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Evaluating the Synergistic Larvicidal Effects of *Pistia stratiotes* (Water Lettuce) and *Azadirachta indica* (Neem) Leaf Ethanolic Extract on *Aedes aegypti* (Yellow Fever Mosquito) Larvae

Audric B. Vitangcol[1*], Rein Erin C. Dalawampu[1], Vianne Germaine D. Gonzalvo[1], Annika Stacey A. Parungao[1], and Shannen Anziel D. Tucay[1]

[1] *De La Salle Medical and Health Sciences Institute*

**corresponding author: audricbv@my.dlshsi.edu.ph*

Ramgie M. Bartolata, *Research Adviser*

[1] *De La Salle Medical and Health Sciences Institute*

Abstract: Mosquito-borne diseases have been a global concern for centuries, and dengue is still a major problem in the Philippines. However, the widespread use of synthetic chemical larvicides has negatively affected the environment, particularly aquatic biodiversity, by killing other organisms and target larvae. This study aims to create an effective but more environmentally friendly alternative using *Pistia stratiotes* (water lettuce) and *Azadirachta indica* (neem). *Aedes aegypti* larvae were subjected to a mortality assay using five different combinations of *A. indica* and *P. stratiotes* leaf extracts and compared to commercial larvicide temephos. The concentration with 100% *A. indica* had the highest mortality rate (65.33%), and a Tukey's test found that it was as effective as temephos. The same concentration was tested again in five varying concentrations to get its LC_{50} and LC_{90}. After further testing, its LC_{50} was found to be 757.762 ppm, while its LC_{90} is 1,347.754 ppm. Thus, pure *A. indica* extract is an effective plant-based larvicide against *A. aegypti* larvae. However, it does not have a synergistic larvicidal effect with *P. stratiotes* because the mixed concentrations had lower mortality rates than their individual counterparts. Regardless, with this knowledge, people can make potent larvicides with readily accessible plants and cheap, common extraction methods, which is becoming more relevant because mosquito larvae are increasingly becoming resistant to larvicides like temephos.

Keywords: larvicide; *Azadirachta indica*; *Pistia stratiotes*; crude extract; ethanolic extract

1. INTRODUCTION

Around 750,000 individuals die yearly from mosquito-borne diseases like dengue, malaria, and yellow fever (CDC, 2016; Pfizer, 2016). Dengue is still of major concern in the Philippines, as the country has had a long history of dengue outbreaks, going back to as early as 1906 (Pettis, 2017). *Aedes aegypti* is a prominent transmitter of diseases like dengue, the most common mosquito species in the Philippines (Mistica et al., 2019).

Larvicides are used to reduce the population of mosquitoes by inhibiting the growth of larvae, ensuring that they do not become adults (CDC, 2020). However, synthetic larvicides pose threats against biodiversity, as they are highly toxic to non-target animals (Abe et al., 2014). Furthermore, cases of resistance against larvicides such as temephos have already been observed in several Southeast Asian countries (Gan et al., 2021).

Pistia stratiotes (water lettuce) is an invasive aquatic plant in the Philippines (Silvestre, 2018). When extracted with ethanol, phytochemicals like steroids, alkaloids, terpenoids, and flavonoids are found to help make an effective larvicide (Ugya et al., 2019). Ma et al. (2019) found that the ethyl acetate of *P. stratiotes* leaves has a low LC_{50} (14.81 ppm) on *Anopheles* mosquito larvae, while also having an LC_{50} of 602.03 ppm on *Artemia salina* (brine shrimp) larvae, so it would be unlikely for this extract to harm other animals in the surrounding ecosystem.

Azadirachta indica (neem) is traditionally cultivated

to make medicine, and it is known for having phytochemicals called limonoids, like azadirachtin, used in insecticides (Hashmat et al., 2012). *A. indica* has also been proven effective against *A. aegypti* larvae, with its leaf ethanolic reflux extract showing an LC_{50} of 50 ppm after 48 hours of exposure (Nour et al., 2012).

Considering the lack of studies in this area, this study aims to establish results on the synergistic larvicidal effects of *P. stratiotes* and *A. indica* on mosquito larvae. Since both plants effectively kill various mosquito larvae, combining them may create an even more effective biodegradable and environmentally friendly larvicide.

2. METHODOLOGY

2.1. Sample Collection

A total of 932 grams of *P. stratiotes* leaves and 1,001 grams of *A. indica* leaves were bought from local sellers. Two thousand (2,000) *A. aegypti* eggs were bought from the University of the Philippines - Institute of Weed Science, Entomology, and Plant Pathology. All plants and eggs were brought to the Angelo King Medical Research Center.

