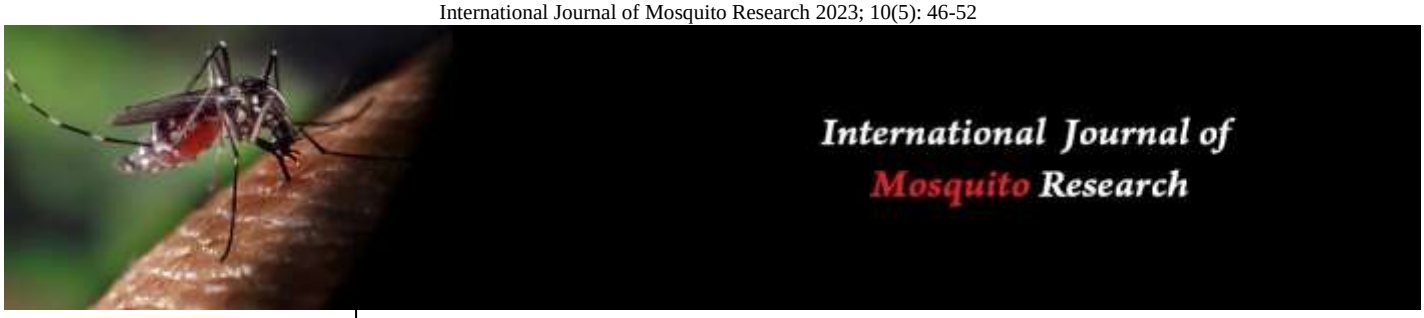

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Effect of the ethanolic extract of *Pachygone ovata* (Poir.) leaf extract on the viability of the egg and larva of *Aedes aegypti* (Diptera: Culicidae) in the laboratory conditions

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Abstract
Numerous plant species are utilised as valuable biological resources because active ingredients are inside them, which have been effectively exploited as insecticides. *Pachygone ovata* (Poir.), a plant species, exhibited characteristics of being predominantly non-aggressive and environmentally benign. However, its capacity to effectively regulate insect populations, namely mosquitoes, remained little explored. Dengue fever is an infectious disease caused by the dengue virus, which is predominantly transmitted by mosquito bites from the Aedes genus, with *Aedes aegypti* being the primary vector. This study examined the effect of an ethanolic extract derived from the leaves of *Pachygone ovata* on the survival rates of *Aedes* (Stegomyia) *aegypti* (Diptera: Culicidae) eggs and larvae in the laboratory of Acharya Nagarjuna University. *Aedes aegypti* is the arthropod vector that transmits the viruses that cause dengue fever, Zika virus, and Chikungunya fever. The vector has proved resistant to organophosphate chemicals used to combat the disease globally. Eggs and fourth-stage larvae of *Aedes aegypti* were exposed to three distinct concentrations of an ethanolic extract derived from the plant of *P. ovata* (C_1 = 0.0017 g/ml, C_2 = 0.0034g/ml, and C_3 = 0.0051 g/ml) in a bioassay. The egg viability was greater than 70% in all three concentrations, whereas the larval mortality rate was greater than 70% in C_3.

Keywords: *Aedes aegypti*, larva**,** eggs, mortality**,** *P. ovata* leaf extract, biological control, ethanolic extract, viability

1. Introduction
The increase in temperature due to the effect of global warming, especially in countries with tropical and subtropical climates, and with it, the presence of atmospheric phenomena such as rain, added to social problems such as sanitary deficiencies, lead to the proliferation of diseases transmitted by insects and other pests [1].
The *Aedes* (Stegomyia) *aegypti* L. 1762 (Diptera: Culicidae) mosquito remains dengue fever's main vector or carrier. The vector of dengue fever is the female *Aedes aegypti* mosquito. This mosquito has special characteristics characterised by silvery white bands or stripes on a black base. The size of the *Aedes aegypti* mosquito ranges from around 3-4 mm with white rings on its legs [2].
This *Aedes aegypti* has proven to be an important vector of various arboviruses, fulfilling its biological cycle in two environments, the aquatic in the first three stages (eggs, larvae and pupae) and the aerial in the adult stage [3]. The adult has mainly urban domestic habits, preferably daily and anthropophilic; The male feeds on the nectar of flowers and the females are hematophagous, a condition that makes it a vector of diseases such as Dengue, Zika, Chikungunya fever, and yellow fever [3-4].
The World Health Organization estimates that 2.5 billion people live in areas at risk of transmission of these diseases due to the presence of the vector and that about 50 million are infected each year, reported in 2019 in the region of the Americas. A total of 3,139,335 cases of dengue, including 1,538 deaths [5]. In India, dengue is one of the infectious pathologies with the greatest impact, being classified as one of the epidemics that is the greatest cause of

