Larvicidal activity of *Citrofortunella microcarpa* (calamansi) peel essential oil against third and early fourth instar *Aedes aegypti*

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Abstract

This study evaluated the larvicidal activity of *Citrofortunella microcarpa* (calamansi) peel essential oil (EO) against third and early fourth instar *Aedes aegypti*. Larvicidal assay was performed against the test organisms to determine the efficacy of the essential oil at 8 ppm, 9 ppm, 10 ppm, and 11 ppm concentrations. Data on the larval mortality after 24 hours of exposure were analyzed using Probit Analysis. Results from the bioassay revealed that calamansi peel EO in 95% ethanol possessed great larvicidal potential with an estimated LC_{50} of 8.89 ppm and LC_{90} of 10.57 ppm. This implies that calamansi peel EO is effective at low concentrations against third and early fourth instar *Ae. aegypti* mosquito larvae and may be used as a potentially safer and alternative biolarvicide posing minimal harmful effects to non-target organisms.

Keywords: *bioassay, biolarvicide, Citrofortunella microcarpa, essential oil, limonene*

Introduction. Mosquitoes transmit many diseases, including but not limited to yellow fever, malaria, several forms of encephalitis, filariasis, and chikungunya. One of the most notable diseases transmitted by mosquitoes worldwide, particularly *Aedes* species, is dengue hemorrhagic fever. It is a viral disease that causes mild to severe fever, which can be potentially life-threatening [1]. In the Philippines, reported cases of dengue from January to August 2019 have reached 271, 480 cases nationwide, 213% higher compared to the same reporting period in 2018 according to a report from the Department of Health (DOH) [2]. Particularly in Region VI, there have been 45, 345 reported dengue cases from January to August 2019, which is 475% higher compared to the previous year.

Controlling mosquitoes, particularly *Aedes* sp. which are vectors of pathogenic diseases that harm humans, has been the predominant subject of several new studies. The life cycle of a mosquito involves four stages: egg, larva, pupa, and adult. The larval stage of a mosquito is subdivided into four substages: first instar, second instar, third instar, and fourth instar. Mosquito control involves targeting the adult mosquito through spraying chemical insecticides or by killing mosquito larvae before entering the adult stage, where they are the most vulnerable, through synthetic larvicides or botanical extracts as an alternative [1]. Insect repellents and/or pesticides containing active ingredients such as N,N-Diethyl-meta-toluamide (DEET) are frequently used. However, adverse effects of DEET have been reported [3,4], with some being severe enough to cause sensory disturbances. In addition, DEET, which is available worldwide in various formulations including aerosols, creams, lotions, and sprays at concentrations ranging from 5% to 100%, is not recommended for children, because exposure to high concentrations of DEET can cause encephalopathy as well as other side effects [5].

Pesticides and insect repellents are common in almost every household. These products do not only contain DEET but other harmful chemicals and substances as well. Continued use of these pesticides against disease-carrying mosquitoes has harmful and adverse effects on the health of the people and the environment as a whole [6]. Two of the basic chemical classes of insect repellents include the following: (1) synthetic chemicals including DEET and picaridin and (2) botanical oils such as citronella oil and eucalyptus oil [5]. Other various types of substances that are both natural and synthetic have also been discovered and used to protect humans from mosquito bites [7].

Essential oil (EO) is one of the natural-based products that are being recently developed because it contains an abundant amount of bioactive compounds that have the potential against the developmental stages of mosquitoes. For example, limonene is a nerve toxin found to be effective against insects by hyperstimulating their motor neurons. Citrus plants, one of the primary sources of EOs, possess insecticidal properties due to the presence of the compound D-limonene in abundant amounts [8,9].

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Among the plants related to citrus, calamansi is common, native, and widely cultivated here in the Philippines. Oftentimes, only the fruit itself is being utilized, then the rest, including the peels, are thrown away [10]. Therefore, to maximize the use of the plant, calamansi peels were chosen for this study.

This study promotes the use of a natural-based product, particularly an alternative biolarvicide, as a means of mosquito control by killing *Ae. aegypti* larvae before it can develop into an adult mosquito and become a vector of dengue. Moreover, this alternative biolarvicide poses little to no harmful effects to the environment as well as non-target organisms unlike synthetic counterparts.

This study aimed to evaluate the larvicidal activity of *Citrofortunella microcarpa* (calamansi) peel essential oil against third and early fourth instar *Aedes aegypti*. It specifically aimed to:

(i) determine the limonene content present in the calamansi peel essential oil by subjecting it to gas chromatography-mass spectrometry;

(ii) evaluate the larvicidal activity of the calamansi peel essential oil against third and early fourth instar *Ae. aegypti* by measuring the mortality rate; and

(iii) compare the results of larval mortality rate using Probit Analysis.