2.2. Larvae Rearing

In the laboratory, the *A. aegypti* eggs were first reared for 7 days. During this time, the larvae were kept in containers at a constant temperature range of 25-29°C and fed with powdered fish flakes and yeast (Ito et al., 2015). A photoperiod of 9D:15N was followed every day. After rearing, the larvae were mostly in the 3rd instar.

2.3. Extraction of Plant Leaves

The leaves were washed with tap water, heated in an oven at 45 °C for 15 hours (Sejali & Anuar, 2011), and ground into a powder using a commercial blender. The resulting powder was then extracted by maceration, following Alibo et al. (2021), soaking the powder in 95% ethanol at a ratio of 1 g:10 mL. Each plant species was soaked separately. The resulting solution was left for 24 hours, and then filtered with coffee filters. The solution was then put in a rotary evaporator at 40 °C until the ethanol fully evaporated. The final extracts were stored at 4 °C (Catapang et al., 2019).

2.4. Preparation of Stock and Test Concentrations

The crude plant extracts were diluted, following Tonk et al. (2006). To create stock solutions, 800 mg and 700 mg of *A. indica* and *P. stratiotes*, respectively, were used. First, 800 mg of *A. indica* leaf extract was dissolved in 8 mL of acetone, and then diluted to 80 mL using distilled water to create a 10,000-ppm stock solution. Similarly, 700 mg of *P. stratiotes* leaf extract was dissolved in 7 mL of acetone, then diluted to 70 mL with distilled water for a 10,000-ppm stock solution. For larvicidal testing, the solutions were diluted to 125 ppm. The remaining stock solution was kept and reserved for LC_{50} and LC_{90} testing.

The chosen concentration, 125 ppm, was based on the results of Nour et al. (2012). In that study, *A. indica* leaves macerated in ethanol at 100 ppm produced a mortality rate of around 30% after 24 hours, based on the graph, while 500 ppm had a mortality rate of around 80%. The use of 125 ppm was done to see whether adding *P. stratiotes* leaf extract to the *A. indica* leaf extract would create a better result than what was previously achieved with just *A. indica*, and produce a mortality rate of at least 50%, while if it had a similar or worse performance, then the extra 25 ppm might still be able to make the mortality rate around 50%.

Distilled water and temephos were used as the negative and positive controls. For temephos, BASF (2015) was followed, and 1-ppm solutions were made.

2.5. Larvicidal Testing

The most recent World Health Organization (WHO) mosquito larvicidal bioassay (WHO, 2005) was used, with slight modifications, following Tonk et al. (2006). Groups of 25 larvae were transferred to disposable cups. Each cup had 100 mL of the concentrations being tested. The cups were left for 72 hours at 25-28°C. Every day, a photoperiod of 9D:15N was observed. Dead and living larvae were counted after 72 hours. Larvae that pupated were removed and not counted. Each test was done thrice to reduce random error, with new solutions and larvae per replicate to ensure uniformity in all tests. After larvicidal testing, the mortality rates were calculated, using the formula for mortality rate, adapted from Colaki et al. (2004), in Equation (1):

$$Mortality\ Rate = \frac{Number\ of\ Dead}{Total\ Sample} \quad (1)$$

2.6. Determination of LC_{50} and LC_{90}

The plant extract concentration that produced the highest mean mortality rate was tested again to get its LC_{50} and LC_{90} using the methods of the WHO (2005).

Pure, 100% *A. indica* extract was tested because it had the highest mean mortality rate among the extracts tested (65.33%). Concentrations of 500 ppm, 250 ppm, 125 ppm, 62.5, and 31.25 ppm were used. Again, this procedure was done thrice per concentration to reduce random error, using 25 larvae and 100 mL of larvicide per cup, with positive control (temephos) and negative control (distilled water).

2.7. Disposal of Larvae

After the experiment was completed, all surviving larvae were placed in temephos until death. The dead larvae, all liquids used, and containers that once had larvae were disposed of in biohazard waste bins. Liquids were put in leak-proof containers before disposal.

2.8. Analysis of Data and Statistical Treatment

First, a Shapiro-Wilk test was done to determine whether the data was normally distributed (Shapiro & Wilk, 1965). Then, to analyze the data from the larvicidal testing, three tests were used: one-way analysis of variance (ANOVA), Kruskal-Wallis H test, and Tukey's HSD test. The ANOVA and Kruskal-Wallis test were used to determine if a significant difference exists in the data (MacFarland & Yates, 2016), and Tukey's test was used to determine where that difference can be found (Lee & Lee, 2018). The non-parametric Kruskal-Wallis H test was only added in case the data was not normal. After LC_{50} and LC_{90} testing, a probit analysis calculated the concentrations of 100% *A. indica* needed to kill 50% and 90% of larvae in 24 hours.

