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Screening for larvicidal activity of ethanolic and aqueous extracts of selected plants against *Aedes aegypti* and *Aedes albopictus* larvae

Michael Russelle Alvarez[1,2], Francisco Heralde III[1], Noel Quiming[2*]

[1]*Department of Biochemistry and Molecular Biology, College of Medicine, University of the Philippines Manila, Manila, Philippines*

[2]*Department of Physical Sciences and Mathematics, College of Arts and Sciences, University of the Philippines Manila, Manila, Philippines*

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ABSTRACT

Objective: To screen for larvicidal activity of aqueous and ethanolic extracts (95% ethanol) from *Selaginella elmeri, Christella dentata, Elatostema sinnatum, Curculigo capitulata, Euphorbia hirta, Murraya koenigii* (*M. koenigii*), *Alpinia speciosa, Cymbopogon citratus, Eucalyptus globulus* (*E. globulus*), *Jatropha curcas* (*J. curcas*), *Psidium guajava, Gliricidia sepium, Ixora coccinea* and *Capsicum frutescens* (*C. frutescens*) against *Aedes aegypti* (*A. aegypti*) and *Aedes albopictus* (*A. albopictus*) 3rd instar larvae.

Methods: Ethanolic and aqueous extracts were screened for larvicidal activity by exposing the *A. aegypti* and *A. albopictus* 3rd instar larvae (15 larvae per trial, triplicates) for 48 h, counting the mortalities every 24 h. Additionally, phytochemical screening for flavonoids, tannins, alkaloids, anthraquinones, anthrones, coumarins, indoles and steroids were performed on active extracts using spray tests.

Results: Against *A. aegypti*, the three most active extracts were *C. frutescens* ethanolic (100% after 24 and 48 h), *J. curcas* ethanolic (84.44% after 24 h and 88.89% after 48 h) and *M. koenigii* ethanolic (53.33% after 24 h and 71.11% after 48 h). On the other hand, against *A. albopictus*, the three most active extracts were *C. frutescens* ethanolic (93.33% after 24 h and 100% after 48 h), *J. curcas* ethanolic (77.78% after 24 h and 82.22% after 48 h) and *E. globulus* ethanolic (64.44% after 24 h and 73.33% after 48 h). Phytochemical screening was also performed on the active extracts, revealing alkaloids, tannins, indoles and steroids.

Conclusios: The results demonstrate the larvicidal activities of ethanolic extracts of *Cymbopogon citratus*, *Euphorbia hirta*, *Ixora coccinea*, *Gliricidia sepium*, *M. koenigii*, *E. globulus*, *J. curcas* and *C. frutescens* against *A. aegypti* and *A. albopictus* 3rd instar larvae. These could be used as potential larvicidal agents for the control of these mosquitoes.

1. Introduction

Dengue is one of the most rapidly spreading vector-borne diseases in the world. It affects many countries, especially the ones in the tropical areas like Philippines, Cambodia, Malaysia, India, Indonesia, Thailand, Brazil, Argentina, Eastern Africa and Nigeria, just to name a few. In the Philippines alone, the reported number of dengue cases as of September 2013 was 117 658 with more than 20 000 cases belonging to the 1–10 age group; of these, 433 deaths were reported[1]. The children are the most susceptible especially those belonging to the poorest class wherein inadequate water supply, improper waste management and other conditions are favorable for the growth and multiplication of dengue's vector, *Aedes aegypti* (*A. aegypti*) and *Aedes albopictus* (*A. albopictus*). Methods for exterminating the adult mosquitoes involve fumigation, bug zappers, mechanical pest control, and use of adulticides, all of which have been shown to be either inefficient, toxic or both. A better way of controlling mosquito populations - and in turn, control dengue spread - is by targeting the larvae instead of the adults. Lavicidal agents are commonly bacterial (*e.g. Bacillus thuringiensis* larvicides) or synthetic (*e.g.* methoprene, pyriproxyfen) in nature. These agents may be efficient and effective, and they still have certain issues regarding quality

*Corresponding author: Noel Quiming, Department of Physical Sciences and Mathematics, College of Arts and Sciences, University of the Philippines Manila, Manila, Philippines.
Tel: +639178920218
E-mail: russellealvarez@gmail.com
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control and acceptance[2].

