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Bioactivity of the aqueous and ethanolic extracts/pellet form of Philippine *Piper nigrum* L. on the duration of egg, larval and pupal development stages of *Aedes aegypti* mosquitoes

Annabelle V Briones and Alice G Garbo

Abstract
Aqueous and ethanolic extracts from Philippine *Piper nigrum* L. and its pelletized form were prepared and determined its effects on the duration of egg, larval and pupal development stages of *Aedes aegypti* mosquitoes. Exposure of 3rd and 4th instars to the aqueous extract at 24 hours gave an LC_{50} of 129.6 mg/L. The LC_{50} of pelletized form of *Piper nigrum* L. is 137.4 mg/L while the ethanolic extract is 19.7 mg/L. Toxicity in egg hatchability and pupae showed the ethanolic extract is the most toxic at 25 mg/L. The ethanolic extract has the highest larval reduction than the aqueous extract and the pelletized form. The development of 1st instar to pupae was prolonged up to 17 days for the extracts and pelletized form as compared to the control which was only 7 days. The ethanolic extract has the lowest emergence of adult *Aedes* mosquitoes as compared to the aqueous and pelletized form and is the most toxic in the inhibition of adult emergence.

Keywords: *Aedes aegypti, Piper nigrum* L., pupae, aqueous extract, ethanolic extract, larvicide

1. Introduction
Aedes aegypti is the primary vector of the dengue virus which infects hundreds of people every year in the Philippines causing dengue fever and the potentially fatal dengue hemorrhagic fever (DHF). In the absence of specific treatment or effective immunization the prevention and control of dengue must rely primarily on the reduction of the population of *Aedes aegypti.* The objective of this study is to determine and compare the biological activity of aqueous and ethanolic extracts as well as the pellet form of Philippine *Piper nigrum* L. on the duration of egg, larval and pupal development stages of *Aedes* mosquito.
Previous studies conducted by Briones *et al*., (2012) [1] showed that Philippine *Piper nigrum* L showed larvicidal property against the 3rd instar *Aedes* mosquito larvae. Larvicidal assay of the aqueous solution showed 100% mortality using 2000 ppm and 98% mortality with 1000 ppm, after forty eight hours exposure to the solution. Briones *et al.,* (2013) [2] field tested the developed plant-based larvicidal solution and pellet in Marikina and Quezon City, Philippines. The field test showed 55% attraction (positive response) of ovitraps to mosquito with larvicidal solution while 53% attraction (positive response) was observed using the larvicidal pellet in both areas. More number of eggs counted in the test solution with more positive counts than the negative control. The larval mortality in both cities was also determined. Larvicidal solution showed around 85% average mortality and about 15% average adult emergence. Larvicidal pellet showed an average larval mortality of 86.2% while average adult emergence is about 13.75%.
Piper nigrum L. is locally cultivated in the Philippines. It is a woody vine with dried, unripe fruit commonly called as black pepper and is used as seasoning [3]. The insecticidal activity of extracts of Philippine ground black pepper was tested against 4 species of Coleoptera, 3 of Lepidoptera and 1 each of Hemiptera and Diptera [4]. Results showed that both crude and semi-purified extracts, applied topically, were more toxic than malathion to larvae of Plutella xylostella and adults of *Musca domestica* [4]. The semi-purified extract was generally more toxic than the crude one, and its toxicity was greatest to *M. domestica* and *Dysdercus cingulatus* and least to *Rhyzopertha dominica* and *Tribolium castaneum* [4]. A lot of studies were also done demonstrating the potential of *P. nigrum* extracts as larvicide and insecticide [5-10].

2. Materials and methods

2.1 Preparation of aqueous extract from *P. nigrum* seeds.

Larvicidal solution was prepared by grinding the dried seeds of *P. nigrum*. The resulting powder was soaked in water overnight at various concentration (300 ppm to 3000 ppm) followed by filtration. The filtrate was set aside for test and evaluation.

