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Inhibition of the growth and development of mosquito larvae of *Culex quinquefasciatus* (Diptera: Culicidae) treated with extract from leaves of *Pseudocalymma alliaceum* (Bignonaceae)

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ABSTRACT

Objective: To determine larvicidal activity of the essential oil, hydrolat and botanical extracts derived from leaves of *Pseudocalymma alliaceum* on mosquito larvae of *Culex quinquefasciatus*. **Methods:** Groups of twenty larvae were used in the larvicidal assays. The mortality, relative growth rate, the larval and pupal duration and viability was estimated. The essential oil was analyzed by solid phase microextraction using gas chromatography coupled to mass spectrometry. **Results:** Essential oil at 800 ppm showed larvicidal activity at 24 h with lethal values of LC_{50} and LC_{90} of 267.33 and 493.63 ppm. The hydrolat at 20% and 10% on 2nd stage larvae showed 100% effectiveness after 24 h. The aqueous extract at 10% had a relative growth index of 0.58, while the ethanolic and methanolic extract obtained values of 0.76 and 0.70 and control reached 0.99. Larvae treated with 10% of methanol, ethanol and aqueous extract showed a reduction in larval duration of 5.00, 2.20 and 4.35 days; ethanol extract at 1% provoke decrease of 2.40 days in the development and exhibited an increment of 3.30 days when treated with 0.01%. Aqueous, ethanol and methanol extracts at 10% reduced in 6.15, 3.42 and 5.57 days pupal development. The main compounds were diallyl disulfide (50.05%), diallyl sulfide (11.77%) and trisulfide di-2-propenyl (10.37%). **Conclusions:** The study demonstrated for the first time, the larvicidal activity of the essential oil and hydrolat of *Pseudocalymma alliaceum*; aqueous, ethanol and methanol extracts inhibited the normal growth and development of mosquito larvae, prolonging and delaying larval and pupal duration.

1. Introduction

Mosquitoes are vectors of disease agents that significantly affect millions of people worldwide[1]. Culex (Diptera: Culicidae) is a genus that is present in tropical and subtropical regions. Some species of this genus like *Culex quinquefasciatus* (*Cx. quinquefasciatus*) (Say) are carriers of *Wuchereria bancrofti*, a nematode parasite wich causes human lymphatic filariasis[2], *Plasmodium relictum* Grassi & Feletti that causes avian malaria and myxomatosis[3,4]. Filariasis is a global public health problem, 120 million people are currently infected by diseases transmitted by mosquitoes and about 1.3 billion are at risk of infection[5]. The traditional way to control this mosquito has been with synthetic insecticides. However, its irrational use has created resistance to the control methods used[6,7], as well as water , air and soil pollution, accumulation of toxic waste and poisoning of users[8]. *Cx. quinquefasciatus* is resistant to a wide range of insecticides[9,10], which limits the choice of chemicals that might be used for its control. These consequences have contributed to find environmentally friendly alternatives, such as plant's extracts and essential oils of ethnopharmacological and ethnobotanical use[11]. *Pseudocalymma alliaceum* (*P. alliaceum*) Sandwith (Bignoniaceae) known by synonyms as *Adenocalymma*

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alliaceum Miers, *Mansoa alliacea* (Lam.) *A. Gentry*, *Bignonia alliacea* Lam, *Pachyptera alliacea* (Lam.) A. H. Gentry and *Pachyptera hymenaea* (DC.) is a woody vine commonly known as "garlic vine" in the region of the Isthmus, Oaxaca, Mexico, because when its leaves are crushed, they release a strong aroma similar to garlic. The Bignoniaceae family includes about 120 genders and 800 species, growing mainly in Africa, Central and South America. This species has been studied to determine their chemical composition and biological activity on various organisms, in addition to its economic importance as a food substitute. The leaves and flowers are widely consumed and used in traditional medicine in South America, including Brazil and Peru[12]. The curative properties attributed are analgesic, anti-arthritic, anti-inflammatory, antipyretic, antirheumatic, depurative, purgative and vermifuge[13-15], also the dried leaves are used to treat colds, pneumonia, strep throat and respiratory disorders[16], nausea and constipation[17]. Based on the above, this paper search aimed to determine the biological activity of the aqueous, ethanol and methanol extracts, as the effectiveness of the essential oil and hydrolat from *P. alliaceum* on mosquito larvae of *Cx. quinquefasciatus*, and in turn determine the chemical compounds present in the volatile essential oil.

