Larvicidal activity of four Philippine plants against Dengue virus vector *Aedes aegypti*(Linn.)

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Abstract -Bioactive compounds, such as the plant-derived allelochemicals, are of growing interest to the scientific community due to their known toxicity against several obnoxious vectors of pervasive diseases like dengue and malaria. Moreover, the development of resistance to synthetic insecticides has diverted the interest of researchers towards insecticides of plant origin. Hence, four Philippine plant species, Citrus microcarpa (Calamansi), Chromolaena odorata (Hagonoy), Nephelium lappaceum (Rambutan), and Jasminum sambac (Sampaguita) of the respective plant families Rutaceae, Asteriaceae, Sapindaceae and Oleaceae were evaluated of their larvicidal activities. The secondary metabolites tannin and citrus, both of which have shown larvicidal and insecticidal activities, have been found in plant families Sapindaceae and Rutaceae. Likewise, Jasminum sambac and Chromolaena odorata have been reported in the literature to contain terpenoids and organophosphates, two major constituents of agricultural pesticides both recognized for the potency. The larvicidal activities of ethanolic extracts in three concentrations (100, 200, 500ppm) from four plants were evaluated against third instar larvae of dengue mosquito, Aedes aegypti in the laboratory. Their activities were compared with black pepper, Piper nigrum (positive control) and the untreated control (distilled water and DMSO). Among the plants bioassayed, the 500 ppm ethanolic extract from C. microcarpa provided 24-hr mortality of 80%, which is slightly lower than the 100% mortality obtained in black pepper. Multiple regression analysis also revealed statistically significant relationship (p-value <0.05) between concentration of the extracts of four plants and mortality rate. Linearity between larval mortality and exposure time is most significant at 500 ppm concentration with a fitted regression model: % mortality = 16.16x – 25.6 X time interval (R^2=0.944). Endpoint analysis of log-probit transformed curve of Citrus microcarpa indicates LC50 and LC90 of 451 and 628 mg/l (500 ppm) respectively. Since C. microcarpa is much cheaper than black pepper, more in depth study should be done in enhancing its activity by slightly increasing its concentration or through the addition of synergist and combination with other potent botanical insecticides. Moreover, the use of C. microcarpa is sage to humans and other non-target organisms and is an environment-friendly method of controlling dengue mosquito wrigglers.

Keywords- bioactive compounds, *Aedes aegypti, Citrus microcarpa, Chromolaena odorata, Nephelium lappaceum, Jasminum sambac,* larvicidal activity

INTRODUCTION

Dengue virus (DV) (genus Flavivirus), the causative agent of dengue fever (DF) and dengue hemorrhagic fever (DHF) consists four distinct serotypes (DEN-1, DEN-2, DEN-3, DEN-4) all capable of attaining full spectrum of the disease (Li et al., 2011) resulting to about 25,000 annual deaths worldwide (World Health Organization (WHO), 2011). The disease burden emerged as one of the major public health concerns especially in Southeast Asia where climate, urban sprawl and deteriorating environmental conditions all promote an easy spread of the virus (Ooi and Gubler, 2008). WHO (2009) further links the same factors for a 30-fold increase in the global incidence rate within the last 50 years. Moreover, the substantial growth in international travels and climate change exacerbates the problem (Hales et al., 2002; Massad and Wilder-Smith, 2009). In 2011, over 70,000 cases with <1% case-fatality rate has been reported in the Philippines, more than 20% of which came from National Capital Region alone (Department of Health (DOH), 2011).

Predation by insect herbivores in their natural habitat drives the positive evolution of plants where better defenses through production of secondary metabolites (allelochemicals), are made to ensure species survival (Metlen et al., 2009). This natural interaction constitutes host-plant resistance and is a function of biologically synthesized chemicals including alkaloids, cyanogenic glycosides, terpenoids, phenolics, organophosphates and many others (de F. Fernandes et al., 2005; Davou and Matur, 2007; Lucia et al., 2008; Morais et al., 2011). Black et al. (2008) claimed these antiherbivory compounds as prototypes for most plant-derived mosquito-control agents with spectrum even encompassing larvicidal and antimicrobial actions.

Although several chemical synthetics against dengue virus vectors have been made commercially available, reports of resistance from these conventional controls have been increasing. Recently, knockdown resistance that gave emergence to two pyrethroid-resistant Culex spp. has been reported in Northern China and Sri Lanka (Song et al., 2007; Wondji et al., 2008). Moreover, researchers in South Carolina and India have also claimed that exploitations of synthetic pesticides on the environment produce detrimental effects to both aquatic and terrestrial food webs (Bhattacharya and Kaviraj, 2008; Williamson et al., 2009). This declining efficacy of commercial insecticides have limited its availability for public use, while those remaining efficient remains beyond the financial capacity of most developing countries including the Philippines. This reality makes the finding of eco-friendly, cheaper and biodegradable alternatives an imperative and important public health goal.

