at 24 hr of methanolic *Z. mauritiana* leaves extract ($p = 0.015$).

Table 2 shows the lethal concentrations of LC_{50} and LC_{90} for each mosquito larvae assayed and time exposure (hr). Of these, mosquito larvae of *Ae. aegypti* was the most susceptible against *Z. mauritiana* leaves extracts. The LC_{50} and LC_{90} in three different time exposure, 24 hrs, 48 hrs, and 72 hr, were the lowest at 122.0, 111.7, 107.7, 200.1, 172.9, and 163.4 mg ml^{-1}, respectively compared to *Ae. albopictus* and *Cx. quinquefasciatus*. On the other hand, *Cx quinquefasciatus* gives the highest values of LC_{50} and LC_{90} of time exposure (hrs.). The lethal concentrations in 24 hrs, 48 hrs, and 72 hr were 246.2, 169.9,

Table 2
Lethal concentrations (LC_{50} and LC_{90}) of Ziziphus mauritiana *extracts against mosquito species*

Mosquito species	Exposure duration (hrs)	LC_{50} (mg ml^{-1})	95% CI (mg ml^{-1})		LC_{90} (mg ml^{-1})	95% CI (mg ml^{-1})		df
			LL	UL		LL	UL	
Aedes aegypti	24	122.0	117.1	126.6	200.1	192.0	209.6	3.5
	48	111.7	107.2	115.9	172.9	166.2	180.9	1.7
	72	107.7	103.3	111.8	163.4	157.1	171.0	1.9
Aedes albopictus	24	189.9	132.5	273.7	421.3	285.8	3044.3	42.2
	48	161.1	116.1	200.2	346.4	258.9	855.9	29.4
	72	150.1	111.4	180.9	306.7	240.5	570.9	25.5
Culex quinquefasciatus	24	246.2	221.0	285.3	496.4	393.5	768.8	8.5
	48	169.9	163.7	176.2	323.3	304.4	347.4	4.1
	72	144.5	137.7	150.9	296.6	278.4	319.8	2.2

Note. Number of studies = 27; Number of effects = 216; Total $N = 5400$. CI = Confidence intervals; df = Degree of freedom; LL = Lower limit; UL = Upper limit

144.5 mg ml^{-1} for LC_{50} and 496.4, 323.3, and 296.6 mg ml^{-1} for LC_{90}, respectively. In relative, lower values of LC_{50} and LC_{90} obtained from methanolic *Z. mauritiana* extracts indicate good effects against tested mosquito larvae.

Morphology Effect

The morphology effect of dead mosquito larvae was observed in different concentrations treatment of control, 0, 100, 200, and 300 mg ml^{-1}, as shown in Figure 1. Figure 1 (a)(i), (b)(i), and (c)(i) showed the

Figure 1. Morphology of mosquito larvae (a) *Aedes aegypti*, (b) *Aedes albopictus*, (c) *Culex quinquefasciatus* photographed at 0.69x by stereo microscope (Model: SZX16-CCD) after treated with different concentrations of *Ziziphus mauritiana* extracts in (i) 0 mg ml^{-1}, (ii) 100 mg ml^{-1}, (iii) 200 mg ml^{-1}, and (iv) 300 mg ml^{-1}

normal morphology features of the well-developed head, thorax, and abdominal region of *Ae. aegypti*, *Ae. albopictus*, and *Cx. quinquefasciatus* larvae with eight segments and anal segments, respectively. *Aedes aegypti* larvae treated with methanolic *Z. mauritiana* leaves extract concentrations of 100 and 200 mg ml^{-1} (Figure 1(a)(ii–iii)) showed ruptured midgut. In the 300 mg ml^{-1} treated concentration (Figure 1(a)(iii)), deformities of *Ae. aegypti* larvae's bodies were observed in which the abdominal segments were indistinguishable, and the head and thorax region were unidentified. On the other hand, observation of the dead larvae of *Ae. albopictus* in treated with methanolic *Z. mauritiana* leaves extract showed a normal morphological appearance with distinguishable head, thorax, and abdominal regions. However, darkening of the thorax, abdomen and anal gill was observed distinctly in larvae exposed to extract concentrations of 100, 200, and 300 mg ml^{-1} (Figure 1(b)(ii–iv)).

The observed morphology of dead *Cx. quinquefasciatus* exposed to 100, 200, and 300 mg ml^{-1} of methanolic *Z. mauritiana* leaves extract concentration after various exposure times were shown in Figure 1(c)(ii–iv). The *Cx. quinquefasciatus* larvae in control (Figure 1(c)(i)) showed normal morphological features with a well-developed head, thorax, eight segments abdominal region, and long siphon. While *Cx. quinquefasciatus* larvae treated with methanolic *Z. mauritiana* leaves extract of 200 mg ml^{-1} (Figure 1(c)(iii)) showed ruptured midgut, indistinguishable abdomen, and anal gill along with poorly developed thorax. On the other hand, larvae treated with 100 and 300 mg ml^{-1} (Figure 1(c)(i) and (iv)) showed well-developed morphology with distinguishable body regions as the larvae in control (Figure 1 (c)(i)). However, the darkening of the thorax and abdomen was notably observed. The results obtained in the morphological observation of dead mosquito larvae after being treated with different concentrations of *Z. mauritiana* leaves extract have confirmed the larvicidal assay findings in which *Ae. aegypti* was the most susceptible and thus exhibited severe deformities effect after being exposed to concentrations of 100, 200, and 300 mg ml^{-1} compared to *Ae. albopictus* and *Cx. quinquefasciatus*.