2.2 Preparation of larvicide in pellet form

Larvicidal admixture in pellet form was prepared by grinding the seeds of peppercorn then mixed with 1% previously cooked starch solution at a ratio of 1:1. The mixture was extruded and formed into pellets using a granulator followed by drying.

2.3 Preparation of alcoholic extract

The alcoholic extract was prepared by macerating the dried ground seeds of peppercorn with ethyl alcohol overnight and continued soaking with alcohol until no color was observed in the solvent during maceration. The mixture was filtered and concentrated in a rotary evaporator. The resulting product produced a syrupy sticky mass.

2.4 Mosquito rearing

The colony of *Aedes* mosquito was maintained in an insectary. Periodic blood meals were provided to female mosquitoes for egg maturation by keeping restrained albino rats in the cages. The eggs were collected in a container lined with Whatman No. 1 filter paper and were allowed to hatch in trays filled with de-chlorinated water. Larvae was fed upon a mixture of yeast powder and ground dog biscuits. The pupae formed was collected and transferred to the cloth cages for adult emergence.

2.5 Larvicidal Assay

Different concentrations (300ppm, 500ppm, 1000ppm & 2000ppm) of aqueous and ethanolic extracts including pellet test solutions were prepared during the study. Water was used as the negative control. The positive control used was the commercially available larvicide (Abate Larvicide). About 25 mL of each solution was placed in a well labeled 250mL beaker. Twenty five (25) healthy 3rd and 4th instar larvae were placed into each solution. Mortality was observed between 24-48 hours. Each test was replicated six (6) times.

2.6 Ovicidal Activity

About twenty five healthy eggs of *Aedes* mosquitoes were collected and placed into 300ppm, 500ppm, 1000ppm, 2000 ppm concentrations of both aqueous test solution, ethanolic extract and pellet solution. Ten replicates were done for this activity. Hatchability and mortality of eggs were observed after 24 hours exposure to the solutions then continued observation for five days. Water was used as the negative control.

2.7 Pupal Assay

Twenty five healthy pupae of *Aedes* mosquitoes were collected and placed into 300ppm, 500ppm, 1000ppm, 2000 ppm concentrations of both aqueous test solution, ethanolic extract and pellet solution. Ten replicates were done for this activity. Mortality was observed between 24-48 hours. Each test was replicated six (6) times.

2.8 Lethal dose concentration

The LC_{50} and LC_{90} values and upper, lower confidence limit and chi-square test were based according to probit methods of Finney [11]. Initially, the mosquito larvae were exposed to a wide range of test concentrations and a control to find out the activity range of the materials under test. After determining the mortality of larvae in this wide range of concentrations, a narrower range (of 4–5 concentrations, yielding between 10% and 95% mortality in 24 h or 48 h) was used to determine LC_{50} and LC_{90} values. Mortality (%) was calculated according to Abbott's formula [12]:

$$\text{Mortality (\%)} = \frac{X - Y}{X} 100,$$

Where X = percentage survival in the untreated control and
Y = percentage survival in the treated sample.

2.9 Effects on growth and development of *Aedes* Mosquitoes

The effects of the different extracts of *P. nigrum*, that were found to be effective as larvicides, were studied as follows:

2.10 Immature stages mortality and adult emergence

Each cup from a larvicidal test, that was still holding living larvae after a 48-hour period, was placed into a separate mosquito cage. All larvae and emergent adults were fed daily. Larval, pupal and adult mortality as well as adult female and male emergence rates were determined.

2.11 Egg and larval number reduction

The method was based on the studies made by Promsiri, *et al.* 2006 [13]. A determination was made of the extracts impact on fertility and of fecundity in emerging adults. After blood feeding and mating, females were isolated in another cage and allowed to lay eggs. The eggs were collected, counted, and recorded daily until all females died, the eggs then being allowed to hatch after being dried for a 3-day period. The number of viable larvae was recorded at the fourth instar stage. The larval reduction in the F1 generation due to exposure to medicinal plant extract was calculated using the following formula by Thangam & Kathiresan, 1992 [14]:

Larval reduction (%) = [(A-B/A)] × 100%,

A = Average number of larvae that hatched per female per cage in the control. (The average was calculated in relation to the total number of mosquitoes tested).