To circumvent the problems posed by the other methods, the usage of plant derived products can be done instead. Several researches have shown the efficacy of plant products as larvicidal agents. Dehydrocostus lactone, costunolide and essential oil isolated from *Saussurea lappa* roots are active against *A. albopictus* larvae, with LC_{50} values of 2.34 μg/mL, 3.26 μg/mL and 12.41 μg/mL[3]. Hexane, chloroform, acetone, methanol and aqueous leaf extrats from Morinda citrifolia were also found to be active against *A. aegypti*, *Anopheles stephensi* (*An. stephensi*) and *Culex quinquefasciatus* (*C. quinquefasciatus*)[4]. *Citrus sinensis*, *Citrus limon* and *Citrus paradisi* essential oils were shown to be toxic against *A. albopictus* larvae, with LC_{50} values ranging from 25.03 mg/L to 37.03 mg/L[5]. Hexane, ethyl acetate, benzene, chloroform and methanol crude extracts of Asparagus racemosus roots were also shown to possess ovicidal, larvicidal and adulticidal effects against *A. aegypti*, *C. quinquefasciatus* and *An. stephensi*[6]. The study demonstrated that the extracts showed high ovicidal activity (0% hatchability for all extracts), moderate larvicidal activity (with methanol extract the most active, LC_{50} = 115.13 mg/L, 97.71 mg/L and 90.97 mg/L for *C. quinquefasciatus*, *A. aegypti* and *An. stephensi*) and moderate adulticidal activity (with methanol extract the highest, LD_{50} = 120.44 mg/L, 135.60 mg/L and 157.71 mg/L for *An. stephensi*, *A. aegypti* and *C. quinquefasciatus*).

For this study, aqueous and ethanolic (95% ethanol) extracts of *Selaginella elmeri* (*S. elmeri*), *Christella dentata* (*C. dentata*), *Elatostema sinnatum* (*E. sinnatum*), *Curculigo capitulata* (*C. capitulata*), *Euphorbia hirta* (*E. hirta*), *Murraya koenigii* (*M. koenigii*), *Alpinia speciosa* (*A. speciosa*), *Cymbopogon citratus* (*C. citratus*), *Eucalyptus globulus* (*E. globulus*), *Jatropha curcas* (*J. curcas*), *Psidium guajava* (*P. guajava*), *Gliricidia sepium* (*G. sepium*), *Ixora coccinea* (*I. coccinea*) and *Capsicum frutescens* (*C. frutescens*) were tested against *A. aegypti* and *A. albopictus*. Mortalities were counted after 24 and 48 h of exposure. The phytochemical constituents of the active extracts were also determined using spray tests.

2. Materials and methods

2.1. Chemicals and reagents

The positive control used was malathion purchased from Leads Agri (Mandaluyong City, Metro Manila, Philippines). The solvents used in the extraction were of analytical grade bought from Chemline Inc. (Quezon City, Metro Manila, Philippines).

2.2. Larvae procurement

Third instar *A. aegypti* and *A. albopictus* larvae were obtained from the Research Institute for Tropical Medicine, Muntinlupa, Philippines.

2.3. Plant sampling and preparation

Plants were obtained from various locales in Luzon, Philippines. *C. dentata, C. capitulata, E. sinnatum* and *S. elmeri* leaves were obtained from Mount Isarog, Camarines Sur. *E. hirta* stems, and *M. koenigii*, *A. speciosa* and *C. citratus* leaves were purchased from the Bureau of Plant Industry, Manila, Philippines. *E. globulus*, *J. curcas*, *P. guajava*, and *G. sepium* leaves and *C. frutescens* fruits were obtained from Echague, Isabela. These plants were identified by the Research Division Office, Bureau of Plant Industry, Manila, Philippines and Botany Division, National Museum, Manila, Philippines.

Plants were air-dried under shade for at least a week until crunchy. Then, these were ground until a coarse fine powder was obtained.