2. Materials and methods

2.1. Collection of eggs and maintenance of larvae

The egg rafts were collected from standing water in the facilities of the Interdisciplinary Research Centre for Regional Integral Development (CIIDIR-OAX) located in Santa Cruz Xoxocotlán, Oaxaca, Mexico (17° 01′ 41.08″ N, 96° 43′ 19.35″ W), the rafts were taken to the laboratory and placed, individually, in plastic trays of 47 cm ×35 cm × 12 cm containing 300 mL of softened water. Mosquito larvae were fed with a sprayed product used to feed fish (Api-barge), until they reached the pupal stage.

2.2. Maintenance of pupae and adults

The pupae were transferred to containers of 30 cm ×20 cm ×6 cm and placed in entomological cages of 60 cm × 60 cm × 60 cm for adult emergence. Adults were provided with 10% sugar solution in a jar with a cotton wick and an immobilized hen was introduced weekly for one night for the female's nourishment. The mass rearing temperatures were kept at (27±2) ℃, 60%-70% relative humidity and a 12:12 photoperiod[18].

2.3. Preparation of plant material

Fresh leaves of *P. alliaceum* were collected in San Pedro Comitancillo (16° 29′ 20.67″ N, 95° 09′ 15.70″ W), from the Isthmus region of Oaxaca, Mexico. *P. alliaceum* was selected for its use in other ethno pharmacological and ethno botanical studies, which reported a distinctive aroma, bitterness and resistance to damage by insect pests in the collection sites. Taxonomic identification was made by the curator of the Herbarium of CIIDIR-OAX and a sample copy was deposited in their research laboratory for future reference. The plant was washed with tap water, then placed on sheets of newspaper to dry them for 16-20 days, then pulverized using a mechanical mill to obtain a powder which was subsequently hydrated[19,20].

2.4. Extraction of essential oil and hydrolat

Essential oil (EO) was obtained by subjecting 500 g of dried plant material to microwave assisted hydrodistillation for 3 h using a Clevenger-type apparatus. A microwave with a frequency output of 2450 MHz was used. The EO layer was separated from the aqueous phase using a separatory funnel and the resulting essential oil was dried using anhydrous sodium sulfate Na_2SO_4 and preserved in an amber colored bottle at 4 ℃ until used. The hydrolat obtained was stored to be evaluated later vs mosquito larvae in several bioassays.

2.5. GC-MS and identification of volatile compounds

The identification of volatile compounds was performed by solid phase microextraction (SPME) in the Posgraduate College, Campus Montecillo, Mexico; the SPME device for manual sampling was obtained from Agilent Technologies (USA). Fibers coated with polydimethylsiloxane (PDMS) of 100 μm were used. The fibers were conditioned before use by heating them in the chromatographic injector port performing a complete run on the system. Whenever necessary the conditioning step was repeated for the cleaning of the fiber. For contacting the volatiles with the fiber, 5 mL capacity amber colored sterilized jars were used. Four μL of essential oil were deposited into the jars and kept inside the fiber by 4 min; subsequently the insertion of the needle of the device in the injector port of the chromatographic system was done. The analysis was performed on a HP 6890-5973 GC-MS system. The compounds were separated on a capillary column HP-5MS. The oven temperature was programmed from 40-250 ℃ with increasing intervals of 10 ℃/min and a total duration of 21 min. The relative percentages of the essential oil compounds were obtained using helium as a carrier gas with a flow rate of 1.0 mL/min. The compounds were identified by GC retention time and mass spectra library NIST 02; also, a comparison of the spectra with those stored in the library Wiley 275 GC/MS and NIST 11.0 was made.

