Larvicidal activity of individual and combined ethanolic extracts of *Annona muricata* (Soursop) seed and *Piper betle* (Betel) leaf against *Aedes aegypti*

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Abstract

The present study investigated the larvicidal activity of individual and combined ethanolic extracts of *Annona muricata* seed and *Piper betle* leaf against third and early fourth instar *Aedes aegypti* larvae. Larvicidal bioassays were done according to the World Health Organization 2005 guidelines for laboratory testing of mosquito larvicides. Individual larvicidal bioassays for *A. muricata* and *P. betle* exhibited LC_{50} values of 16.45 ppm and 847.86 ppm, respectively. Between the two combined extract test ratios, the 70:30 (*A. muricata*:*P. betle*) ratio was more effective with an LC_{50} of 116.51 ppm compared to the 152.16 ppm LC_{50} value of the 50:50 (*A. muricata*:*P. betle*) ratio. Results reveal *A. muricata* extract in this study exhibits greater larvicidal effect compared to previous larvicidal studies using similar extract. *A. muricata* seed and *P. betle* leaf ethanolic extracts are effective larvicides both used individually and in combination against third and early fourth instar *A. aegypti* larvae.

Keywords: *Annona muricata, Piper betle, Aedes aegypti, larvicidal bioassay, ethanolic*

Introduction. The danger posed by mosquito-borne diseases has been a long-time problem for people inhabiting the tropical regions of the world. Dengue fever, dengue hemorrhagic fever, yellow fever, and chikungunya are among the diseases *Aedes aegypti* is the primary vector of. There is no effective and widely accepted vaccine to prevent dengue infections; hence, the primary measure taken is to reduce the population of *A. aegypti.*

The current global strategy for the control of vector-borne diseases is based on vector control achieved through larvicides, adulticides, and bite prevention. Among the means of vector control, larvicides have been proven to be the most effective since they are target specific and the overall usage of pesticides for mosquito control is minimized [1]; not to mention mosquitoes are in their most vulnerable state when they are in the larval stage.

Synthetic pesticides are the most common method used in regulating mosquito populations, at the expense, however, of adverse effects to the environment. Not only that, but it has been reported that multiple mosquito species have developed resistance against these synthetic pesticides [2]. This is why pesticides from botanical sources are deemed more advantageous as they can be much less toxic, less prone to the development of resistance, and degrade easier [3].

Combining larvicides to exhibit synergistic effects has economic and ecological benefits by reducing cost and increasing toxicity. However, more studies have been conducted to investigate the synergistic effects of combining synthetic larvicides compared to combining plant-synthetic and plant-plant larvicides [4]. The use of plant extracts with different modes of action against larvae may exhibit synergistic effects by reducing the concentration of the individual extracts [5] and the chance of mosquito populations developing resistance to the larvicidal formulation [6].

The larvicidal activity of *Annona muricata,* commonly known as “Guyabano”, has been well documented along with the compounds responsible for its larvicidal activity. *A. muricata* seed extracts have shown insecticidal activity [7] due to its content of chemical compounds such as alkaloids, fatty acids and acetogenins. The reason behind the larvicidal activity of acetogenins found in *A. muricata* lies in its mode of action being the blocking of energy generation in the mitochondria [5].

A review article has also shown that plants from the Piperaceae family have potential as larvicidal agents [8]. *Piper betle,* locally known as “Buyo” or “Ikmo”, is a common plant in the Philippines that contains insecticidal compounds such as piperamides, particularly piperine and piperlonguminine [9]. The primary mode of action of piperamides on insects is neurotoxicity [5].