2.4. Extraction

At least 20 g of each plant powder were serially extracted with 95% ethanol and then double distilled in a 1 g material: 10 mL solvent. The plant materials were extracted twice by soaking with 95% ethanol at room temperature for 48–72 h and with double distilled water at 4 °C for 48–72 h. These were subsequently filtered after extraction. The ethanolic filtrates were concentrated using a rotary evaporator and then dried on a water bath at 45 °C while aqueous filtrates were frozen and lyophilized.

2.5. Screening for larvicidal activity

Larvicidal assay was based on the World Health Organization Guidelines for Laboratory and Field Testing of Mosquito Larvicides with slight modifications[7]. The extracts were tested against *A. aegypti* and *A. albopictus* larvae separately. Prior to assaying, larvae were allowed to acclimatize in double distilled dechlorinated water ($dddH_2O$) for 2–3 h. Glass assay cups containing 60 mL $dddH_2O$ (for aqueuous extracts) or 1% dimethyl sulfoxide (for ethanolic extracts) were prepared as negative controls while 60 mL of 0.1 mg/L malathion was used as positive control. Ethanolic extracts were reconstituted in 1% dimethyl sulfoxide while aqueous extracts were reconstituted in $dddH_2O$, making the final concentrations of the extracts to 800 mg/L. There were 3 replicates for each treatment - positive control, extracts and negative controls. Batches of 15 third instar larvae were put into the assay cups containing 60 mL of 800 mg/L reconstituted extracts and left for 48 h. Throughout the assay, the ambient temperature was kept at (30 ± 2) ˚C. Dead larvae were counted after 24 and 48 h. Larvae were counted as dead if they cannot be induced to move when the cups were shaken, or prodded with a needle or if they are moribund. After statistical analysis using One way ANOVA with Tukey's and Dunnett's *post-hoc* tests, the extract with the significantly highest mortality was subjected to partial purification and dose-response assay.

2.6. Phytochemical screening

Phytochemical analysis was done on the active extracts that exhibited significantly high mortality. The extract was screened for the presence of flavonoids, steroids, tannins, alkaloid, anthraquinone, anthrones, coumarins and indoles via spray tests.

2.7. Statistical analysis

Corrected percent mortality was computed for both crude extracts and fractions from Abbott's formula:

$$\text{Corrected mortality (\%)} = \frac{\text{Mortality treatment - Mortality in control}}{\text{100 - Mortality in control}} \times 100$$

And percent mortality was computed using the following formula:

$$\text{Mortality (\%)} = \frac{\text{Percent survival}_{\text{control}} - \text{Percent survival}_{\text{treatment}}}{\text{Percent survival}_{\text{control}}} \times 100$$

Mean and variance were also calculated. One-way ANOVA with Dunett't *post-hoc* test was used to determine the significance of the difference between the treatments and control.

3. Results

Table 1 shows the larvicidal activities of the ethanolic and aqueous extracts against *A. aegypti*, while Table 2 shows the activities against *A. albopictus*. Of the 14 ethanolic plant extracts, only 8 plants exhibited significant larvicidal activity. Against *A. aegypti*, *M. koenigii* (53.33% after 24 h and 71.11% after 48 h), *E. globulus* (11.11% after 24 h and 62.22% after 48 h), *C. frutescens* (100.00% after 24 h and 100.00% after 48 h), *I. coccinea* (2.22% after 24 h and 42.22% after 48 h), *J. curcas* (84.44% after 24 h and 88.89% after 48 h) and *E. hirta* (22.22% after 24 h and 48.89% after 48 h) all had significantly higher percent mortality results compared to the negative control. On the other hand, against *A. albopictus*, *C. citratus* (2.22% after 24 h and 15.56% after 48 h), *G. sepium* (0.00% after 24 h and 13.33% after 48 h), *M. koenigii* (8.89% after 24 h and 40.00% after 48 h), *E. globulus* (64.44% after 24 h and 73.33% after 48 h), *J. curcas* (77.78% after 24 h and 82.22% after 48 h) and *C. frutescens* (93.33% after 24 h and 100.00% after 48 h) showed significantly higher percent mortalities compared to the negative control. The rest of the ethanolic plant extracts and all of the aqueous extracts showed no larvicidal or otherwise statistically insignificant activity at 800 mg/L.