2.6. Preparation of the essential oil and hydrolat concentrations

From the stock solution of essential oil, 0.008 mL were taken and diluted in 10 mL distilled water with 0.01% polysorbate 20 which served as an emulsifier to the solution, thereby obtaining the concentration of 0.08% (800 ppm), and subsequently by volumetric dilution series concentrations of 400, 200, 100, 50 and 25 ppm were prepared and used for a bioassay on early 4th instar mosquito larvae. To prepare the different concentrations of hydrolat, 4 mL were taken from the stored material, and diluted in 10 mL of distilled water to obtain a concentration of 40.00%; from this concentration 20.00%, 10.50%, 2.50%, 1.25% and 0.65% solutions were prepared. To determine the mortality effect on early 2nd instar larvae, concentrations of 20.00% to 0.65% were used, while for 4th instar larvae concentrations of 40.00% to 1.25% were used.

2.7. Preparation of crude extracts

For preparing the crude extracts 100 g of the dried-ground plant were added to 300 mL of solvent contained in a flask and allowed to stand for 24 h for aqueous extraction and 72 h for less polar solvents; the solid was separated from the liquid using filter paper and the remainder was discarded. The solvent was removed with reduced pressure on a rotary evaporator, obtaining the crude extract for each solvent. The crude extract was stored in amber colored bottles at 8 ℃, 1 g of crude extract was taken and diluted in 10 mL of distilled water with 0.01% of polysorbate 20, then centrifuged for 10 min and filtered using tricot loom to avoid lumps, obtaining a 10% solution, which through volumetric dilution series produced treatments of 1.00%, 0.10% and 0.01% for the growth inhibition bioassay.

2.8. Larvicidal activity

The mortality effect on early 2nd and 4th instar larvae of *Cx. quinquefasciatus* was determined for three consecutive days after application of the treatments[21]. For the establishment of bioassays, groups of 20 larvae were selected and placed in a plastic beaker with 99 mL of distilled water and 1 mL of treatments. When the larvae had not normal movements compared to the control, the larvae were considered to be dead; as well as when the larva was disturbed with a brush in the siphon in the cervical region and didn't show any reaction. Each treatment was replicated four times.

2.9. Growth Inhibition

Early 2nd instar larvae were used. The experimental unit consisted of a plastic beaker of 125 mL capacity with 99 mL of distilled water and 20 larvae. Each experimental unit received 1 mL of the concentrations used with four replications. Concentrations were evaluated at 10.00%, 1.00%, 0.10% and 0.01%. Each bioassay included a control treatment with 0.01% of polysorbate 20 and one without application of any treatment. When the control treatments presented between 90%-93% of formed pupae, dead and alive pupae were counted in each stage and the number of adults emerged. Adult was considered dead identifying those who were trapped in the pupal exuviae and dead larvae and pupae those that didn't show normal movements when disturbed with a dissecting needle[22]. With the information gathered, a growth inhibition index (GII) was estimated[23].

$$GII = \frac{(\sum_1^4 \text{No.alive insects} \times (\text{insect phase}) + \sum_1^4 [\text{No. dead insects} \times (\text{insect phase} - 1)]}{(\text{No. total evaluated insects} \times \text{total insects phases})}$$

where 1, 2, 3 and 4 are 2nd, 3rd, 4th instar and pupae of the insect formed respectevily. The number of insects used per concentration was 80, the total number of stages of the insect were four (three larval and pupae). The relative growth rate (RGI) was determined by RGI = GII treatment / GII control without application.

2.10. Larval and pupal viability and durability

The larval and pupal duration was obtained by multiplying the percentage of pupae and adults developed by the number of days in that developed in each replicate, these values are summed and the total is divided by the total percentage of pupae and adults developed. The larval and pupal viability was estimated by counting the number of individuals who came to pupae and adult and expressed in percentage according to the initial number of individuals treated.

2.11. Statistical analysis

Bioassays were established under a completely randomized design. An analysis of variance and Duncan mean comparison ($P<0.01$) was performed. To compare the larvicide potency of treatments and the susceptibility of the larvae, the LC_{50}, LC_{90} and confidence intervals were calculated by Probit analysis using SAS 9.0. In all tests, no control mortality was detected after exposure, so no correction was required based on Abbott's formula.