A. muricata and *P. betle* each have a different mode of action towards mosquito larvae. The combination of the two plant extracts is expected to produce a more cost-effective and more toxic formulation against *A. aegypti.*

Methods. *Annona muricata* seeds and *Piper betle* leaves were collected, air dried, powdered, then macerated in 95% ethanol. The extracts were concentrated using a rotary evaporator and stored in a -20°C freezer until use. Test setups were prepared by filling beakers with 50 ml of deionized water and 20 third and early fourth instar larvae each. Three replicates were done for each test setup. Larvicidal bioassays on the individual *A. muricata* and *P. betle* extracts were carried out according to the WHO 2005 guidelines for laboratory and field testing of mosquito larvicides. Two combined extract test ratios were used

for the larvicidal bioassay on combined *A. muricata* and *P. betle* extracts according to the same guidelines. Percent mortality was calculated from each larvicidal bioassay. The collected data was analyzed for the LC50 values of the individual and combined *A. muricata* and *P. betle* extracts using Probit Analysis through SPSS software.

Mosquito Rearing. Third and early fourth instar *Aedes aegypti* larvae used in the study were cultured in the Standards and Testing Division laboratory of the Industrial Technology Development Institute, Taguig, Metro Manila. The mosquito colony was maintained at 65±10% relative humidity, 26±2°C temperature, and 12h:12h light and dark photoperiod cycle. The larvae were reared in plastic trays filled with deionized water and fed with powdered biscuits.

Collection and Extraction. One kilogram of *A. muricata* seeds and *P. betle* leaves were collected from Iloilo City Terminal Market and Arevalo, Iloilo, respectively and verified for its species by the Department of Agriculture, after which the plant materials were air dried with no direct contact to sunlight in a laboratory at room temperature for seven days. The plant material was finely powdered using a commercial powdering machine and macerated by using Erlenmeyer flasks covered with aluminum foil where the plant material was submerged in 95% ethanol for two days in a dark environment at the Biology laboratory of Philippine Science High School Western Visayas. The liquid extracts were filtered using Whatman No. 1 filter paper then transported in amber bottles kept in an icebox to the Department of Science and Technology - Region 6 where the extracts were then concentrated in an EYELA rotary evaporator. The *A. muricata* seeds and *P. betle* leaves yielded 30 ml and 20 ml of crude ethanolic extracts respectively. The extracts were subsequently transferred to amber bottles for storage in the -20°C freezer of the Gregor Mendel Laboratory at the University of San Agustin until use.

Stock Solution. The stock solutions for individual larvicidal bioassay testing were prepared by weighing 1 g of crude plant extract in a beaker. One ml of 95% ethanol was added to the collected crude extract to ensure a homogenous solution once it was mixed with 24 ml of deionized water.

Larvicidal Bioassay. Larvicidal bioassays were conducted in the Standards and Testing Division of the Industrial Technology Development Institute according to the World Health Organization (WHO) guidelines [10] for mosquito larvicides. Preliminary testing of the extracts was carried out to establish the effective range of test concentrations. Different concentrations of *A. muricata* extract (5 ppm, 10 ppm, 20 ppm, 30 ppm, 40 ppm) and *P. betle* extract (300 ppm, 600 ppm, 900 ppm, 1200 ppm, 1500 ppm) were prepared during the study. All experimental exposures were carried out using 100 ml beakers filled with 50 ml of deionized water. Twenty third or early fourth instar larvae were then added into each beaker and subjected to testing of the different test concentrations. Three replicates were carried out simultaneously for each concentration. Mortality was recorded after 24 hours during which no food was offered to the larvae. The number of dead larvae was counted by transferring the larvae from the used beaker for testing onto a petri dish. A pasteur pipette was used to prod the larvae while observing for signs of movement. Larvae which were unresponsive to the prodding of the pasteur pipette were considered dead, whereas, responsive yet weak larvae were considered to be moribund. Mortality was recorded by counting the total number of dead and moribund larvae in a setup. Negative and positive controls were conducted alongside the established test setups with the negative control being 5 ml of 95% ethanol and the positive control being Abate.

Synergistic Activity. Two combined extract ratios of *A. muricata* and *P. betle* extracts (*A. muricata*:*P. betle*) were used, namely, 50:50 and 70:30. Five different concentrations were each prepared for the 50:50 ratio (100 ppm, 200 ppm, 300 ppm, 400 ppm, 500 ppm) and for the 70:30 ratio (40 ppm, 80 ppm, 120 ppm, 160 ppm, 200 ppm). The synergistic factor (SF) was calculated using the following equation [11]:

$$Synergistic\ Factor\ (SF) = (LC50\ of\ plant\ extract\ alone\ /\ LC50\ of\ combined\ plant\ extract)$$

A synergistic factor with a value more than 1 indicates synergism; the greater the synergistic factor, the higher the synergistic activity.