Based on the phytochemical screening of the active extracts, most if not all, contained tannins, alkaloids, indoles and steroids (Table 3). Only the ethanolic extracts of *M. koenigii*, *E. globulus* and *C. frutescens* contained flavonoids, while only ethanolic extracts of *C. citratus* and *M. koenigii* contained anthrones. Only the ethanolic extracts of *E. globulus*, *J. curcas* and *C. frutescens* contained coumarins.

Table 1

Results of the larvicidal screening against *A. aegypti*.

Plant species and treatment	Exposure (h)	Ethanolic extracts			Aqueous extracts		
		Average mortality (%)	Variance (n = 3)	P value	Average mortality (%)	Variance (n = 3)	P value
Positive control (0.1 mg/L malathion)	24	100.00	0.000	0.000	100.00	0.000	0.000
	48	100.00	0.000	0.000	100.00	0.000	0.000
S. elmeri	24	0.00	0.000	0.968	0.00	0.000	0.968
	48	4.44	1.333	0.599	0.00	0.000	0.968
C. dentata	24	0.00	0.000	0.968	0.00	0.000	0.968
	48	0.00	0.000	0.968	0.00	0.000	0.968
E. sinnatum	24	0.00	0.000	0.968	0.00	0.000	0.968
	48	0.00	0.000	0.968	0.00	0.000	0.968
C. capitulata	24	2.22	0.333	0.671	0.00	0.000	0.968
	48	2.22	0.333	0.852	0.00	0.000	0.968
C. citratus	24	0.00	0.000	0.968	2.22	0.333	0.671
	48	4.44	1.333	0.599	2.22	0.333	0.852
P. guajava	24	0.00	0.000	0.968	0.00	0.000	0.968
	48	4.44	1.333	0.599	0.00	0.000	0.968
G. sepium	24	0.00	0.000	0.968	0.00	0.000	0.968
	48	6.67	1.000	0.301	0.00	0.000	0.968
M. koenigii	24	53.33	1.000	0.000	2.22	0.333	0.671
	48	71.11	4.333	0.000	2.22	0.333	0.852
J. curcas	24	84.44	0.333	0.000	0.00	0.000	0.968
	48	88.89	0.333	0.000	0.00	0.000	0.968
E. globulus	24	11.11	0.333	0.000	0.00	0.000	0.968
	48	62.22	0.333	0.000	0.00	0.333	0.968
C. frutescens	24	100.00	0.000	0.000	0.00	0.000	0.968
	48	100.00	0.000	0.000	0.00	0.000	0.968
I. coccinea	24	2.22	0.333	0.671	0.00	0.000	0.968
	48	42.22	0.333	0.000	0.00	0.000	0.968
E. hirta	24	22.22	0.333	0.000	2.22	0.333	0.671
	48	48.89	0.333	0.000	2.22	0.333	0.852
A. *speciosa*	24	0.00	0.000	0.599	2.22	0.333	0.671
	48	4.44	0.333	0.968	2.22	0.333	0.852

P-values were taken from Dunnett's *post-hoc* test, comparing the extracts against the negative controls at $\alpha = 0.05$. $P < 0.05$ implies statistically significant average percent mortality compared to the negative control.

Table 2

Results of the larvicidal screening against *A. albopictus*.