Methods. This was an experimental type of research which was composed of two phases. The first phase involved the purchasing of commercially available steam distilled calamansi peel essential oil (EO) and the verification of the purchased EO. The second phase involved the actual experiment and conduct of the test. This phase included the collection of the test organisms, its acclimatization process, the conduct of the preliminary testing, and the final confirmatory testing. The preliminaries were necessary to establish the range of concentrations that would give larval mortality from 10% to 90%. Each of the treatments: the calamansi peel EO in acetone, calamansi peel EO in 95% ethanol, positive control, and negative controls had four replicates. The positive control used for this study was Abate® 1SG while the negative controls included 95% ethanol, acetone, and dechlorinated water. The preparation of the set-ups was done in this phase. After the preparation, the concentration of the EOs were applied to the set-ups. Larvicidal activity of the calamansi peel EO was evaluated by the measurement of the mortality rate of the test organism introduced. The results obtained were tabulated. It was then analyzed using Probit Analysis. The duration of the data gathering was one week.

Acquiring of Essential Oils. One hundred (100) mL of Calamansi Essential Oils extracted from the fruit's peels via steam distillation was commercially purchased. A Material Safety Data Sheet (MSDS) was issued upon purchasing.

Gas Chromatography. Twenty (20) mL of the essential oil was subjected to Gas Chromatography - Mass Spectrometry Test for Limonene in order to determine the limonene content present in the product.

Collection of Mosquito Larvae. The total number of larvae was determined from the range of concentrations obtained from the preliminary testing. However, 20 larvae (a combination of third and early fourth instar) were used for each set-up both during the preliminary testing and confirmatory testing.

Acclimatization Process of Mosquito Larvae. The *Ae. aegypti* larvae used in the study were cultured in the DOST-ITDI Entomology Section Insectary and were reared according to their standard procedures following the guidelines provided by the World Health Organization (WHO). The larvae were reared at a laboratory condition of 25 ± 2°C and a relative humidity of 70% ± 10%.

Preparation of Mosquito Larvae Set-ups. Using a pasteur pipette, batches of 20 third and early fourth instar *Ae. aegypti* larvae were transferred to 100 mL glass beakers each containing 50 mL of dechlorinated water. Small, unhealthy, or damaged larvae were removed and replaced as they are not considered to be valid test organisms, following the guidelines of World Health Organization.

Preparation of Stock Solution. Two stock solutions of 10,000 ppm each (0.1 mL extract in 10 mL acetone and 0.1 mL in 10 mL 95% ethanol) were prepared. Ethanol and acetone were used as solvents because the essential oil is not miscible in water, if applied directly.

Preparation of the Positive and Negative Controls. Abate® 1SG was used as a positive control, whereas set-ups with dechlorinated water, acetone, and 95% ethanol were used as negative controls. These controls were then tested against third and early fourth instar *Ae. aegypti* mosquito larvae.

Bioassay. In the preliminary testing, the third and early fourth instar *Ae. aegypti* mosquito larvae were exposed to a wide range of test concentrations to establish a set of concentrations that would give larval mortality from 10% to 90%. The results were also compared to the negative controls to determine whether the dilution of the extract with the solvents had an effect on the mortality of the test organisms. For each concentration, at least four replicates were prepared.

For the final confirmatory test, the test organisms were exposed to concentrations ranging from 8 ppm to 11 ppm. After 24 hours, the mortality of the mosquito larvae for each set-up was recorded. The mortality rate was calculated using the following formula:

$$Mortality\ Rate = \frac{Number\ of\ dead\ larvae}{Number\ of\ larvae\ introduced} \times 100$$

The larvae were probed with a pasteur pipette and if there was no response from the larvae, it was considered dead. In calculating the percentage mortality, moribund larvae which is the larvae that is approaching death, was counted too and was added

to the total number of dead larvae. Moribund larvae were qualified as those incapable of rising to the surface. They did not show any reaction when the water was disturbed.

Data Analysis. Probit Analysis is a type of regression that was used to analyze the obtained results. The recorded values were plotted in a spreadsheet, wherein all the values of concentration used in the final confirmatory test were converted into the logarithm of the concentration and all the mean percentage larval mortality were transformed into probit values in order to obtain the linear equation that would estimate the LC_{50} and LC_{90} values.