Data Analysis. Data from all replicates were pooled for analysis. Standard deviation of the means of percent mortality was calculated. A test series was valid if the relative standard deviation (or coefficient of variation) was less than 25%. The lethal concentrations to kill 50% and 90% of the larval population (LC_{50} and LC_{90}) were calculated through Probit Analysis using SPSS software.

Safety Procedure. *A. muricata* and *P. betle* extracts were kept in sealed glass amber bottles. Biohazards with regards to the *A. aegypti* larvae were cleared as they were provided by the laboratory institution and conduct of the larvicidal bioassay was supervised by professional personnel. Laboratory gowns, safety gloves, and face masks were worn during the conduct of the study as biosafety procedures. The stock solutions used for the larvicidal bioassay were disposed of in a separate container specifically for chemical waste. Hot water was poured over all the beakers after testing. Dead larvae were disposed of in biohazard containers, per the standard procedures of the institution.

Results and Discussion. The LC_{50} values of *A. muricata* and *P. betle* extracts were determined to be 16.45 ppm and 847.86 ppm, respectively. A positive correlation between the extract concentration used and the rate of mortality was observed, with the percent mortality being directly proportional to the extract concentration. The results of the larvicidal bioassays on both plant extracts show that there is a distinct difference between the toxicity of the *A. muricata* extract and *P. betle* extract on *A. aegypti* larvae, with the former evidently being the more effective larvicide. The LC_{50} of Abate, the positive control, was determined to be 0.84 ppm. Meanwhile, the negative control exhibited 0% mortality which guaranteed that

the solvent used did not contribute to the overall mortality of the larvae during the bioassay.

Two combined extract ratios were tested, namely 50:50 (*A. muricata*:*P. betle*) and 70:30 (*A. muricata*:*P. betle*), with the reason behind the latter ratio being the results of the larvicidal bioassays on the individual *A. muricata* and *P. betle* extracts which revealed the *A. muricata* extract to be the more toxic larvicide. Both 50:50 (*A. muricata*:*P. betle*) and 70:30 (*A. muricata*:*P. betle*) combined test ratios proved to be more toxic than the individual *P. betle* extracts but not as toxic as the individual *A. muricata* extracts with their LC_{50} values being 152.16 ppm and 116.51 ppm, respectively. The synergistic factors of *A. muricata* and *P. betle* for the 50:50 (*A. muricata*:*P. betle*) combined test ratio were calculated to be 0.1081 and 5.5722, respectively. For the 70:30 (*A. muricata*:*P. betle*) combined test ratio, the synergistic factors of *A. muricata* and *P. betle* were calculated to be 0.1411 and 7.2771, respectively.

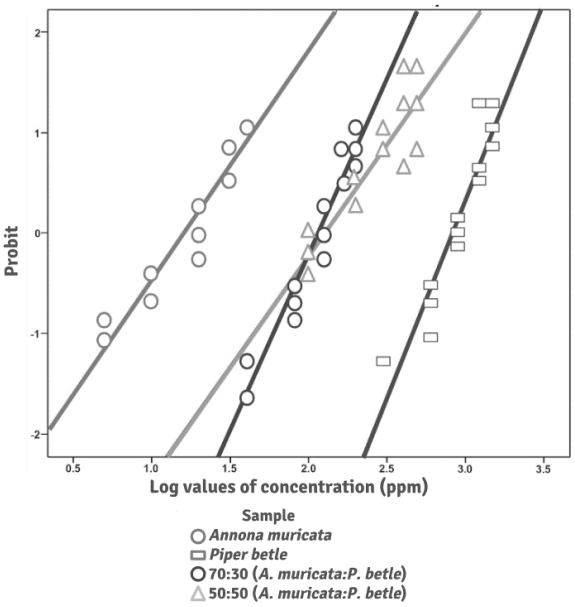

Figure 1. Linear regression of larvicidal activity of different samples against *Aedes aegypti* 3rd instar/early 4th instar mosquito larvae.