In recent years, development of vaccine against dengue has been the focal point of research (Durbin et al., 2005; Melino and Paci, 2007; Whitehead et al., 2007). While in fact several clinical trials of tetravalent vaccine by some pharmaceutical firms (Acambis, GSK Biological, Hawaii Biotech, InViragen,

Sanofi Pasteur, Shanta Biotechnics) and government health agencies (FDA, WRAIR) have begun (Hombach, 2007), the use of botanical bioactive compounds remains the prevailing model to curtail disease transmission. Farrar and Whitehorn (2010) further stress their significance as useful adjuncts to future vaccines with marked synergistic effects between the two. Biological control that utilizes Cry and Cyt toxins of Bacillus thuringiensis israelensis (Bti), is another novelty found to be both effective and efficient in killing larvae of many Dipterans. However, emergence of Bti-resistant Culex spp. (Wirth et al., 2010) and adverse trophic effects on breeding birds have been noted (Poulin et al., 2010).

While several studies have already proven the efficacy of botanical bioactive compounds (Jang et al., 2002; Cavalcanti et al., 2004; Pushpanathan et al., 2006; Jung and Moon, 2011; Lima et al., 2011; Medeiros et al., 2011; Raghavendra et al., 2011), studies that investigate the larvicidal potentiality of Philippine plants are lacking. Thus, the ethanolic extracts prepared in various concentrations (100, 200, and 500ppm) were used against the laboratory-reared early fourth instar Aedes aegypti larvae (Diptera: Culicidae), the major mosquito vector of dengue virus in the country.

MATERIALS AND METHOD

Insects

Second-instar larvae of the laboratory-reared A. aegypti were provided by Dr. Pio A. Javier, Research Professor, Crop Protection Cluster, College of Agriculture, University of the Philippines Los Banos, College, Laguna. The obtained larvae were reared at polyene plastic containers (12 x 6) filled with 300 ml dechlorinated aged tap water and aerated through lid punctures. They were fed intermittently once a day with brewers yeast and flake fish food (30% to 40% protein) with increasing amounts for successive instars. Formation of residual food artifacts detrimental to the larvae development was closely monitored and periodically removed. This prohibited scum formation (biofilm) that deprives larvae of atmospheric oxygen (Asahina, 1964). Optimal conditions were sustained at 25 ± 2 °C with 70 ± 5% relative humidity (RH), and a photoperiod of 16 : 8 (Light : Dark) h (Jang, et.al., 2002).

Plant Materials

The peels (exocarp) of Citrus microcarpa, seeds of Nephelium lappaceum, flowers of Jasminum sambac and leaves of Chromolaena odorata were gathered from the parent plants during the flowering and fruiting seasons at separate locations in Batangas City (Table 1). These respective parts represented the highest concentrations and yield of bioactive compounds (Amusan et al., 2005; Ragasa et al., 2005; Joy and Raja 2008). Dr. William Gruezo of the College of Arts and Sciences, Institute of Biological Sciences, UPLB, taxonomically identified the plants.

TABLE 1
General information of four Philippine plants tested

Plant Species	Family	Part Collected	Yield[*](% wt/wt)
Citrus microcarpa	Rutaceae	Peel	12.77
Chromolaena odorata	Asteriaceae	Leaf	6.60
Jasminum sambac	Oleaceae	Flower	5.00
Nephelium lappaceum	Sapindaceae	Seed	6.93

[*]Yield (%) = (Dried weight of ethanol extract\dried weight of sample) x 100

Preparation of Stock Solution

The samples were washed three times with 500 ml distilled water to remove impurities, shade-dried and finely powdered. Each sample (150 grams weighed through analytical balance) was extracted twice with 300 ml ethanol (boiling point 78°C) in an airtight bottle at room temperature (26 + 3 °C) for three days and filtered. The combined filtrates (ethanolic extract) were then concentrated into a semisolid state for three days under laminar hood. The residues were weighed and made into stock solution (10% w/v) using distilled water as a solvent and 1% dimethyl sulfoxide (DMSO) as penetrating agent. Stock solution was kept inside the refrigerator set at 2-6 °C until use.