Biosafety Procedures and Waste Disposal. All laboratory protocols were strictly observed throughout the conduct of the experiment, which included the wearing of laboratory gowns, safety gloves, face masks, and proper handling of laboratory equipment. Proper waste disposal was observed and done according to the institution's rules. The stock solution was disposed as chemical waste. Hot water was poured on the mosquito larvae prior to its disposal as biohazard waste.

Results and Discussion. This study aimed to evaluate the larvicidal activity of calamansi peel EO against third and early fourth instar *Ae. aegypti*. Larvicidal bioassay was performed with concentrations ranging from 8 to 11 ppm. Results were observed after 24 hours. After the data analysis, the lethal concentration values obtained were compared to determine which treatment achieved the lowest value, indicative of high efficacy of the treatment.

Gas Chromatography. Results from the Gas Chromatography-Mass Spectrometry test showed that the limonene content of calamansi peel EO is 83.1%w/w, indicating a substantial amount of limonene. The limonene present caused the larvicidal activity of calamansi peel EO. A study by Cheong et al. [9] reported that calamansi peels were composed of limonene. Limonene is known to cause the plants' larvicidal activity. Thus, it entails that it caused the death of the mosquito larvae [12,13,14]. This active compound is a nerve toxin that kills insects on contact by acting upon their sensory cells, leading to hyperstimulation of motor neurons [8]. One of the factors accounting for differences between this study's and related study's results may be the species of plant and the plant part used. Plant extracts have various insecticidal and medicinal values depending on the compound present [16]. In addition to that, Mahanta et al. [11] stated that major compounds of the essential oil along with its quality and quantity is one of the significant factors that can determine the insecticidal activity of a different plant essential oil.

Larvicidal Activity. Table 1 shows the mean percent larval mortality after 24 hours of exposure from varying concentrations of the calamansi peel EO in acetone and calamansi peel EO in 95% ethanol at 8 ppm, 9 ppm, 10 ppm, and 11 ppm, the LC_{50} and LC_{90}, and the positive control and negative control group for comparison. As the dose per treatment increases, mean % mortality also increases. As shown in Table 1, 11.0 ppm of the calamansi peel EO in acetone has the highest mean % mortality having a value of 92.14%. Then, it was followed by 10.0 ppm, 9.0 ppm and lastly 8.0 ppm. The same trend was observed in the calamansi peel EO in 95% ethanol, having a value of 94.28% mortality at 11.0 ppm. The larval mortality in the positive control, Abate® 1SG Mosquito Larvicide, was observed at a concentration ranging from 0.1 ppm to 0.5 ppm with the larval mortality rate from 8.0 ± 5.7% to 94.95 ± 3.54% within exposure period of 24 hours (Table 1). No larval death was observed in the negative controls using acetone alone, 95% ethanol alone, and dechlorinated water alone within an exposure period of 24 hours.

Table 1. Larvicidal activity of calamansi peel EO against third and early fourth instar *Ae. aegypti* after 24 hours (n=20).

Treatments	Dose (ppm)	Mean % Mortality ± SD
Calamansi Peel EO in Acetone	8.00	11.43 ± 4.76
	9.00	52.85 ± 7.56
	10.00	84.29 ± 9.76
	11.00	92.14 ± 6.36
Calamansi Peel EO in 95% Ethanol	8.00	22.14 ± 13.18
	9.00	50.88 ± 8.26
	10.00	82.14 ± 8.59
	11.00	94.28 ± 5.34
Positive Control (Abate® 1SG mosquito larvicide)	0.10	8.00 ± 5.70
	0.20	41.00 ± 8.90
	0.30	77.00 ± 7.58
	0.40	91.00 ± 7.41
	0.50	94.95 ± 3.54
Negative Control (Dechlorinated Water)	0.00	0.00
Negative Control (Acetone)	11.00	0.00
Negative Control (95% Ethanol)	11.00	0.00

Calamansi peel EO possessed significant toxicity based on the mortality of the third instar and early fourth instar *Ae. aegypti* mosquito larvae. It was reported in a previous study by Pansit et al. [1] that calamansi is a more potent larvicide compared to other plant extracts. In line with this, according to the results of this study, calamansi peel EO showed promising larvicidal activity against *Ae. aegypti* mosquito larvae.