B = Average number of larvae hatched per female per cage for mosquitoes treated with a respective plant extract.

2.12 Growth retardation and prolongation of development

Groups of 25 of the first, second, third and fourth instar larvae of *A. aegypti* were each exposed to LC_{50} doses of the different extracts of *P. nigrum.* There was no fixed duration of exposure. It was sustained from the onset of egg hatching until larval death or else of successful adult emergence. The larvae were fed with ground mouse feed for the duration of their exposure. Data on growth and survival percentage were collected and the Harley index, 1967 [14] was used for comparing the relevant effects of the various extracts, the Harley formula [15] being:

Mean index = Percentage of individuals pupation + Percentage of individuals reaching adulthood/median day of pupation.

2.13 Data Analysis

Analysis of data for the bioassay was based on the WHO Guidelines for Laboratory and Field Testing of Larvicides, 2005 [16]. Data from all replicates were pooled for analysis. LC_{50} and LC_{90} values were calculated from a log dosage–probit mortality regression line using computer software programs, or estimated using log–probit paper. Bioassays were repeated at least three times, using new solutions or suspensions and different batches of larvae each time. Standard deviation or confidence intervals of the means of LC_{50} values were calculated and recorded on a form (Table.1). A test series was valid if the relative standard deviation (or coefficient of variation) was less than 25% or if confidence limits of LC_{50} overlap (significant level at $P < 0.05$). The potency of the chemical against the larvae of a particular vector and strain were then compared with the LC_{50} or LC_{90} values of other insecticides.

Table 1: Laboratory evaluation of the efficacy of larvicides against mosquito larvae (blank form)

Experiment No: _____ Investigator: _____ Location: _____ Treatment date: _____
Material: _____ Formulation: _____ Temp: _____ Lighting: _____
Species: _____ Larval instar: _____ Larvae/cup or vessel: _____
Water: Tap/Distilled Volume of water: _____ ml Food: _____ Date stock solution made: _____

		No of dead larvae at various conc. (mg/L) post exposure (hr.)													
		24 hr							48 hr						
Date	**Replicate**	**0.00**							**0.00**						
	1														
	2														
	3														
	4														
	5														
	6														
	7														
	8														
	9														
	10														
	11														
	12														
	Total														
	Average														
	% Mortality														
LC50 (CL 95%): _____ LC90 (CL 95%): _____ LC99: _____ Slope: _____ Heterogeneity: _____									LC50 (CL 95%): _____ LC90 (CL 95%): _____ LC99: _____ Slope: _____ Heterogeneity: _____						

Analysis of data for immature stages mortality and adult emergence from all replicates of each concentration were combined. Total or mean emergence inhibition was calculated on the basis of the number of third stage larvae exposed. The overall emergence of adults reflects activity. IE% was calculated using the following formula [17]:

$$IE\ (\%) = \left\{100 - \frac{T \times 100}{C}\right\}$$

Where T = percentage survival or emergence in treated batches and C = percentage survival or emergence in the control.

If adult emergence in the control was less than 80%, the test was discarded and repeated. Where the percentage is between 80% and 95%, the data were corrected using Abbott's formula [12]. IE values obtained at each concentration were be subjected to probit regression analysis to determine IE_{50} and IE_{90} values (using computer software programs or estimated from log–probit paper). Data were entered on a form (Table 2).