Larvicidal Bioassays

The larvicidal activities of the different ethanol plant extracts were bioassayed against third instar larvae of Aedes aegypti following the WHO standard protocols (WHO, 2005) with slight modifications. A 10% stock solution was prepared for each ethanol plant extract using 1% dimethyl sulfoxide (DMSO). Three concentrations (100, 200, and 500 ppm) of each of the ethanol extract was prepared by adding the required volume of stock solution in 50 ml of distilled water using plastic cups (100 ml). In each plastic cup, 15 A. aegypti third instar larvae were introduced. A separate control set-up using 1% DMSO and distilled water was used in each experiment. Meanwhile, black pepper (piper nigrum) concentrate was utilized as the positive control. The treated and control (positive and negative) larvae were held at same conditions. All treatments were replicated three times. Mortality of wrigglers was monitored starting at 0.5, 1, 2, 3, 4, 14, 28 and 72 hrs after exposure to the ethanol extract during which one gram of yeast was added and conditions maintained 25 + 2 °C. Larvae were considered dead when they fail to move following tapping with separate applicator sticks for a minimum of five times (Lucia et al., 2008).

Data analysis

The effectiveness of the ethanolic extracts from different plants was determined by regression analysis of the log-probit transformed linear curve

using SPSSv18® (Finney, 1981). For Citrus microcarpa, toxicity was further evaluated using the median lethal concentration (LC50) and LC90 obtained from the regression line (see Table 4). For the remaining plants, Nephelium lappaceum, Chromolaena odorata and Jasminum sambac, LC50 and LC90 could not be reliably computed because the highest mortality was lower than 27% (recommended mortality should be close to 10 to 90%), besides, their regression line is statistically incompatible for LC derivation.

RESULTS AND DISCUSSION

Ethanolic extract

The percent yield (Yield % wt/wt) for each ethanol extract is provided in Table 1. As seen in the table, Calamansi (C. microcarpa) provided the highest yield of 12.77% whereas Sampaguita (J. sambac) gave the lowest yield of 5. The degree of pigmentation of the plants does not correlate with amount yield. Solubility of the compounds with ethanol may explain the variability but other contributing factors must also be taken into consideration (e.g. plant part).

Statistical Findings

Scheffe's test revealed statistically significant relationship (p-value <0.05) between the mortality rate and the species of plants bioassayed using three different concentrations (100, 200, 500 ppm). The dose-mortality relations for 100, 200 and 500 ppm have corresponding p-values of 0.041, 0.104 and 0.037. This indicates that dose-mortality relationship is most significant at 500 ppm concentration (p-value 0.037) with a confidence interval of 96.3%. This high p-value implies that mortalities tend to improve with higher extract concentrations and thus is most predictable at 500ppm. Although mortality at 500ppm is not solely attributed to the plant extract, it is the concentration to which the plant extract as a factor of larval mortality was most profound (data not shown).
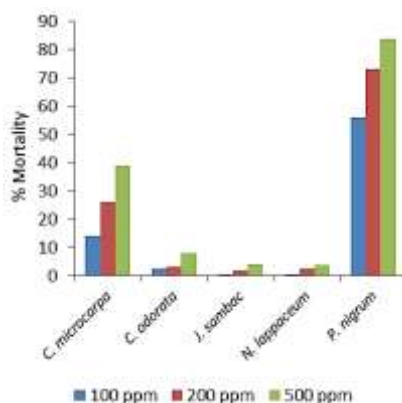

Figure 1

Relationship of % mortality of *Aedes aegypti* larvae against different ethanolic concentrations (100ppm, 200ppm and 500ppm) of the five treated plants