3. RESULTS AND DISCUSSION

3.1. Normality of the Data

Table 3.1 shows the results of a Shapiro-Wilk test used to determine the normality of the experimental data. Since the p-value is below the 0.05 level of significance, the data is not normal, so Kruskal-Wallis tests will also be considered for significance.

Table 3.1

Test of Normality using Shapiro-Wilk

	Mortality Rate	Mortality Rate No Negative Control	Mortality Rate Transformed
Shapiro wilk	0.756	0.786	0.756
p-value	0.0000..	0.001	0.001
alpha	0.05	0.05	0.05
normal	no	no	no

3.2. Comparison of Mortality Between Test Concentrations and Temephos

Table 3.2 shows the results of a one-way ANOVA and Kruskal-Wallis test done to determine whether the test concentrations used in the larvicidal testing are significantly different from temephos.

Table 3.2

One-Way Analysis of Variance and Kruskal-Wallis Test on Aedes aegypti Larvae Mortality between Pistia stratiotes and Azadirachta indica Extracts and Temephos

Test of Significance	ANOVA	Kruskal-Wallis
p-value	0.000000229	0.014661

Since the p-value is less than 0.05, a significant difference exists between the plant concentrations and temephos, making it possible to analyze further with a post-hoc test. Because the ANOVA and Kruskal-Wallis test agree, Tukey's test was used to determine where exactly the significant differences can be found.

Figure 3.1

Comparison of the Mortality (%) of Aedes aegypti Larvae in Pistia stratiotes and Azadirachta indica Extracts as Compared to Temephos

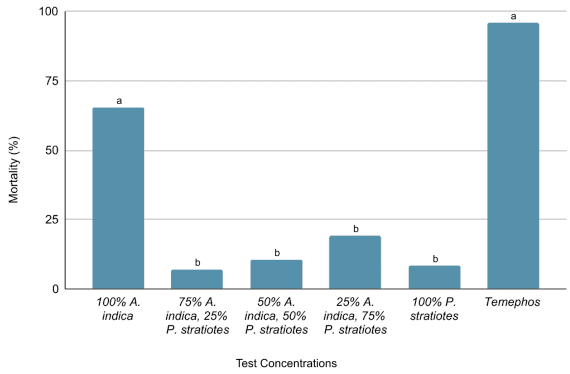

Figure 3.1 shows the results of the Tukey's test. Bars with the same letter indicate no significant difference (p-value > 0.05). Based on the results, temephos is significantly different from the concentrations of: 75% *A. indica*, 25% *P. stratiotes*; 50% *A. indica*, 50% *P. stratiotes*; 25% *A. indica*, 75% *P. stratiotes*; and 100% *P. stratiotes*. Because the mean mortality rate of temephos is greater than the mortality rates of all the plant extracts, temephos is significantly more effective than these.

On the other hand, 100% *A. indica* does not have a significant difference with temephos, meaning that these have a similar level of effectiveness. This shows that pure *A. indica* is a good potential substitute for temephos.

3.3. Comparison of Mortality Percentage of Different Concentrations

Table 3.3 shows the results of a one-way ANOVA and Kruskal-Wallis test to determine whether the test concentrations used in the larvicidal testing are significantly different. The ANOVA p-value is below 0.05, claiming a significant difference among the plant concentrations, while the Kruskal-Wallis test p-value is above 0.05, showing no significant difference. However, this is still below the 0.10 level of significance, so a Tukey's test was done to determine where the differences lie.

Table 3.3

One-Way Analysis of Variance and Kruskal-Wallis Test on Aedes aegypti Larvae Mortality between Pistia stratiotes and Azadirachta indica Concentrations

Test of Significance	ANOVA	Kruskal-Wallis
p-value	0.001	0.056

Figure 3.2

Comparison of Pistia stratiotes and Azadirachta indica Extracts on Mortality (%) of Aedes aegypti Larvae

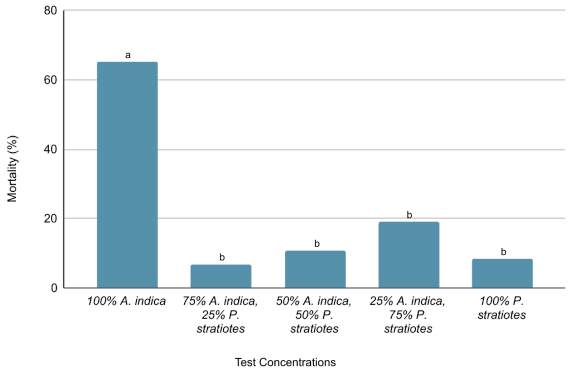

Figure 3.2 represents the results of the Tukey's post-hoc test. Bars with the same letter indicate no significant difference (p-value > 0.05). Based on the results, 100% *A. indica* is significantly different from all the other test concentrations. Since its mortality rate is the greatest among the plant concentrations, it can be said that 100% *A. indica* is significantly more effective than all of these.