Table 2: Laboratory evaluation of the efficacy of insect growth regulators against mosquito larvae (blank form)

Experiment No: _____Investigator: _____Location: _____ Treatment date: _____
Material:_____ Formulation:_____ Sampling technique: _____
Species: _____ Larval instar: _____ No. of larvae released/exposed: _____ Setting date: _____
Cumulative number of dead / alive mosquitoes after treatment (date or days pre or post treatment or setting) L=larvae, P=pupae, A=adults

Date:																														Grand Total					
Conc. (mg/)		Alive			Dead			Alive			Dead			Alive			Dead			Alive			Dead			Alive			Dead			Alive			Dead		
	Rep	L	P	A	L	P	A	L	P	A	L	P	A	L	P	A	L	P	A	L	P	A	L	P	A	L	P	A	L	P	A	L	P	A	L	P	A
0.0	1																																		
	2																																		
	3																																		
	4																																		
	Total																																		
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T5	1																																		
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	3																																		
	4																																		
	Total																																		
	Mean																																		

3. Results

The results of the efficacy of the different extracts prepared from *Piper nigrum* L. as larvicides against *Aedes* mosquito larvae is shown in Tables 3. At 24 hours exposure of 3rd and 4th instars to the aqueous extract, the LC_{50} was 129.6 mg/L and 337 mg/L for the LC_{90}. After another 24 hours exposure for a total of 48 hours, to the aqueous extracts, the results showed an LC_{50} of 125.5 mg/L and LC_{90} of 329.5 mg/L. The efficacy of the pelletized form of *Piper nigrum* L. as larvicide at 24 hours in 3rd and 4th instars of *Aedes* mosquitoes is 137.4 mg/L or the LC_{50} and 411.4 mg/L for the LC_{90}. It decreases to 128.74 mg/L for the LC_{50} and 377.4 mg/L for the LC_{90} after another 24 hours exposure. Among the three extracts from *Piper nigrum* L., the ethanolic extract is the most toxic. After 24 hours exposure, the LC_{50} is only 19.7 mg/L while the LC_{90} is 31.6 mg/L. The LC_{50} decreases to 19.3 mg/L and LC_{90} to 31.3 mg/L after 48 hours exposure (refer to Table 3). The regression equation for all the extracts is also reflected in Table 3.

Table 3: Summary of the toxicity of the different pepper extracts in 3rd and 4th instars of *Aedes aegypti* mosquitoes.

	24 hours			48 hours		
	LC_{50} (95%CL), mg/L	**LC_{90} mg/L**	**Regression Equation**	**LC_{50} (95% CL), mg/L**	**LC_{90} mg/L**	**Regression Equation**
Ethanolic Extract	19.7 (18.0 – 21.4)	31.6	Y=6.23x-3.06	19.3 (18.1 -20.4)	31.3	Y= 6.07x-2.80
Aqueous Extract	129.6 (124.8 -133.8)	337.0	Y= 3.07x-1.48	125.5 (121.4 -129.6)	329.5	Y=3.07x-1.45
Pellet Form	137.4 (132.5 -142.3)	411.4	Y=2.67x-0.7	128.74 (123.9 – 133.6)	377.4	Y=2.67x-0.60
Abate 1SG	-	-	-	0.025	0.070	Y=2.88x + 9.62
Sumilarv	-	-	-	0.096	0.62	Y= 1.58x +6.61

Results of the toxicity of the pepper-based extracts and the pelletized form on the egg hatchability of *Aedes aegypti* are shown in Table 4. Again the ethanolic extract was the most toxic than the aqueous extract and the pelletized form.

Table 4: Toxicity of *P. nigrum* extracts/ pellet form on the eggs hatchability of *Aedes aegypti* mosquitoes

	LC_{50} (95% CL)	LC_{90}	Regression Equation
Ethanolic Extract	24.8 (21.5-28.1) ~25 mg/L	52.0 mg/L	Y= 3.96x-0.52
Aqueous Extract	3234.9 (3233.4-3236.4)~ 3235 mg/L	4734.3 mg/L	Y= 7.76x-22.24
Pellet Form	3396.7 (3395 -3398.1) ~ 3397 mg/L	4882.5 mg/L	Y= 8.19x-23.93
Abate 1SG	0.90 mg/L	2.11 mg/L	Y=1.96x + 5.08
Sumilarv	1.648 mg/L	3.66 mg/L	Y= 3.69x +4.20

The pepper-based extracts and pelletized form were investigated its toxicity to the pupae of *Aedes aegypti* mosquitoes. The results are tabulated in Table 5 and the ethanolic extract was the most toxic.