The data on percent mortality of A. aegypti due to different concentrations of five plant materials during the seven different time intervals are provided in Table 2. On the other hand, the relationship between % mortality and ethanolic plant extract can easily be gleaned in figure 1. Among the test plants, only C. microcarpa provided significantly highest toxicity against the 3rd-instar larvae of Aedes aegypti. As early as 30 mins of exposure to 500 ppm, 2% mortality was observed (figure 2). This further increases to 80% after 24 hours whereas for 100 and 200ppm, mortalities were not observed until 2-hour post exposure. There was no significant difference between the 24-hour and 72-hour mortality rates in which 91.3% of the larvae died at 500 ppm. The ethanolic extract of Piper nigrum, the positive control used in the experiment, provided 100% mortality as early as 4 hr after exposure to 500 ppm. The same mortality rates were observed after 24 hour of exposure to 100 and 200ppm. This results collaborates with the reports regarding the effectiveness of black pepper against the wrigglers of A. aegypti (Bhuyan and Guswami, 2006; Vasudevan et al., 2009). Further increase in mortality rate of C. microcarpa was most dramatic between 4 and 24-hour intervals (51.3-80%). The remaining plants (C. odorata, J. sambac and N. lappaceum) displayed the similar inclinations, (Figure 2) however the mortality rate of <20% deemed it insignificant. No mortality was obtained from the controls until the 72-hour exposure confirming the non-toxicity of distilled water and 1% DMSO to the larvae (Figures 1 and 2). Moreover, this indicates that the wrigglers were properly handled such that the mortalities observed when exposed to the ethanolic plant extracts are primarily due solely to the activity of the extracts.
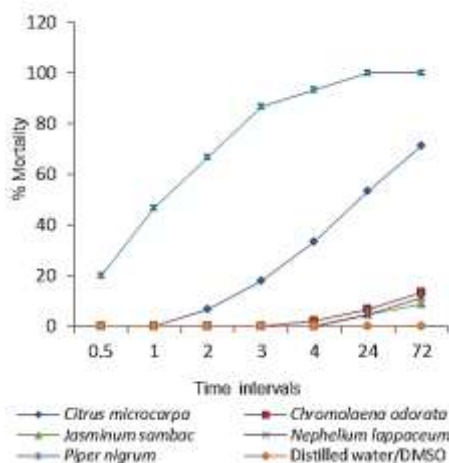

Figure 2
Relationship between Aedes aegypti larval mortality and time intervals.
Each point represents the mean of three replicates of the four Philippin plants

and their corresponding mortalities at 200ppm

On the other hand, the ethanolic extracts of the remaining three plants, Chromolaena odorata, Jasminum sambac and Nephelium lappaceum caused mortality lower than 9% even at very high concentration of 500 ppm (Table 2) suggesting that these plants in the absence of synergist or any potentiators have no potential activity for the control of Aedes aegypti larvae.

TABLE 2
Percentage mortality in time of five Philippine plant ethanolic extracts
on 15 *A. aegypti* 3rd instar larvae in three replicates

Concentration (ppm)		0.5hr		1hr		2hrs		3hrs		4hrs		24hrs		72hrs	
		N	%	N	%	N	%	N	%	N	%	N	%	N	%
Citrus microcarpa	100	0	0	0	0	0.3	2	1	6.7	1.7	11.3	4.7	31.33	7	46.7
	200	0	0	0	0	1	6.7	2.7	18	5	33.3	8	53.3	10.7	71.3
	500	0.3	2	1	6.7	2	13.3	4.3	28.7	7.7	51.3	12	80	13.7	91.3
Chromolaena odorata	100	0	0	0	0	0	0	0	0	0	0	0.7	4.7	1.7	11.3
	200	0	0	0	0	0	0	0	0	0.3	2	1	6.7	2	13.3
	500	0	0	0	0	0.3	2	1	6.7	1	6.7	2	13.3	4	26.7
Jasminum sambac	100	0	0	0	0	0	0	0	0	0	0	0	0	0.3	2
	200	0	0	0	0	0	0	0	0	0	0	0.7	4.7	1.3	8.7
	500	0	0	0	0	0	0	0	0	0.3	2	1.3	8.7	2.7	18
Nephelium lappaceum	100	0	0	0	0	0	0	0	0	0	0	0	0	0.3	2
	200	0	0	0	0	0	0	0	0	0	0	0.7	4.7	1.7	11.3
	500	0	0	0	0	0	0	0	0	0.3	2	1	6.7	2.7	18
Piper nigrum	100	1	6.7	4	26.7	6	40	8	53.3	10	66.7	15	100	15	100
	200	3	20	7	46.7	10	66.7	13	86.7	14	93.3	15	100	15	100
	500	7	46.7	10	66.7	12	80	14	93.3	15	100	15	100	15	100

= Number of death recorded; % = % mortality

As can be seen on table 2, there were no significant increases in mortalities after 72 hours of exposure. It is unlikely that the ethanol extracts of the three plants accounted for the deaths, however this claim is too premature to be decisive. The cause of the death may be inherent to the larvae itself as there were no reliable means to ensure the consistency in vitality of the experimented larvae. However, the absence of dead wrigglers in the control indicates that the

larvae are healthy and were properly handled although this does not resolve the issue of vitality. The accepted mortality rate in the control is 10% but corrected mortality should be computed as indicated by Finney (1981). If larvae are weak then majority of them will die in 72 hrs, a finding not observed for the three plants. All the other variables (temperatures, ventilation, humidity and lightings) did not have significant deviations during the three-day exposure, ruling them out as factors for the larval death. Besides, the control set up composed of 50% distilled water and 1% DMSO exhibited consistent 0% mortality all throughout (Figure 2).