mortality in the country [6]; However, it recorded the highest rates of cases and deaths in 2017 due to multiple factors, including the coastal child phenomenon, showing an increase only in dengue cases of 55.6% between the years 2016 and 2017, with 41 reported deaths of 46,950 cases presented [7].

Currently, the Ministry of Health is adopting measures to control the proliferation of this insect, based on the elimination of potential mosquito breeding sites, in addition to intensifying surveillance and vector control campaigns in which organophore products are used and the so-called growth regulators with larvicidal action, such as Temefós 1% GR and Pyriproxyfen 0.5%, respectively. Its prolonged and excessive use generates resistance, environmental pollution, and toxic effects on wildlife and humans due to the presence of residues in water and food and unpleasant odours that are even inaccessible to the population. low-income [8].

As alternative methods for vector control, the use of biological controllers such as predators, entomopathogenic fungi and natural plant extracts have been evaluated in the laboratory and in the field, which has proven to be effective in controlling mosquito populations and preventing the development of resistance [9]. In a study carried out with the flower buds of *Syzygium aromaticum*, the larvicidal capacity was demonstrated on fourth-stage larvae of *Aedes albopictus*, reporting a lethality greater than 50% after 17 hours [10]. Other evaluations carried out under laboratory conditions of the effective insecticidal activity of ethanolic extracts of *Gliricidia sepium*, *Sapindus saponaria* and *Annona muricata* on third instar larvae of *Aedes aegypti* produced mortality in them after 1 day (24 hrs) of exposure [11].

The Andhra Pradesh region has a great diversity of plants with medicinal properties that repel and control insects. There are also introduced climbing plants such as the *P. ovata* (*Pachygone ovata* (Poir.)), a very well-adapted non-endemic wild plant with no special requirements for its growth. In its chemical composition, more than 20 compounds are reported, mainly terpenoid active metabolites, among them N-methylerotsparine, reticuline, reticuline N-oxide, (+)-quercitol, liriodenine, trilobine, coclaurine, nuciferine etc., Pachygone, a member of the family Menispermaceae, is a small genus of scandent shrubs which are distributed mainly in the Asian region. No insecticidal work on any of the *Pachygone* species has yet been reported [12].

The effectiveness of bioinsecticides can be increased by mixing various parts of plants that have active compounds that are toxic to pests [13]. Indian traditional medicine practitioners have successfully employed Menispermaceae species [14] to treat various medical conditions, including diabetes, edema, pain, rheumatoid arthritis, bone fracture, nephritis, pyrexia, and hypertension. The Menispermaceae plant species *Pachygone ovata* has mainly remained undiscovered and underappreciated. *P. ovata* is a genus of plants native to the southern regions of India, specifically Tamil Nadu, Karnataka, and Andhra Pradesh. The ancients recognised the medicinal value of the entire plant, from the dried fruit (which they used as fish poison and vermicide) to the leaves (which they used to lower their core body temperature and boost their fertility). Unfortunately, no supporting evidence exists for *P. ovata* purported pharmacological effects [7].

From an environmental point of view, it is a promising active material due to its rapid degradation that minimises the waste problem, its low toxicity in mammals and its benignity in most natural enemies. Given this problem and considering the search for alternative solutions that minimise these problems, this research work was carried out whose objective was to determine the effect of the ethanolic extract of Fish berry "*P. ovata* " on the viability of the egg and larvae of *Aedes aegypti* in the laboratory, as an alternative for biological control to provide a new framework of strategies in the control of this vector, contributing to the increase of experimental models in the epidemiological context of diseases.

2. Materials and Methods

2.1 Geographic location

The work was carried out in the Department of Biochemistry at Acharya Nagarjuna University from May 2020 to June 2021, as described by WHO [5]. The temperature was measured with a RH/Temp Datalogger DTR-305 sensor and fluctuated between 27±1 °C and a relative humidity of 60%, which was maintained with the help of an air conditioning system.