DISCUSSION

Theoretically, the higher the concentration, the more active compounds will directly contact the tested larvae. Askur et al. (2021) showed that ethanolic *Z. mauritiana* extracts cause greater mortalities against *Ae. aegypti* larvae with higher concentrations. These findings agreed with the present study conducted on methanolic extracts. Even though the solvent and concentrations used in each study were different, it has been shown that the *Z. mauritiana* extracts have larvicidal effects against *Ae. aegypti* if proper extractions technique and solvents are used. Another parameter observed in this study was the lethal concentration (LC) values. These values were analysed to evaluate the association between lethal concentrations and mortality. The lower

LC obtained indicated, the more effective the extracts used. Therefore, the larvicidal effects generally have good potential when the extract causes high mortality at a low LC value. Meyer et al. (1982) and Santos Pimenta et al. (2003) have classified the larvicidal effectiveness based on the ranges of LC values obtained: LC_{50} > 200 µg ml^{-1} (0.2 mg ml^{-1}) very weak (inactive), LC_{50} 20–200 µg ml^{-1} (0.02–0.2 mg ml^{-1}) as moderate, and LC_{50} < 200 µg ml^{-1} (0.2 mg ml^{-1}) as very good. The lowest LC_{50} values recorded of *Ae. aegypti* in this present study were 121.975, 111.726, and 107.734 mg ml^{-1}, while the LC_{90} were 200.100, 172.923, and 163.357 mg ml^{-1} for 24, 48, and 72 hr, respectively. These results obtained, however, are considered average in comparison with a study by Amaliyah et al. (2021), which reported a lower LC_{50} (46.97 mg ml^{-1}) and LC_{90} (86.48 mg ml^{-1}) values of *Z. mauritiana* leaves extract against *Ae. aegypti* for 24 hr.

The different larvicidal activities of different plant extracts are probably due to the extraction method and types of solvents used to extract the plants' leaves. According to Zhang et al. (2018), the solvent selection is crucial for plant extraction. The performance of solvent extraction can be enhanced by choosing solvents that have a polarity value near the polarity of the solute. In addition, the most crucial element in producing the best extract quality is the samples' phytochemicals themselves. A study by Velázquez-Martínez et al. (2022) has shown that the content and antioxidant activity of plant extracts remained stable across different extraction processes; however, different geographical regions of the plants obtained showed differences in the abundance of secondary metabolites in which related to the amount received of UV radiation, nutrient components, temperature, and water stress.

Morphological changes in all treated *Ae. aegypti*, *Ae. albopictus*, and *Cx. quinquefasciatus* larvae were demonstrated in which the most obvious deformities appear with poorly developed body regions. Most of the dead *Ae. aegypti* and *Cx. quinquefasciatus* larvae have ruptured midgut, and damaged tissues, possibly caused by the presence of chemical compounds in methanolic *Z. mauritiana* leaves extract that targets the larvae's osmoregulation system (Wang et al., 2019). For instance, the presence of tannins in methanolic *Z. mauritiana* leaves extract can cause disruption of membrane integrity and/or function of the midgut (Procópio et al., 2015). There were few notable observations on the body deformities of the *Ae. aegypti* and *Cx. quinquefasciatus* larvae, such as indistinguishable abdomen segments and poorly developed head and thorax, suggest that methanolic *Z. mauritiana* leaves extract has certain chemical properties that can cause structural distortion of larvae. Larvae of *Ae. albopictus* showed a typical appearance, but darker pigmentation was also observed, especially at the thorax, abdomen, and anal gills. The methanolic Z. mauritiana possibly causes the pigmentation leaves extract, which is dark green.

Montell and Zwiebel (2016) discussed that mosquito larvae usually do not discriminate against what they ingest. Thus, the high number percentage of mortality in larvae of *Ae. aegypti* compared to *Ae. albopictus* and *Cx. quinquefasciatus* were probably due to their feeding behaviour. According to Merritt et al. (1992), larvae of *Ae. aegypti* feed by swimming or diving to substrates in their habitats and thus are commonly known as "bottom feeders". Most methanolic *Z. mauritiana* leaves extract precipitation was found to be sediment or deposited on the bottom of the test cups, while the larval food powder was usually found. Thus, higher mortalities in *Ae. aegypti* larvae could be attributed to their feeding behaviours of "bottom feeders".

CONCLUSION

The results revealed that methanolic *Z. mauritiana* leaves extract at all tested concentrations cause mortality and morphology changes in *Ae. aegypti*, *Ae. albopictus*, and *Cx. quinquefasciatus* larvae associated with a higher concentration of the methanolic leaf extract and longer exposure time. It is concluded that these methanolic leaves extract of *Z. mauritiana* can induce morphological changes among larvae mosquito vectors. This study has also suggested that methanolic *Z. mauritiana* leaves extract can be a potent larvicide to reduce the mosquito population by controlling the larval stage. However, further studies on the active compounds, mechanisms, and mode of action of methanolic extracts of *Z. mauritiana* leaves against vector mosquitoes are required to understand the nature of its killing properties. Future initiatives of larvicidal bioassay using methanolic *Z. mauritiana* leaves extract against field strain mosquitoes are necessary. It is also crucial to further evaluate any toxicity and hazards established by *Z. mauritiana* extract towards the non-target organisms and environment. Different extraction methods associated with various solvents and *Z. mauritiana* plant parts can be considered against different mosquito species.

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