TABLE 3

Bioequivalence of the lethal concentrations (LC_{50} and LC_{90}) of the ethanolic extracts of *Citrus microcarpa* and *Piper nigrum* on third instar larvae of *Aedes aegypti*

Ethanolic extract concentrations		Larvicidal Activity (mg/l)			
		LC_{50}	CI 95%	LC_{90}	CI 95%
Citrus microcarpa	100ppm	757	610 – 1034	1315	977 – 2285
	200ppm	581	481 - 731	1009	788 – 1581
	500ppm	451	372 - 548	628	628 – 1151
P. nigrum	100ppm	240	175 – 337	891	592 – 1641
	200ppm	124	87-152	406	282-671
	500ppm	62	40-102	231	160-366

CI=Confidence Interval

Figure 3
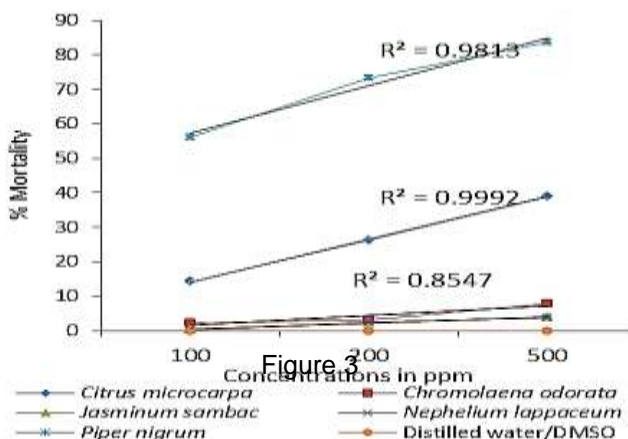
Regression analysis of the relationship between % mortality of Aedes aegypti larvaeand concentration (ppm). Each point represents the mean of three replicatessimultaneous with the controls

Moribund larvae predominated during 48-hr post treatment for Citrus microcarpa and Nephelium lappaceum and were usually deceiving unless tapped. As mentioned, only Citrus microcarpa demonstrated sufficient toxicity after 24 hour of treatment (80%, N=12 mortality) besides the positive control (Black pepper). Moribund larvae on the other hand are excluded in the mortality records. Meanwhile, some emergence of adults ensues after 72 hours of treatment. This failure to inhibit adult emergence, in addition to larvicide mortality suggest the nontoxicity of the extracts (all plants except black pepper) against the pupa stage of Aedes aegypti. On the other hand, dead and moribund larvae fail to pupate and emerged as adults. This aids in the enumeration of deaths and survivors, thus reducing the risk of miscounting. It also serves as a reliable control for inspecting uniformity in larval age. Percentage of adult emergence was not recorded.

Regression analysis showed a statistically significant relationship (p<0.01) between the mortality rates of the third-instar Aedes aegypti larvae and ethanolic extract concentrations of the five plants (treated and positive control) (figure 3). The fitted regression models for C. microcarpa and J. sambac were: larval mortality = 12.4x + 1.7 X extract concentration in ppm and 1.9x – 1.566 respectively. Both have same R2-values of 0.99 indicated that the fitted model explains 99% of the variability in larval mortality. Contrarily, for Chromolaena odorata and Nephelium lappaceum, R2-values of 0.854, 0.878 respectively indicate that only 85.4 and 97.8 % of the variability can be explained by the model (Figure 3). The remaining 14.6 and 2.2% might be inherent in nature i.e. larvae vitality is not uniform or may be caused by lurking variables such as contaminated applicator sticks or water temperature which were not standardized prior to the study. Suspicion is high that these variables could be random in nature. Thus, the researchers advise the standardization of such variables should one opt to conduct a similar study. It is important to note that while high R2-value indicates minimum interference from variables, this does not underestimates their influence or rules them out entirely.

Probit Analysis

A p-value of <0.05 (Chi-square=28.652) was derived from the log-probit transformed curve of Citrus microcarpa suggesting the existence of statistically significant relationship between its ethanolic extract concentration and larval mortality (Figure 4).
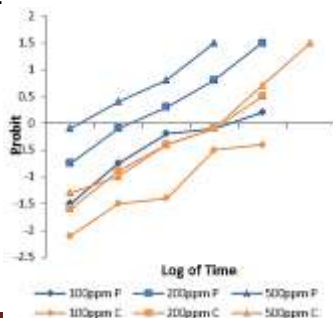
Figure 4