Comparing the Larvicidal Activities. As shown in Table 2, the larvicidal activity of calamansi peel EO in acetone and calamansi peel EO in 95% ethanol of this study were compared to the larvicidal activity of different plant extracts of the other studies. Comparing each of the treatments' lethal concentrations, the lowest LC_{50} and LC_{90} values were obtained by the calamansi peel EO in 95% ethanol having 8.89 ppm for its LC_{50} and 10.57 ppm for its LC_{90}. Then, it was followed by the calamansi peel EO in acetone having an LC_{50} value of 9.08 ppm and LC_{90} value of 10.58 ppm. Other citrus species as reported by similar studies, *Citrus grandis*, *Citrus*

aurantium peel EO and *Citrus paradisi* and other plant extracts such as *Hyptis suaveolens* in acetone, *Hyptis suaveolens* in ethanol and *Leucas aspera* obtained lethal concentrations greater than 30.0 ppm [11,13,15,16].

Table 2. Lethal concentrations of the different treatments.

Treatments	LC_{50} (ppm)	LC_{90} (ppm)
Calamansi Peel EO in Acetone	9.08	0.58
Calamansi Peel EO in 95% Ethanol	8.89	0.57
Positive Control (Abate® 1SG mosquito larvicide)	0.21	0.40
Negative Control (Dechlorinated Water)	0.00	0.00
Negative Control (Acetone)	0.00	0.00
Negative Control (95% Ethanol)	0.00	0.00
Citrus grandis peel [11]	61.04	-
Citrus aurantium peel EO [13]	31.20	73.83
Citrus paradisi peel EO [13]	35.71	70.23
Leucas aspera [15]	44.02	73.24
Hyptis suaveolens in Acetone [16]	95.66	196.76
Hyptis suaveolens in Ethanol [16]	78.88	193.49

Calamansi peel EO in acetone and calamansi peel EO in 95% ethanol were able to obtain low concentrations compared to the other plant extracts because of the abundance of limonene. Plants with limonene are more efficient than plants without limonene when it comes to larvicidal activity [11,12,13]. This study has proven that the kind of major compounds present in the extract determine the differences in insecticidal activity of plant. The dominant compounds found in *C. grandis* are nootkatone and eudesmol [11] in contrast with the findings of this study where limonene is found to be dominant. In the study done by Sanei-Dehkordi et al. [13], it was found that *C. aurantium* contains 94.81% limonene and it obtained LC_{50} value of 31.20 ppm and LC_{90} value of 73.83 ppm. This is in line with this study because Sanei-Dehkordi et al. [13] also stated that high presence of limonene showed effective larvicidal activity. Other studies by Oumarou et al. [16] and Elumalai et al. [15] did not use citrus species, thus, limonene is not present in their plant sample. This means that a different extract was used which affected the larvicidal activity of the plant. Furthermore, the difference in results with previous studies may also be due to difference in the species of mosquito used. The mosquitoes used in other studies were not limited to third and early fourth instar *Ae. aegypti*, as in the study by Sanei-Dehkordi et al. [13] for example *Anopheles stephensi* was used. This may account for the lethal concentration being higher in the said study than that found in this one. In the study of Oumarou et al. [16], they used *Anopheles gambiae*, the resulting lethal concentration being 78.88 ppm in contrast with this study's lowest lethal concentration which is 8.89 ppm. It is important to note the factors that can affect the findings of the study which include the test organism and the plant used since the differences may have an impact in the results obtained.

Limitations. This study was conducted primarily for the purpose of evaluating the larvicidal activity of *Citrofortunella microcarpa* (calamansi) peel EO against third and early fourth instar *Aedes aegypti*. The conduct of the study was limited only to the third and early fourth instar larval stages of the *Ae. aegypti* mosquito considering that these are stages where the mosquito larvae are most vulnerable. This study was also limited to the essential oil from peels of *C. microcarpa* fruit. The calamansi peel EO used in the study was commercially purchased. The EO was specifically tested to determine the amount of limonene present in the product, hence, other components of the EO were not discussed.

Conclusion. The present study which evaluated the larvicidal activity of *C. microcarpa* (calamansi) peel essential oil is found to be effective at low concentrations against third and early fourth instar *Ae. aegypti* mosquito larvae. Therefore, it can be used as an alternative to the commercially available larvicide.

Recommendations. It is recommended to increase the number of replicates for each concentration in order to eliminate outliers. A smaller range of intervals between concentrations may also be tested for larvicidal activity observation for more accurate results. It is also recommended to test the calamansi peel EO against other types of mosquitoes. The EO that was used in this study was commercially purchased, therefore it is also suggested to perform the manual extraction of the EO via steam distillation. Only the limonene content of the EO was determined, thus it is also recommended to subject it to other tests to determine other compounds also present in the product.

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