Thus, the results indicate that among the test concentrations, the most potent plant extract larvicide is 100% *A. indica.* When adding some *P. stratiotes* (75% *A. indica*, 25% *P. stratiotes*), the mean mortality rate sharply declined from 65.33% to 6.83%. The same results were observed in other mixed extracts of the plants, with the mortality rate increasing with more *P. stratiotes*, then declining again when pure *P. stratiotes* was used.

All mixtures tested had mortality rates significantly lower than 100% *A. indica*, so *A. indica* and *P. stratiotes* do not have a synergistic larvicidal effect on *A. aegypti* larvae. This may be caused by differences in phytochemicals between the two plants, since *P. stratiotes* does not have limonoids, while *A. indica* does. This would also explain why 100% *A. indica* was more effective than 100% *P. stratiotes*. This is supported by Rubio and Lubos (2016), who found that adding *A. indica* decreased the mortality rate of *Carica papaya*. Still, their pure *A. indica* extracts all had a mortality rate of 0%, which contradicts the mortality rate found in the current study.

Still, this provides insights into the effectiveness of *P. stratiotes* as a larvicide against *A. aegypti*. With a mean mortality rate of 8.46% at 125 ppm after 72 hours, this is substantially lower than the results found by Ito et al. (2015), who got an LC_{50} of 0.11 ppm for 3rd instar *A. aegypti* larvae, and 0.22 ppm for 4th instar *A. aegypti* larvae, after 72 hours.

3.4. Lethal Concentration 50 and Lethal Concentration 90 of the Most Effective Concentration

Among the plant extracts used in the larvicidal testing, 100% *A. indica* had the greatest mean mortality rate, so it was used for LC_{50} and LC_{90} testing. Data from this LC_{50} and LC_{90} testing was used, specifically the mortality rates observed in various concentrations of 100% *A. indica*. Then, the mortality rates after 24 hours were put through a probit analysis to find the LC_{50} and LC_{90} of the extract. Table 3.4 shows the results of this analysis.

The results show a significant increase in mortality as concentration increases, with an LC_{50} of 757.762 ppm and an LC_{90} is 1,347.754 ppm. Both are higher than the highest concentration tested, 500 ppm, but they still have a significant larvicidal effect on *A. aegypti*.

Table 3.4

LC_{50} and LC_{90} of 100% Azadirachta indica Extract on Aedes aegypti

Time	Treat-ment	LC_{50} (ppm) with 95% confidence interval	LC_{90} (ppm) with 95% confidence interval	Regre-ssion Equation
24 Hours	100% *A. indica* extract	757.762 (592-1147.29)	1347.754 (1013.591-2164.861)	Y = -1.646 + 0.002 X

It can be said that the mortality rate of *A. indica* is dependent on its concentration, as significantly higher mortality rates were observed as concentration was increased. This confirms the findings of past literature, such as the study of Nour et al. (2012). However, this experiment still provides new insights on the larvicidal effects of *A. indica* on *A. aegypti* larvae, since the calculated LC_{50}, 757.762 ppm, is considerably higher than that found by the mentioned study.

4. CONCLUSIONS

In tropical countries like the Philippines, where mosquito-borne diseases are prevalent, the still-widespread use of synthetic chemical larvicides raises environmental concerns. The main purpose of this research is to develop an effective alternative larvicide and test it on *A. aegypti* larvae and harness the full potential of two plants that already have larvicidal effects on their own. After testing concentrations of leaf ethanolic extracts of *A. indica* (neem) and *P. stratiotes* (water lettuce), findings demonstrated a clear, significant difference between each extract and temephos, a common synthetic larvicide.

Pure, 100% *A. indica* is the most effective among the plant extract larvicides tested, and it is the only one shown to have larvicidal effects similar to temephos. Additionally, mixing the two plant extracts made the mortality rates lower than those of 100% *A. indica*, demonstrating a synergistic larvicidal effect does not exist between *A. indica* and *P. stratiotes*.

Because of the effectiveness of 100% *A. indica*, it was tested for its LC_{50} and LC_{90}, which turned out to be 757.762 ppm and 1,347.754 ppm, respectively. It is recommended that future researchers replicate the procedures used in this study to verify its results.

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