Probit-transformed responses curve of Citrus microcarpa and Piper nigrum

The lethal concentrations (LC50 and LC 90) for Citrus microcarpa and Piper nigrum were derived from the log-probit transformed curve shown in figure 4 (Table 3). Their bioequivalence proved the notable claim regarding P. nigrum's larvicidal potential. If 200ppm was used as a basis, it will only take 124 mg/l for it to kill 50% of the larvae and only 406 mg/l to eradicate the whole test population. For Chromolaena odorata, Jasminum sambac and Nephelium lappaceum, LC50 and LC90 were no longer derived since their larval mortalities were below 50% post-24 hour exposure. This may either imply that these plants have in reality no larvicidal activity against Aedes aegypti 3rd-instar larvae or the LC50 too high to be derived. In case the latter proposition is true, we encourage the retesting of these three plants using higher increments. Thus, for plants exhibiting no to low larvicidal activity, it would be pointless and unwise to further ascertain the toxicity profile.

The fitted regression models for the corresponding increments (100, 200, and 500 ppm) were: larval mortality = 7.5736x − 16.29, 12.396x − 23.5 and 16.161 x − 25.6 X time intervals. The correlation coefficients vary between each concentration and tend to progress with dose. For 500ppm, as much as 94.4% (R^2=0.944) of the variability can be explained by the model while only 81.6% for 100ppm. This indicates that the influence of the extraneous factors irrelevant to the study tends to decline as the concentration increases. Thus, for 500ppm, only 5.6% of the variables were outliers against 18.4% scattering seen in 100ppm.
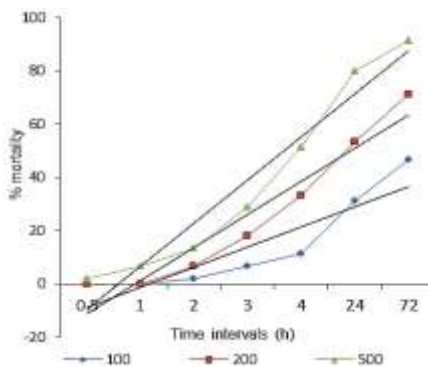

Figure 5

Regression analysis depicting the relationship between time and % mortality of Aedes aegypti third instars against different concentrations of Citrus microcarpa ethanolic extract.

The larvicidal bioassay pointed out the dependency of larval mortality against the concentration, type of plant and time of exposure. Overall, the connections between these variables are linear, provided the uniformity and standardization of the assay and variables. Statistical analyses also revealed the

existence of confounding and misaligned variables, reflected by the regression model and R2 values. This signified that some significant variables may have been missed in the study (e.g. vitality of the larvae, temp of the water and contamination). For Calamansi and black pepper, however, the confidence levels are very high (Figure 2-4) whereas the influence of variables are most explicit for C. odorata, J. sambac and N. lappaceum.

CONCLUSION

The findings of this study provide the preliminary report of the four Philippine plants larvicidal activity against Aedes aegypti larvae. For C. microcarpa, regression analysis showed that 50% and 90% mortality can be attained at 581 and 1009 mg/l respectively (200ppm; CI=95%). Thus, the plant extract may be considered as a possible larvicide substitute to Aedes aegypti if other biological means are not available. Although it lags behind black pepper with regards to potency (table 3), studies complementing the extract with an affordable or accessible agonist may even proved superior considering its nontoxicity to environment. This conforms to the study establishing the genus Citrus (family: Rutaceae) larvicidal activity, with the monoterpene Tepinoline noted as the bioactive compound responsible for the larvicidal property (Amusan et.al 2005). These findings is of significant contribution to the Philippines, especially that studies outlining the larvicidal toxicity of Philippine plants are still currently limited, despite being a hotspot for floral diversity. The larvicidal profiles of the tested plants may also be used to predict the larvicidal activity of the experimented plants. The model of this study to devise greener approach could be of interest in view of the fact that resistance and health hazards against man-made insecticides are on the increase.

REFERENCES

Amusan, A. A., Idowu, A. B., Arowolo, F. S., (2005). Comparative toxicity effect of bush tea leaves (*Hyptis suaveolens*) and orange peel (C*itrus sinensis*) oil extract on larvae of the yellow fever mosquito *Aedes aegypti*. *Tanzania Health Research Bulletin ,7, 174*

Asahina, S. (1964).Food Material and Feeding Procedures for Mosquito Larvae.*Bull. Org. mond. Santé, 31,* 465-466

Black, L., Keily, M., Kroon, P., Plumb, J., & Gry, J. (2008).Development of EuroFIR-BASIS – a composition and biological effects database for plant-based bioactive compounds. *Nutrition Bulletin, 33*, 58-61.