Table 5: Toxicity of *P. nigrum* Extracts/pellet form on the pupae of *Aedes aegypti* mosquitoes

	LC_{50} (95% CL)	LC_{90}	Regression Equation
Ethanolic Extract	3546.1(3542.7-3549.5) mg/L	7,129.8 ~ 7,130 mg/L	Y= 4.22x-9.98
Aqueous Extract	6677.5 (6674.5 – 6680) mg/L	10,508.4 mg/L	Y= 6.5x- 19.86
Pellet Form	6918.3 (6916.1-6920.5 mg/L	11,550.7 ~ 11,551 mg/L	Y=5.75x-17.08
Abate 1SG	0.08 mg/L	0.43 mg/L	Y=1.70x + 6.88
Sumilarv	0.16 mg/L	1.33 mg/L	Y=1.38x + 6.11

The results of the test for the effects of the pepper extracts and pelletized form on the immature stages and adult emergence expressed as egg and larval reduction is shown in Table 6. The ethanolic extract has the highest larval reduction than the aqueous extract and the pelletized form of pepper.

Table 6: Effects of *P. nigrum* on immature stages and adult emergence

	Conc. mg/L	Total Number of survival in all replicates within 48 h	Number of adult emergence			Ave. number of eggs per female	Ave. number of hatched eggs per female	Larval Reduction (%)
			Adult	Male	Female			
Ethanolic	20	50	41	16	25	33	30	27
Aqueous	150	33	27	12	15	39	36	12
Pellet form	150	31	27	11	16	41	37	10
Control	-	80	76	29	47	43	41	0
Abate 1SG	1	0	0	0	0	0	0	0
Sumilarv0.5G	1	0	0	0	0	0	0	0

The relevant effects of the various extracts of pepper and its pelletized form on the duration of larval stages, pupal development and adult emergence is shown in Table 7. The mean index in percentage represents the percentage of individual pupation plus percentage of individuals reaching adulthood divided by the median day of pupation using the Harley index, 1967 [15]. All extracts and pelletized form has lower mean indices as compared the control (water alone). As observed in the results, development of 1st instar to pupae was prolonged up to 17 days for the extracts and pelletized form as compared to the control which is only 7 days.

Table 7: Effects of *P. nigrum* extracts on the duration of larval stages, pupal development and adult emergence

	Conc. mg/L	Larval instar	*Mortality ± SD within 24-48h (%)	*Pupation ± SD (%)	*Adult Emergence ± SD (%)	Larval to pupal duration (days)	Median day Pupation (days)	Mean Index (%)	Inhibition of Emergence (%)
Ethanol Extract	20	1st instar	74 ± 2.5	9 ± 6	8 ± 5	25	17	1	92
		2nd instar	59± 5	38 ± 6	35 ± 4	23	16	5	61
		3rd instar	49 ± 5	40 ± 4	40 ± 4	15	12	7	60
		4th instar	41 ± 5	55 ± 6	55 ± 6	9	8	14	59
Aqueous Extract	130	1st instar	68 ± 5	9 ± 6	9 ± 6	24	15	1	90
		2nd instar	55 ± 4	35 ± 7	34 ± 5	19	16	4	63
		3rd instar	49 ± 4	36 ± 7	35 ± 6	16	12	6	64
		4th instar	42 ± 3	45 ± 6	44 ± 8	9	8	11	56
Pellet Form	130	1st instar	68 ± 5	9 ± 2	9 ± 2	22	14	1	90
		2nd instar	54 ± 2	38 ± 3	36 ± 2	19	15	5	60
		3rd instar	46 ± 2	35 ± 4	35 ± 4	15	12	5	64
		4th instar	39 ± 2	38 ± 3	38 ± 3	9	8	10	62
Control (Water alone)	0	1st instar	0	90 ± 4	90 ± 4	12	7	26	
		2nd instar	0	92 ± 3	91 ± 2	10	6	30	
		3rd instar	0	98 ± 3	98 ± 3	10	6	33	
		4th instar	0	100	100	8	6	33	
Sumilarv 0.5G	0.1	1st instar	100	0	0	-	-	-	100
		2nd instar	84±11	12.5±5	5±0	14	7	3	95
		3rd instar	55.8±7.1	20±4.1	8.8±2.5	12	6	5	91
		4th instar	51.25±4.8	15±2.5	10±0	1	6	4	90