3. Results

3.1. Identification of volatile compounds

The microwave assisted hydrodistillation of 500 g of

powdered dried leaves of *P. alliaceum*en in 1 000 mL of water, showed dark brown oil with a strong aroma of garlic with 0.44% yield. Volatile compounds are shown in Table 1. By comparing the mass spectra of each compound with the data reported in the library Wiley 275 GC/MS and NIST 11, 26 compounds were identified representing 99.98%. The most abundant compounds were diallyldisulfide (50.05%), diallyl sulfide (11.77%), trisulfide, di-2-propenyl (10.37%) and 1-Octen-3-ol (4.89%). The remaining compounds represented between 2.27% and 0.21%.

Table 1
Chemical composition of volatile extracted from leaves of *P. alliaceum*.

Peak#	Retention time	Compound	Composition (%)
1	3.00	2-Butenal, 2-methyl-	0.66
2	4.32	1,5-Cyclooctadiene, 3,4-dimethyl	1.42
3	4.54	Diallyl sulfide	11.77
4	5.39	Catecholborane	1.89
5	6.11	3-Chloropropionic acid, 2-phenylet	1.71
6	6.41	1-Octen-3-ol	4.89
7	6.54	1-Ethynylcyclopentanol	0.56
8	6.63	3-Octanol	2.27
9	7.11	Pyridine, 5-ethyl-2-methyl-	0.99
10	7.29	1,3-Dithiolane, 2,2-dimethyl-	1.22
11	7.60	γ -Terpinene	0.62
12	8.29	Diallyl disulphide	50.05
13	8.39	Crotonic acid, 4-mercapto-3-(methy	1.46
14	8.45	1-Oxa-4,6-diazacyclooctane-5-thione	2.01
15	8.98	(Methylthio)-acetonitrile	1.14
16	9.74	Thiophene, 2,4-dimethyl-	1.80
17	9.96	Hydrazinecarbodithioic acid, 1-met	0.35
18	10.10	3-Vinyl-1,2-dithiacyclohex-5-ene	1.35
19	10.78	Isobutyl isothiocyanate	0.29
20	11.14	Safrole	0.44
21	11.39	Trisulfide, di-2-propenyl	10.37
22	12.38	5,6-Diamino-1,3-dimethyluracil	0.41
23	14.52	Tetrasulfide, di-2-propenyl	1.52
24	17.01	5-Ethylthiazole	0.31
25	17.30	2-Mercapto-4,5-dimethylthiazole	0.21
26	17.64	Propanedioic acid, methyl-, bis(trimethylsilyl) ester	0.27
Total (%)			99.98

3.2. Larvicidal activity

Table 2 shows the results of susceptibility of 4th instar larvae of *Cx. quinquefasciatus* to the essential oil of *P. alliaceum*; concentrations of 800 to 25 ppm were evaluated, showing at 24 h post treatment lethal values of LC_{50} and LC_{90} of 267.33 and 493.63 ppm respectively. Toxic effects were observed on the larvae treated with concentrations from 800 to 100 ppm, 800 ppm concentration showed more than 80% effectiveness before the first registration day, causing 100% mortality at 24 h. At 48 h the concentration of 400 ppm caused over 92% mortality with LC_{50} and LC_{90} of 201.90 and 385.29 ppm respectively. On the third day the 200 ppm concentration treatment showed effectiveness above 50%.

Table 2
Larvicidal activity for three consecutive days of the essential oil of *P. alliaceum* on 4th instar larvae of *Cx. quinquefasciatus*.

Concentration (ppm)	Mortality (%)		
	24 h	48 h	72 h
800	100.00±0.00^a	100.00±0.00^a	100.00±0.00^a
400	73.75±13.14^b	92.50±8.66^b	100.00±0.00^a
200	35.00±4.08^c	43.75±4.78^c	58.75±12.50^b
100	0.00±0.00^d	11.25±2.50^d	28.75±4.78^c
50	0.00±0.00^d	0.00±0.00^e	5.00±0.00^d
25	0.00±0.00^d	0.00±0.00^e	0.00±0.00^d
Polysorbate 20	0.00±0.00^d	0.00±0.00^e	0.00±0.00^d
Control	0.00±0.00^d	0.00±0.00^e	0.00±0.00^d
LC_{50}	267.33	201.90	146.69
(LCL-UCL)	(243.08 – 293.89)	(183.17 – 222.73)	(132.12 – 163.10)
LC_{90}	493.63	385.29	312.22
(LCL-UCL)	(434.04 – 586.04)	(337.29 – 458.72)	(269.61 – 377.43)

**Data for columns with different letters are significantly different P <0.01. LC: Lethal concentration, LCL: Limit minimum fiducial; ULC: Limit maximum fiducial.