BRAZIL; Dengue fever vaccine. (2009, July 19). *The Adelaide*. Retrieved from http://web.ebscohost.com

Cavalcanti, E. S. B., de Morais, S. M., Lima, M. A., & Santana, E. P. (2004). Larvicidal Activity of Essential Oils from Brazilian Plants against *Aedes aegypti* L. *Mem Inst Oswaldo Cruz, 99*(5), 541-544.

Chung, L. P., (2008). Situation of Chinese Medicine in China.*Asia Pacific Biotech News, 13*(6), 22-30. Retrieved from http://www.asiabiotech.com

Clinical trial for dengue vaccine held in Laguna. (2011, August 13). Retrieved from ABS-CBN News website, http://www.abs-cbnnews.com/nation/regions/08/12/11/clinical-trial-dengue-vaccine-held-laguna

de Morais, S. M., Facundo, V. A., Bertini, L. M., Cavalcanti, E. S. B., dos Anjos, J. F., Junior., Ferreira, S. A., et al. (2007). Chemical composition and larvicidal activity of essential oils from *Piper* species.*Biochemical Systematics and Ecology, 35,* 670-675. doi: 10.1016/j.bse.2007.05.002

Dengue and the Aedes aegypti mosquito.(n.d.). Retrieved from Centers for Disease Control and Prevention website, http://www.cdc.gov

Durbin, A. P., Whitehead, S. S., McArthur, J., Perreault, J. R., Blaney, J. E., Jr., Thumar, B., et al. (2005). rDEN4D30, a Live Attenuated Dengue Virus Type 4 Vaccine Candidate, Is Safe, Immunogenic, and Highly Infectious in Healthy Adult Volunteers.*Journal of Infectious Diseases, 191*, 710-718.

Fernandes, F. D. F., Freitas, E. D. P. E. S., da Costa, A. C., & da Silva, I. G. (2005). Larvicidal potential of *Sapindus saponaria* to control the cattle tick *Boophilus microplus.Pesquisa Agropecuária Brasileira, 40*(12), 1243-1245.

Finney D. J. (1981). *Probit analysis.* (3rd ed.) New Delhi: S. Chand & Company

Hales, S., de Wet, N., Maindonald, J., & Woodward, A. (2002). Potential effect of population and climate changes on global distribution of dengue fever: an empirical model. *Lancet, 360,* 830-34. Retrieved from http://www.thelancet.com

Hombach, J. (2007). Vaccines against dengue: a review of current candidate vaccines at advanced development stages*. Pan American Journal of Public Health, 21*(4), 254-260.

Human clinical trial of NIH-developed dengue vaccine begin. (2010, August 9). Retrieved from NIH News website, http://www.nih.gov/news/health/aug2010/niaid-09.htm

Inviragen Initiates a Phase 2 Clinical Study of DENVax, a Recombinant, Tetravalent Dengue Vaccine. (2011, November 28). Retrieved from http://web.ebscohost.com/

Jang, Y-S., Kim, M-K., Ahn, Y-J., & Lee, H-S. (2002) Larvicidal Activity of Brazilian Plants against *Aedes aegypti* and *Culex pipiens pallens* (Diptera: Culicidae). *Agric. Chem. Biotechno, 45*(3), 131-134.

Jung, J-C., & Moon, H-I.(2011). Larvicidal activity of 4-hydroxycoumarin derivatives against *Aedes aegypti.Pharmaceutical Biology, 49*(2), 190-193. doi: 10.3109/13880209.2010.507769

Li, S., Fang, M., Zhou, B., Ni, H., Shen, Q., Zhang, H., et al. (2011). Simultaneous detection and differentiation of dengue virus serotypes 1-4, Japanese encephalitis virus, and West Nile virus by a combined reversetranscription loop-mediated isothermal amplification assay. *Virology Journal, 8*(360). Retrieved from http://www.virologyj.com/content/8/1/360

Lima, M. A. A., de Oliveira, F. M., Gomes, G. A., Lavor, P. L., Santiago, G. M. P., Nagao-Dias, A. T., et al. (2011) Evaluation of larvicidal activity of the essential oils of plants species from Brazil against *Aedes aegypti* (Diptera: Culicidae). *African Journal of Biotechnology, 10*(55), 11716-11720. Retrieved from http://www.academicjournals.org/AJB

Lucia, A., Licastro, S., Zerba, E., & Masuh, H. (2008). Yield, chemical composition, and bioactivity of essential oils from 12 species of *Eucalyptus* on *Aedes aegypti* larvae.*Entomologia Experimentalis et Applicata, 129,* 107–114. doi: 10.1111/j.1570-7458.2008.00757.x