Plant species and treatments	Exposure (h)	Ethanolic extracts			Aqueous extracts		
		Average mortality (%)	Variance (n = 3)	P value	Average mortality (%)	Variance (n = 3)	P value
Poitive control (0.1 mg/L malathion)	24	100.00	0.000	0.000	100.00	0.000	0.000
	48	100.00	0.000	0.000	100.00	0.000	0.000
S. elmeri	24	0.00	0.000	0.968	0.00	0.000	0.968
	48	0.00	0.000	0.968	0.00	0.000	0.968
C. dentata	24	0.00	0.000	0.968	0.00	0.000	0.968
	48	0.00	0.000	0.968	0.00	0.000	0.968
E. sinnatum	24	0.00	0.000	0.968	0.00	0.000	0.968
	48	0.00	0.000	0.968	0.00	0.000	0.968
C. capitulata	24	0.00	0.000	0.968	0.00	0.000	0.968
	48	0.00	0.000	0.968	0.00	0.000	0.968
C. citratus	24	2.22	0.333	0.705	2.22	0.333	0.705
	48	15.56	0.333	0.000	2.22	0.333	0.627
P. guajava	24	0.00	0.000	0.968	0.00	0.000	0.968
	48	4.44	0.333	0.130	0.00	0.000	0.968
G. sepium	24	0.00	0.000	0.968	0.00	0.000	0.968
	48	13.33	0.000	0.000	0.00	0.000	0.968
M. koenigii	24	8.89	2.333	0.002	0.00	0.000	0.968
	48	40.00	1.000	0.000	0.00	0.000	0.968
J. curcas	24	77.78	0.333	0.000	0.00	0.000	0.968
	48	82.22	0.333	0.000	0.00	0.000	0.968
E. globulus	24	64.44	0.333	0.000	0.00	0.000	0.968
	48	73.33	1.000	0.000	0.00	0.000	0.968
C. frutescens	24	93.33	1.000	0.000	0.00	0.000	0.968
	48	100.00	0.000	0.000	0.00	0.000	0.968
I. coccinea	24	4.44	0.333	0.235	0.00	0.000	0.968
	48	4.44	0.333	0.130	0.00	0.000	0.968
E. hirta	24	0.00	0.000	0.968	0.00	0.000	0.968
	48	0.00	0.000	0.968	0.00	0.000	0.968
A. speciosa	24	0.00	0.000	0.968	0.00	0.000	0.968
	48	0.00	0.000	0.968	0.00	0.000	0.968

P-values were taken from Dunnett's *post-hoc* test, comparing the extracts against the negative controls at $\alpha = 0.05$. *P*-value < 0.05 implies statistically significant average percent mortality compared to the negative control.

Table 3
Phytochemical screening summary.

Plant extract	Flavonoid	Tannin	Alkaloid	Anthraquinone	Anthrone	Coumarin	Indole	Steroid
E. hirta ethanolic	-	-	-	-	-	-	-	+
C. citratus ethanolic	-	+++	++	-	+	-	+	+
I. coccinea ethanolic	-	+++	++	-	-	-	++	++
G. sepium ethanolic	-	+++	++	-	-	-	++	++
M. koenigii ethanolic	+	+++	++	-	+	-	++	++
E. globulus ethanolic	+	+	++	-	-	+	++	+
J. curcas ethanolic	-	++	-	-	-	+	-	++
C. frutescens ethanolic	+	+++	+	-	-	+	++	++

-: No reaction; +: Weak intensity reaction; ++: Intense reaction; +++: Strong intensity reaction.

4. Discussion

Only *C. frutescens*, *J. curcas*, *M. koenigii*, *E. globulus*, *I. coccinea*, *C. citratus*, *E. hirta* and *G. sepium* ethanolic extracts exhibited significant larvicidal activity against either *A. aegypti* or *A. albopictus*, with *C. frutescens* having the highest activity against both. The rest of the ethanolic plant extracts showed no larvicidal or otherwise statistically insignificant activity at 800 mg/L. This is probably because the plant in question had no previous studies demonstrating any larvicidal or insecticidal activity, *e.g. A. speciosa, C. dentata, C. capitulata, E. sinnatum*, and *S. elmeri*, or that the larvicidal activity was only observed when other solvent systems are used.

Whereas most of these active extracts showed larvicidal activity after 24 h. *G. sepium* and *I. coccinea* ehtanolic extracts showed delayed action, killing larvae only after 48 h. The two mosquito species also exhibitied different susceptibilities to the plant extracts, but generally, *A. albopictus* showed higher resistance to the plant extracts compared to *A. aegypti*.