Origin of the biological material and establishment of the experimental *Aedes aegypti* colony larvae were provided by the Surveillance and Vector Control area of the Vijayawada Auto Nagar, Krishna District, Andhra Pradesh, India, which were collected from the vases of some cemeteries in Vijayawada in which no prior chemical treatment had been applied. These were identified using the taxonomic keys of González *et al*. (2008) [15] and were raised under laboratory conditions (temperature 25±3 °C and relative humidity 60%). The emerged adults were sexed using the taxonomic key. Subsequently, six couples were selected, whose first generations (F1) were used as a homogeneous population, from which the eggs and larvae were obtained to start the test.

2.2 Management of the biological cycle of *Aedes aegypti*

It was developed based on the methodology used by Quispe-Pretel *et al*. (2015) [16]. To do this, the six pairs of Ae were placed aegypti, each in a plastic box (30 cm x 30 cm) that acted as breeding chambers for the adults, which had a hole on one of the side faces with tulle sleeves through which the insects were fed. Inside each box, a black plastic container (12 cm x 6 cm) was placed as an ovitrap, which was covered around the edges with filter paper and filled with clean, dechlorinated water.

- **Eggs:** The ovitraps were checked every two days. Those that contained eggs were carefully removed from the filter paper and placed in a plastic tray labelled with a label containing the date and number of eggs. The trays containing eggs were divided into two groups: those that contained eggs for hatching and obtaining larvae and those used for the bioassay.
- **Larvae:** When the larvae emerged, they were counted to take into account the living space, the water in the container and the amount of food. For this, the formula used based on [17], placing 200 larvae per 750 cm^3 tray in 800 ml of dechlorinated water.
- Brewer's yeast was added as food. Water and feed were changed every two days to prevent fungal formation.
- **Pupae:** After the pupae emerged, they were placed in plastic containers covered with tulle fabric to later transfer said containers to the adult rearing chambers.
- **Adults:** Sexual dimorphism was determined from the emerged adults, and they were moved in pairs with the help of a capture tube to the breeding boxes.

Inside the boxes, for feeding males, cotton wool moistened with sugar water (5g of sugar in 100 ml of water) was placed, and the substrate was changed every two days to prevent the formation of fungi. To feed the females, they were offered human blood every two days for 15 minutes, and the hand was inserted up to arm height.

2.1 Plant material collection and authentication

The raw material for *Pachygone ovata* (Poir.) Miers ex Hook. f. & Thomson (Menispermaceae family) [18] was authenticated by the Head Department of Botany at Acharya Nagarjuna University and was obtained from Tirupati in the Eastern Ghats of Andhra Pradesh in October 2018 (Figure 1). The voucher for the herbarium is ANBH 121/2018. Before using the samples (leaf), we allowed them to dry for an entire month at room temperature in the shade.

Fig 1: Leaves of *Pachygone ovata* (Poir.) Miers ex Hook. f. & Thomson

2.2 Preparation of Plant Extracts

1000 g of *Pachygone ovata* leaves were weighed, washed with distilled water to remove adhered particles, allowed to air dry for 24 hours, and the sample was ground for ten minutes with Magic Bullet equipment. The sample was then separated into two topaz flasks with 250 ml of 96% ethanol for maceration at ambient temperature and humidity for seven days, with periodic shaking three times a day. Next, a first filtration was carried out using a cotton cloth, where larger particles were separated, and then a second filtration was carried out, this time with a vacuum system with a 0.45 µm pore membrane filter and 40 mm diameter; said filtrate was placed on a Petri dish and the alcohol was allowed to evaporate for 3 days (72 hrs). At the end of the period, a rotary evaporator at 37 ºC was used to remove the extract dissolved in distilled water from the solvent. The extract, the solvent of which was separated, was stored in dark bottles at +4 °C degrees [9].

2.3 Preparation of solutions

After the evaporation of the ethanol from the plate, the solidified extract was removed with a spatula, then the quantities of 0.340 g, 0.680 g and 1.020 g were weighed on an A&D Company analytical balance of 0.0001 g precision. These quantities were dissolved in 200 ml of dechlorinated water, obtaining the concentrations of the biological assay (Table 1).

Table 1: Solutions used during the bioassay.