The results of the individual larvicidal bioassays for both plant extracts show that there is a distinct difference between the toxicity of the *A. muricata* extract and the *P. betle* extract on *A. aegypti* larvae, with the former evidently being the more effective larvicide.

The *A. muricata* ethanolic seed extracts in the present study were found to have a lower LC_{50} value compared to similar larvicidal studies on *Aedes aegypti* where results showed *A. muricata* to have LC_{50} values of 93.48 ppm [5] and 244.27 ppm [12].

Meanwhile, the LC_{50} of *P. betle* ethanolic leaf extracts in the present study was found to be higher in value when compared to a similar larvicidal study where the LC_{50} value of *P. betle* was determined to be 236.73 ppm [13]. The different modes of action of the bioactive compounds present in each plant may have contributed to the difference in their efficiency. Acetogenins from *A. muricata* inhibit mitochondrial energy generation (ATP) while piperine and related amides from *P. betle* act as neurotoxins [5]. The lower LC_{50} value of *A. muricata*, indicating a more effective larvicide, may be due to the higher potency of acetogenin, the compound present in *A. muricata*. The difference in the LC_{50} values may also be due to differences in the quality of plant material given that the collection site of similar studies varies. Compared to current commercially available synthetic larvicides such as Abate which has an LC_{50} of 0.84 ppm when tested as a positive control in the current study, larvicide formulations derived from plant extracts still need to be used at higher concentrations to be as effective as their synthetically produced counterparts [14]. This may be due to difficulties in standardizing the amount of bioactive compounds present in each botanical source given that the efficacy of phytochemicals responsible for larvicidal activity may vary depending on the plant parts used and their maturity [3]. Deviation in the resulting LC_{50} of the same plant species in different studies may also be attributed to differences in the efficiency of extraction procedures and length of plant extract storage prior to the conduct of the larvicidal bioassay.

The substantial difference between the toxicity levels of the *A. muricata* seed and *P. betle* leaf extracts is one of the main factors that led to the toxicity of the combined extracts to fall between the LC_{50} values of the two individual plant extracts and fail to exhibit an overall synergistic effect.

The combined extract formulations were successful in reducing the amount of *P. betle* extracts used while increasing its toxicity, making the combination exhibit a synergistic effect from this perspective. On the contrary, the combined formulations reduced the amount used for the *A. muricata* seed extracts but lessened its toxicity, exhibiting an antagonistic effect. Similar studies that involved larvicidal plant extract combinations have also reported the toxicity level of the individual plant extracts to be higher than that of the combined plant extracts, indicating antagonism [15,16]. The different modes of action exhibited by each of the combined plant extracts did not exhibit a synergistic effect and the reduced concentration of the individual extracts weakened the larvicidal activity of the combined extract formulation. There is a possibility that the combination of *A. muricata* seed and *P. betle* leaf extracts resulted in unforeseen chemical reactions between the bioactive compounds of both plant extracts that weakened the larvicidal activity of the combined plant extract formulations.

Error Analysis. Prolonged storage of extracts may cause bioactive compounds to degrade. The time of collection for leaves may have affected the presence of bioactive compounds due to factors such as photosynthesis; moreover, failure to follow standard protocol of washing the leaves may have had an indirect influence on the data.

Conclusion. *Annona muricata* seed and *Piper betle* leaf ethanolic extracts are effective as larvicides both when used individually and in combination against third and early fourth instar *Aedes aegypti* larvae.

Recommendations. It is recommended to use more combined extract test ratios in order to determine a larger number of ratios that may exhibit synergism. Phytochemical screening of the extracts may also be done to further support the acquired results since this will determine the present bioactive compounds responsible for the larvicidal activity. Checking the quality of soil where plant material was collected can provide some insight into other possible factors that could have affected its larvicidal activity. Future studies are also recommended to investigate the structural changes in the bioactive compounds of plant extracts that resulted from the chemical interaction of compounds from both plant extracts upon combination.

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