Madhuban, Bhattacharya, & Kaviraj, A. (2009). Toxicity of the pyrethroid pesticide fenvalerate to freshwater catfish *clarias gariepinus*: Lethality, biochemical effects and role of dietary ascorbic acid. *Journal of Environmental Science and Health Part B, 44*, 578-583. doi: 10.1080/03601230903000602

Massad, E., & Wilder-Smith, A., (2009).Risk Estimates of Dengue in Travelers to Dengue Endemic Areas Using Mathematical Models. *Journal of Travel Medicine, 16*(3), 191-193. doi: 10.1111/j.1708-8305.2009.00310.x

Matur, B. M., and Davou, B. J., (2007).Comparative Larvicidal Property of Leaf Extract of *Chromolaena odorata L* (Composidae) and Chlopyrifos (Organophosphorus Compound) on *Simulium* Larvae.*Biomedical and Environmental Sciences, 20*, 313-316.

Medeiros, E. S., Costa, F. M., Rodrigues, I. B., Tadei, W. P. (2011). Larvicidal activity of the aqueous extract of the clove (*Eugenia caryophyllata*) against *Aedes aegypti* (Diptera, Culicidae) under laboratory conditions.*Journal of Research in biology, 1*(7), 518-523. Retrieved from http://www.jresearchbiology.com

Melino, S., & Paci, M. (2007).Progress for dengue virus diseases Towards the NS2B–NS3pro inhibition for a therapeutic-based approach.*The Febs Journal, 274,* 2986–3002. doi:10.1111/j.1742-4658.2007.05831.x

Metlen, K. L., Aschehoug, E. T., & Callaway, R. M. (2009).Plant behavioural ecology: dynamic plasticity in secondary metabolites. *Plant, Cell and Environment, 32*, 641-653. doi: 10.1111/j.1365-3040.2008.01910.x

Newman, J., (2009, October 6). Vaccine companies merge. *The Madison.* Retrieved from http://web.ebscohost.com/

Ooi, E-E., & Gubler, D. J. (2008). Dengue in Southeast Asia: epidemiological characteristics and strategic challenges in disease prevention. *Cad. Saúde Pública, 25,* 115-124.

Poulin, B., Lefebvre, G., & Paz, L. (2010). Red flag for green spray: adverse trophic effects of Bti on breeding birds. *Journal of Applied Ecology, 47*, 884-889. doi: 10.1111/j.1365-2664.2010.01821.x

Priya, J., & Raja, D.P. (2008, July 13). Anti-Bacterial Activity Studies of *Jasminum grandiflorum* and *Jasminum sambac. Ethnobotanical Leaflets, 12*, 481-483.

Pushpanathan, T., Jebanesan, A., & Govindarajan, M. (2006).Larvicidal, ovicidal and repellent activities of *Cymbopogan citratus* Stapf (Graminae) essential oil against the filarialmosquito *Culex quinquefasciatus* (Say) (Diptera : Culicidae). *Tropical Biomedicine, 23*(2), 208-212.

Raghavendra, B. S., Prathibha, K. P., & Vijayan, V. A. (2011). Larvicidal Efficacy of *Eugenia jambolana* Linn.Extracts in Three Mosquita Species at Mysore.*Journal of Entomology, 8*(5), 491-496. doi: 10.3923/je.2011.491.496

Seppa, N. (2011, December 31). Dengue vaccine tests advance. *Science News, 190*(14), 17. Retrieved from http://web.ebscohost.com/

Sivagnaname, N., & Kalyanasundaram, M. (2004). Laboratory Evaluation of Methanolic Extract of Atlantia monophylla (Family: Rutaceae) against Immature Stages of Mosquitoes and Non-target Organisms. *Mem Inst Oswaldo Cruz, 99*(1), 115-118.

Song, F., Cao, X., Zhao, T., Dong, Y., and Lu, B. (2007).Pyrethroid resistance and distribution of *kdr* allele in *Culex pipiens pallens* in north China.*International Journal of Pest Management, 53*(1), 25-34. doi: 10.1080/09670870601002625

Thitilertdecha, N., Teerawutgulrag, A., Kilburn, J. D., & Rakariyatham, N. (2010). Identification of Major Phenolic Compounds from *Nephelium lappaceum* L.

and Their Antioxidant Activities. *Molecules, 15*, 1463-1465. doi: 10.3390/molecules15031453

Verdon, D.R. (2001, September). Dengue fever vaccine shows promise after clinical trials. *DVM Newsmagazine*, 60. Retrieved from http://www.dvm360.com/news