	Treatment 1	**Treatment 2**	**Treatment 3**
Extract	0.340g	0.680g	1.020g
Dechlorinated water	200ml	200ml	200ml
Concentrations	0.0017g/ml	0.0034g/ml	0.0051g/ml
(C)	(C_1)	(C_2)	(C_3)

2.4 Bioassay

We worked with a total of 300 eggs. There was one Control (T) per repetition, to which no treatment was applied, and a positive control (P) to which 0.1 g of Pyriproxyfen (Pyrilarv 0.5%) was applied as a reference. The larvae and eggs were kept with their respective treatments in inadequate conditions for the cycle to develop correctly. At all times, the protocol was considered to determine the susceptibility or resistance to insecticides of mosquitoes of the *Ae. aegypti* species. To evaluate the effect of the extract on the viability of the eggs, the number of viable eggs, that is, those that hatch, was quantified [19], comparing it weekly for 4 weeks with the control group and the control group. To evaluate the effect of the extract on the viability of the larvae, the number of non-viable larvae was quantified, that is, those that did not pass to the pupal stage [19], due to cause of death, comparing it with the control group and the control group, measured every 12 (0.5 days) hours until 5 days (120 hrs).

2.5 Analysis of data

With the data obtained, a matrix belonging to the F of the adult pairs was created in the Microsoft Excel 2016 program of the insectary. Three repetitions were carried out; for each one, 100 eggs that were a maximum of seven days old were removed on the same filter paper on which they had been oviposited, which was cut into segments that grouped 20 eggs randomly. Subsequently, each group was placed in a transparent plastic container containing 200 ml of dechlorinated water. The five containers, in total, were allowed to acclimatise for one day. The next day, the respective treatments of 0.340 g (C_1), 0.680 g (C_2) and 1.020 g (C_3), Positive Control (P) and Control (T) were applied to the containers, all duly labelled with date and time.

In the case of the larvae, we worked with 300 larvae of the IV stage of *Aedes aegypti* taken directly from eggs hatched in the insectary; these were also placed in the containers one day prior to application so that they could acclimatise. Three repetitions were carried out, randomly taking 20 larvae for each of the 5 containers (C_1, C_2, C_3) Positive Control and Control), duly labelled with date and time. (Microsoft Corp, USA), and with the statistical package IBM SPSS Statistics 24, descriptive statistics were obtained.

Both the dose-response graphs for eggs and larvae and the hatching-non-hatching graphs of eggs and mortality-viability of larvae were made using the Microsoft Excel 2016 program.

Finally, two-way ANOVA was used to evaluate differences within and between treatments. In addition, Tukey's mean comparison test was used for groups with significant differences.

3 Results

3.1 Egg viability

The cumulative average egg hatching of *Aedes aegypti* for each treatment is seen in Fig. 2. C_3 had the lowest average number of eggs hatched in the first two weeks. C_2 and C3 were the concentrations in which the eggs hatched for longer.

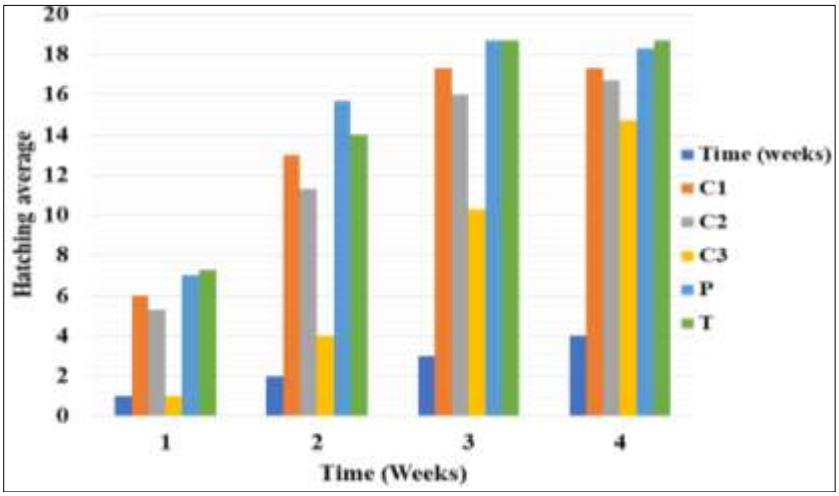

Fig 2: Cumulative average of Ae egg hatching per treatment. aegypti.