The subproduct obtained from the hydrodistillation of *P. alliaceum* was evaluated on 2nd instar larvae at doses of 2.00% to 0.65% (Table 3), and at 40.00% to 1.25% on the 4th instar larvae (Table 4). Concentrations of 20% and 10% on 2nd stage larvae showed 100% effectiveness at 24 h post treatment. The treatment applied to 5% concentration showed more than 50% toxicity on the third day of registration. In the assessment of early 4th instar larvae, it was obtained nule pupae formation at concentrations of 40% and 20%, registering 100% mortality at 24 h and 72 h respectively. The 5% concentration decreases their effectiveness on 4th instar larvae by 22.5% compared to the 2nd stage. Lethal concentrations were estimated for fourth instar larvae with LC_{50} and LC_{90} values for 24 and 72 h of 10.20%, 15.60% and 7.51%, 11.47% respectively.

Table 3
Larvicidal activity for three consecutive days of hydrolat of *P. alliaceum* on 2nd instar larvae of *Cx. quinquefasciatus*

Concentration (%)	Mortality (%)		
	24 h	48 h	72h
20	100.00±0.00^a	100.00±0.00^a	100.00±0.00^a
10	100.00±0.00^a	100.00±0.00^a	100.00±0.00^a
5.0	38.75±7.50^b	47.50±6.45^b	50.00±7.07^b
2.5	6.25±2.50^c	6.25±2.50^c	6.25±2.50^c
1.25	0.00±0.00^d	0.00±0.00^d	0.00±0.00^d
0.65	0.00±0.00^d	0.00±0.00^d	0.00±0.00^d
Control	0.00±0.00^d	0.00±0.00^d	0.00±0.00^d

**Data by columns with different letters are significantly different P <0.01.

3.3. Growth Inhibition

Plant extracts inhibited larval growth of *Cx. quinquefasciatus*

when applied at the concentrations used, the treatment of 10% aqueous extract showed a 0.58 relative growth index RGI, while the ethanol and methanol extract was obtained 0.76 and 0.70 respectively; the control witness recorded 0.99 of RGI. Treatments at concentrations of 1.00%, 0.10% and 0.01% showed a reduction in larval growth compared with the control witness (Table 5). When formed between 90% and 93% of pupae in the control without application, the number of dead and alive larvae and pupae were counted, the larvae of *Cx. quinquefasciatus* were more susceptible to the aqueous and ethanol extracts compared with the methanol extract. Table 5 shows the significance between the concentrations employed. The aqueous extract of dried leaves of *P. alliaceum* recorded 73.75% mortality and methanol extract 46.25% when 10% concentrations was used. However the ethanol extract at 1% showed higher biological effectiveness with 46.25% compared with 35.00% of aqueous extract and extract elaborated with methanol produced 37.50%. All concentrations and plant extracts used showed toxic effect.

3.4. Larval and pupal viability and durability

The viability of 2nd instar larvae of mosquito treated with a 10% of aqueous extract showed a 28.75% reduction in the formation of larvae, which represents a decrease of 30% when compared with the control treatment, which showed a 96.25%. In the study, with the decrease of concentration treatments larval viability increased for all extracts, the

Table 4

Larvicidal activity for three consecutive days and the percentage of formed pupae of 4th instar larvae of *Cx. quinquefasciatus* treated with hydrolat of *P. alliaceum*.