*mean % data rounded off to nearest whole number

The overall emergence of adult *Aedes aegypti* mosquitoes after exposure to different pepper-based extracts and pelletized form is shown in Figure 1. The ethanolic extract has the lowest emergence of adult *Aedes* mosquitoes as compared to the aqueous and pelletized form and is the most toxic in the inhibition of adult emergence (see Table 8).

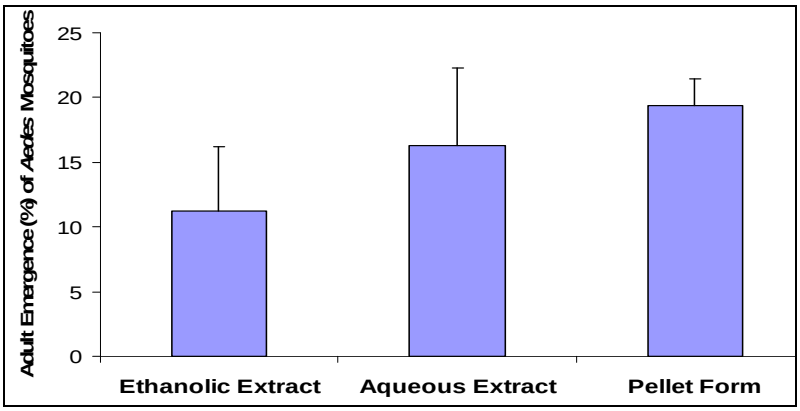

Fig 1: Overall emergence of *Aedes aegypti* mosquitoes in *P. nigrum* extracts/pellet

Table 8: Lethal concentration (LC) of the extracts for 50% and 90% inhibition of adult emergence (IE50 and IE90)

	IE50 (95% CL)	IE90 (95%CL)	Regression Equation
Ethanolic Extract	18.5 mg/L(16.3-20.7)	32.9	Y= 5.12x-1.49
Aqueous Extract	125.4 mg/L (120.6-130.2)	340.6	Y=2.95x-1.19
Pellet Form	132.7 mg/L (128.1-137.3)	344.5	Y+3.09x-1.56

4. Discussion

The extracts of *Piper nigrum* L. are effective larvicides and retards growth and development of *Aedes* mosquitoes. Among the extracts, the ethanolic extract showed the lowest % overall emergence (IE) of adult *Aedes* mosquitoes as compared to the aqueous and pelletized form. It is the most toxic among the larvicides since using ethanol in preparing the crude extract may obtain most of the bioactive compounds present in the fruit than by water. Santiago VS *et al.* 2015 [18] have reported that *P. nigrum* ethanolic extracts exhibited potential larvicidal activity to 3rd to 4th *A. aegypti* instar larvae of which they identified oleic acid as the active compound in one of the ethanolic fractions prepared. Oleic acid is soluble in ethanol than in water thus making the ethanolic extract more toxic than the aqueous extract. Further, Park *et al*. 2002 [19] have studied the biologically active constituents of *P. nigrum* fruits that have larvicidal activity against *A. aegypti*. They were able to characterized the compounds as the isobutylamide alkaloids such as pellitorine, guineensine, pipercide, and retrofractamide a of which these are mostly soluble in ethanol-water mixtures.

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