These plants have been previously shown to possess larvicidal activities, albeit against different species or that different solvent was used. The larvicidal and pupicidal activity of *E. hirta* against 1st, 2nd, 3rd and 4th instar larvae and pupae of *An. stephensi* have been shown by Panneerselvam *et al.*[8]. When used alone, the LC_{50} values were 137.40 mg/L against 1st instar, 172.65 mg/L against 2nd instar, 217.81 mg/L against 3rd instar, 269.37 mg/L against 4th instar and 332.39 mg/L against pupae. When used in field trials, there was an 84% reduction in *An. stephensi* larval density after 72 h. *C. citratus* has also been shown to possess larvicidal activity. *C. citratus* essential oil was found to be active against *A. aegypti*, with LC_{50} values of 123.30 mg/L and 94.31 mg/L, after 24 and 48 h of exposure, respectively[9]. Also, the LC_{95} values 242.69 mg/L and 163.0 mg/L, after 24 and 48 h of exposure, respectively, were also determined. On the other hand, a study by Suryawanshi *et al.* showed that *I. coccinea* leaf and flower extracts are effective against *A. aegypti* (LC_{50} = 150.7 mg/mL for leaf and 139.7 mg/mL for flower) and *An. stephensi* (LC_{50} = 160.3 mg/mL for leaf and 218.9 mg/mL for flower)[10]. *G. sepium* have also been shown to possess larvicidal activity against other mosquito species. Krishnappa *et al.* studied the larvicidal, ovicidal and pupicidal activities of ethyl acetate and ethanol extracts of the dried leaves against *An. stephensi*[11]. The ethanol extract showed significant larvicidal activity with LC_{50} = 121.79 mg/L and LC_{90} = 231.98 mg/L. When pupae were treated with 25 mg/L extract, 58.10% emerged as adults. Hatchability was also tested; at 100 mg/L, 0% of the eggs hatched. Tennyson *et al.* screened 25 plant extracts against *C. quinquefasciatus* and found that at 1 000 mg/L, the hexane extract of *M. koenigii* exhibited maximum percent mortality after 24 h exposure[12]. Larvicidal carbazole alkaloids, namely, mahanimbine, girinimbine, murrayacine, murrayanine, murrayafoline A and 3-methylcarbazole, have also been isolated from *M. koenigii*[13]. These have larvicidal activities against *A. aegypti*, with LC_{50} values less than 3 μg/mL. For *E. globulus*, its essential oil have been tested against *A. aegypti*; at 0.1 mg/mL, the oil showed > 80% larval mortality against *A. aegypti*[14]. Additionally, another study tested *E. globulus* essential oil against 3rd instar *A. aegypti* larvae, and found out that at 1 000 mg/L, 20% mortality was observed[15]. *J. curcas* is another plant with larvicidal activity. *J. curcas* hexane, chloroform, ethyl acetate and methanol leaf extracts were shown to be effective against 3rd instar *C. quinquefasciatus* larvae, with LC_{50} values of 230.32 mg/L, 212.85 mg/L, 92.07 mg/L and 113.23 mg/L, respectively[16]. Field trials show 46.78%, 71.7% and 89.9% reduction in larvae density after 24, 48 and 72 h of exposure. The larvicidal activity of *C. frutescens* leaf and fruit methanol extracts against second and third instar *A. aegypti* larvae have been demonstrated by Vinayaka *et al.*[17]. At 100 mg/mL, the percent moralities were 92% and 99% after 24 h of the leaf and fruit extracts, respectively, against second instar larvae and 99% and 99.3% after 48 h of the leaf and fruit extracts, respectively. On the other hand, at 100 mg/mL, the percent mortalities were 41.3% and 91.6% after 24 h of the leaf and fruit extracts, respectively, against third instar larvae and 66% and 99.6% after 48 h of the leaf and fruit extracts, respectively.

Based on the results of the phytochemicals screening, most - if not all - plants contained tannins, alkaloids, indoles and steroids. Alkaloids, tannins and indoles have been shown to possess toxic activities against insects; hence, their presence in the extracts can be the cause of their larvicidal activity[18-21].

In conclusion, the study screened 28 plant extracts including 14 ethanolic and 14 aqueous and of these 28, 8 ethanolic extracts had

significant activity against *A. aegypti* and *A. albopictus*. The most active of these was *C. frutescens* which exhibited 100% mortality after 24 h against both mosquito species.

Conflict of interest statement

We declare that we have no conflict of interest.

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