Concentration (%)	Mortality (%)			(%) Pupae Formed
	24 h	48 h	72h	
40	100.00±0.00 [a]	100.00±0.00 [a]	100.00±0.00 [a]	–
20	96.25±4.78 [a]	98.75±2.50[a]	100.00±0.00 [a]	–
10	52.5±10.40[b]	68.75±8.53[b]	80.00±7.07[b]	5.00
5.0	0.00±0.00[c]	0.00±0.00[c]	11.25±2.50[c]	38.75
2.5	0.00±0.00[c]	0.00±0.00[c]	0.00±0.00[d]	52.50
1.25	0.00±0.00[c]	0.00±0.00[c]	0.00±0.00[d]	53.75
Control	0.00±0.00[c]	0.00±0.00[c]	0.00±0.00[d]	43.75
LC50	10.20	9.05	7.51	
(LCL–UCL)	(9.41 – 11.05)	(8.37 – 9.71)	(6.94 – 8.13)	–
LC90	15.60	12.77	11.47	
(LCL–UCL)	(14.08 – 18.03)	(11.68 – 14.61)	(10.35 – 13.21)	–

**Data by columns with different letters are significantly different P <0.01. LC: Lethal concentration, LCL: Limit minimum fiducial; UCL: Limit maximum fiducial.

Table 5

Percentage of larvae and pupae dead and RGI of 2nd instar larvae of *Cx. quinquefasciatus* treated with extracts of *P. alliaceum*.

Treatments	Concentration (%)	Mortality (%) by instar					RGI
		2do	3ro	4to	Pupae	Total	
Methanol	10.0	5.00	6.25	32.50	2.50	46.25[a]	0.76±0.03[c]
	1.0	3.75	5.00	25.00	3.75	37.50[ab]	0.83±0.07[bc]
	0.1	5.00	7.50	23.75	2.50	38.75[ab]	0.79±0.06[bc]
	0.01	1.25	6.25	18.75	8.75	35.00[b]	0.85±0.08[b]
	Polysorbate 20	0.00	0.00	1.25	1.25	2.50[c]	1.02±0.00[a]
	Control	0.00	0.00	1.25	2.50	3.75[c]	0.99±0.04[a]
Ethanol	10.0	0.00	5.00	46.25	21.25	72.50[a]	0.70±0.08[b]
	1.0	1.25	5.00	40.00	0.00	46.25[b]	0.77±0.05[b]
	0.1	1.25	20.00	21.25	0.00	42.50[b]	0.73±0.16[b]
	0.01	1.25	8.75	30.00	0.00	40.00[b]	0.76±0.11[b]
	Polysorbate 20	0.00	0.00	1.25	1.25	2.50[c]	1.02±0.00[a]
	Control	0.00	0.00	1.25	2.50	3.75[c]	0.99±0.04[a]
Aqueous	10.0	10.00	18.75	42.50	2.50	73.75[a]	0.58±0.07[c]
	1.0	2.50	3.75	25.00	3.75	35.00[c]	0.83±0.10[b]
	0.1	2.50	2.50	22.50	2.50	30.00[c]	0.87±0.04[b]
	0.01	1.25	3.75	28.75	16.25	50.00[b]	0.79±0.07[b]
	Polysorbate 20	0.00	0.00	1.25	1.25	2.50[d]	1.02±0.00[a]
	Control	0.00	0.00	1.25	2.50	3.75[d]	0.99±0.04[a]

**Data for treatment with different letters are significantly different P<0.01.

methanol and ethanol extracts showed 48.75% and 56.25% larval formation by employing 10% concentrations. The viability in the pupal stage showed statistically significant differences when employing a 10% ethanol extract which caused a14.10% inhibition in the mosquitoe development. The control treatment without application showed a larval duration of 16.25 days. Larvae treated with 10% of methanol, ethanol and aqueous extract showed a reduction in larval duration of 5.00, 2.20 and 4.35 days, respectively. The methanol extract at concentrations of 1.00% to 0.01% exhibit no significant reduction in larval duration.

Larvae treated with 1% ethanol extract showed a decrease in the development of 2.40 days, and there is a prolongation of 3.30 days when treated with 0.01%. The aqueous extract prolongs 0.85 and 2.47 days the larval duration when using concentrations at 0.10% and 0.01% respectively and it reduces in 1.18 days when treated with 1.00%. The polysorbate 20 used as an emulsifier prolongs in 0.87 days larval development without statistical significance when compared to the untreated application. Similar results were obtained to the pupal duration by applying 10% treatments reducing 6.15, 3.42 and 5.57 days the mosquitoe development when treated with aqueous, ethanol and methanol extract respectively. The control witness recorded a pupal duration of 19.17 days. Pupae treated with ethanol and aqueous extract showed a prolongation in the durability of 3.73 and 2.33 days when treated with the concentration of 0.01% respectively (Table 6).

4. Discussion

The crude extracts and essential oils of plant species have a complex mix of chemical elements; these secondary metabolites have been used empirically in vector control and causal agents of deseases. This study agree with results of previous studies with nonpolar solvents in demonstrating the larvicidal activity of plant extracts of *P. alliaceum* on *Cx. quinquefasciatus*, obviously this plant species is a rich source of natural products with potential for control of this mosquito species, but further studies are required to assess the toxicity of this species to determine the risks to human health before its use is generalized. It was demonstrated that the essential oil extracted from dry leaves of *P. alliaceum* can be a natural larvicide of *Cx. quinquefasciatus* and it is well known that essential oils have been a rich source of insecticides[24,25]. The mean value for control of 4th instar larvae (LC_{50}) of essential oil was estimated 24 hours post treatment at 267.33 ppm (243.08-293.89). The effectiveness of the subproduct obtanined during the extraction of the essential oil of *P. alliaceum* was demonstrated using concentrations of 20% and 10% on 2nd larval instar and 4th instar larvae when treated with the 40% hydrolat presenting 100% of biological effectiveness, which allows us to consider that the effective chemical elements in the essential oil are in the subproduct and that it can be used as alternative for the control of this mosquito species. The prolongation of larval duration when applying aqueous and ethanol extract of *P. alliaceum* shows the effect on the normal development of the insect; similar effects were reported by Sagar and Sehgal[26] when using acetone extract of *Azadirachta indica* on mosquitoe larvae and pupae of *Culex pipiens* and *Aedes aegypti*. Singh[27] used a methanol extract of neem seed where he observed an increased in the duration of the larval instar of *Cx. quinquefasciatus*. Also, Ndung'u *et al*[28] observed growth inhibition effects after exposure of *Anopheles gambiae* Giles larvae to root bark extracts of five *Meliaceae* species.

Table 6
Durationand larvaland pupal formationand total mortality of larvae of *Cx. quinquefasciatus* treated with plant extracts of *P. alliaceum*.

Tretaments	Concentration (%)	Larval (Larvae to pupae)		Pupal (Pupae toadult)	
		Formed (%)	Duration (d)	Formed (%)	Duration (d)
Methanol	10	56.25^{c}	11.25^{b}	23.30^{a}	13.02^{b}
	1	66.25^{bc}	16.65^{a}	23.58^{a}	18.77^{a}
	0.1	63.75^{bc}	15.77^{a}	24.02^{a}	17.75^{a}
	0.01	73.75^{b}	15.20^{a}	21.61^{a}	17.15^{ab}
	Polysorbate 20	96.25^{a}	17.12^{a}	24.68^{a}	20.10^{a}
	Control	96.25^{a}	16.25^{a}	23.05^{a}	19.17^{a}
Ethanol	10	48.75^{b}	14.05^{b}	14.10^{b}	15.75^{b}
	1	51.25^{b}	13.85^{b}	25.00^{a}	15.52^{b}
	0.1	53.75^{b}	15.92^{ab}	24.42^{a}	17.97^{ab}
	0.01	58.75^{b}	19.55^{a}	24.47^{a}	22.90^{a}
	Polysorbate 20	96.25^{a}	17.12^{ab}	24.68^{a}	20.10^{ab}
	Control	96.25^{a}	16.25^{ab}	23.05^{ab}	19.17^{ab}
Aqueous	10	28.75^{c}	11.90^{c}	22.82^{a}	13.60^{c}
	1	68.75^{b}	15.07^{b}	23.63^{a}	17.05^{bc}
	0.1	72.50^{b}	17.10^{ab}	24.13^{a}	19.70^{ab}
	0.01	66.25^{b}	18.72^{a}	18.86^{a}	21.50^{a}
	Polysorbate 20	96.25^{a}	17.12^{ab}	24.68^{a}	20.10^{ab}
	Control	96.25^{a}	16.25^{ab}	23.05^{a}	19.17^{ab}

**Data for treatment with different lettersare significantly different P <0.01.

The regulatory effect on insect growth is attributed to compounds that mimic juvenile hormone in arthropods[29] delaying or prolonging their development or causing malformations that lead to death of the insect. In this paper, the larvicidal effect of polar extracts of water and ethanol was apparent, although there are reports of effectiveness with other less polar extracts such as those by Shrankhla *et al*[30] in which lethal concentrations were determined obtaining values of 2.49, 15.06 and 1.16, 8.45 ppm of LC_{50} and LC_{90} at 24 and 48 h post treatment respectively when they evaluated the hexane extract of *P. alliaceum* on larvae of *Cx. quinquefasciatus*. A LC_{50} of 8.70 ppm for larvae of *Anopheles stephensi* (Liston) was reported by Shrankhla *et al*[31]. In the present study it was observed that the essential oil and methanol extract of *P. alliaceum* caused a dark brown necrotic formation on the abdomen of the larvae treated which killed the insect and the adults were trapped in the exuviae of pupae. Tabassum *et al*[32] observed that plant extracts affect the morphology of *Cx. quinquefasciatus* larvae causing pigmentation and alterations in the abdomen and head. Murty *et al*[33] reported that adults of *Cx. quinquefasciatus* emerged deformed from pupae getting caught in the outer shell of the insect, and also have reported inhibition of adult emergence when treated with a leaf extract of *Polyalthia longifolia* Sonn.

However, the effectiveness of the essential oil and hydrolat of *P. alliaceum* and the polar effect of plant extracts on the growth and development of larvae of this mosquito has not been fully investigated. In the literature no records were found of the growth inhibitory effect of plant extracts and larvicidal activity of essential oil and hydrolat of *P. alliaceum* presented in this study, therefore those results represent the first report of this kind on mosquito larvae of *Cx. quinquefasciatus*. Larval stage extension is reported with exposure of the larvae of *Anopheles stephensi* to sub-lethal dose of neem[34,35]. Shaalan *et al*[36] said that secondary metabolites of many plant species show effects on growth and development in various life stages of mosquitoes, causing a wide range of effects such as delays and extensions of larval and pupal development, molting inhibition, morphological abnormalities and mortality; especially during the molting process and melanization. The chemical composition of this plant species has been studied by Verma *et al*[37] which analyzed volatile compounds from leaves through gas chromatography coupled to mass spectrometry (GC-MS) and determined that the main compounds are diallyl disulfide (65.9%) and diallyl trisulfido (29.6%), allicin-derived organosulfur compounds that give the characteristic aroma of garlic. Rao *et al*[38] analyzed the essential oil of leaves through GC-MS and determined the presence of diallyl trisulfide (44%), diallyl disulfide (37%), I-octen-3-ol (5%) and diallyl tetrasulfide (4%). Volatile compounds in this study were identified by SPME. The strong garlic aroma emanating from the leaves of *P. alliaceum* is caused by naphthaquinones derived from lapachol and secondary compounds as allyl disulfide, alliin, allicin or diallyl sulfide among the most representative, some naphthaquinones may be cytotoxic, this explains the insecticidal properties of these compounds[39,40]. Das Graças *et al*[41] mentioned the presence of allyl polysulfide in the essential oil of this species. Itokawa *et al*[42] report that a methanol extract of leafstalk produced cytotoxic activity against colon cancer cells. Rugkeart *et al*[43] reported antioxidant and antimicrobial activityes with extracts of petroleum ether and ethanol. Rattanachaikunsopon and Phumkhachorn[44] documented that diallyl sulfide was able to inhibit the growth of food-borne bacterial strains.

In conclusion, the study demostrated, for the first time, the larvicidal activity of the essential oil and hydrolat of *P. alliaceum* against mosquitoe larvae of *Cx. quinquefasciatus*; the aqueous, ethanol and methanol extracts of leaf inhibited the normal growth and development of the insect prolonging and delaying larval and pupal duration. The chemical analysis showed that the major constituents of the essential oil from dried leaves were diallyl disulfide (50.05%) and diallyl sulfide (11.77%). These results demonstrate the potential of this plant species to be included as an alternative to control for *Cx. quinquefasciatus*.

Conflict of interest statement

We declare that we have no conflict of interest.

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