All treatments presented egg hatching percentages greater than 70%; However, within the unhatched eggs, the highest percentage was for C3 at 26.67%, followed by C_2 at 16.67% and C_1 at 13.33%, finally, the Control (Pyriproxyfen) with 8. 33% and the Witness with 6.67% (Fig. 3)

The Analysis of Variance of two factors for the hatching of the eggs was not statistically significant for the Concentrations since the significance value is greater than 0.05 (Sig.= 0.757); therefore, it was not necessary to perform Tukey's statistical test.

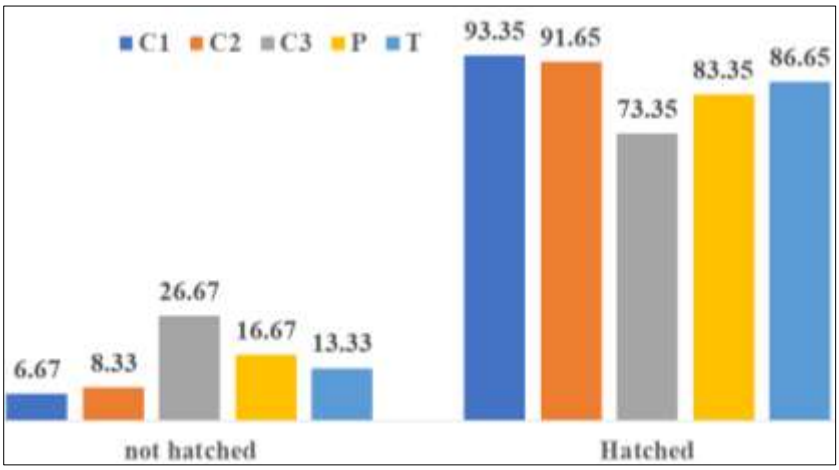

Fig 3: Percentage of hatching vs. non-hatching of Ae eggs aegypti.

3.2 Larval viability

The accumulated averages of mortality of the larvae in each treatment follow the following order: with C_1 they began to die after 48 h (0.33 larvae), with C_2 at 36 h (0.33 dead), with C3 at 24 h (6.33 deaths), while with the positive control (P) they began to die at 48 h (0.67 deaths); Furthermore, C3 generated physical damage to the morphology of the larva (Fig. 4) and presented the highest mortality rates (Fig. 5). The highest mortality percentage was obtained by C3 (73.35%), followed by the Positive control (38.35%), C_2 (36.65%), C_1 (11.65%) and finally the Control (10%) (Fig. 6). The Analysis of Variance of two factors for larval mortality (Table 2) was statistically significant (Sig.<0.005) for the variable Concentrations (Sig.= 0.000). In the Tukey statistical test, significant differences were found between C3 and the other concentrations (Sig. $\leq$ 0.002) (Table 3). An optical microscope (Leica USA) with a magnification range of 40-400x was used to observe early third and fourth instar larvae of *Aedes aegypti*. The morphological photogram of the midgut following food ingestion, as depicted in Figure 4 of this study, provides valuable insights into the impacts of larvicidal mechanisms.

Fig 4: Dead larvae of *Aedes aegypti*. A) Control, B) Positive control. C) Concentration 3.

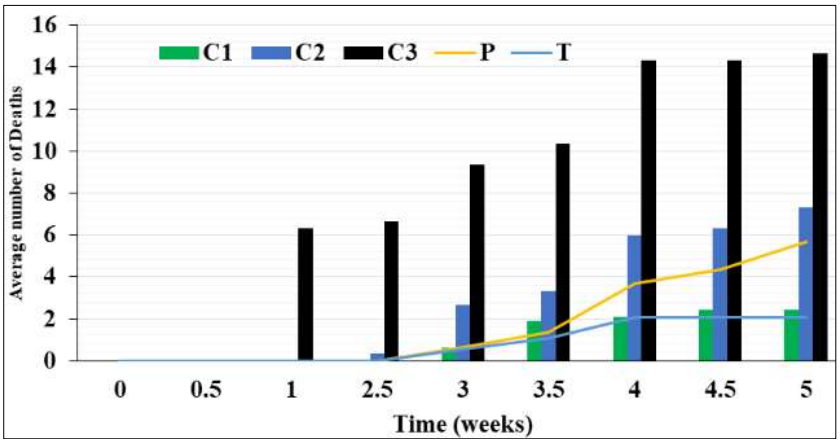

Fig 5: Cumulative average mortality of Ae larvae per treatment. Aegypti.

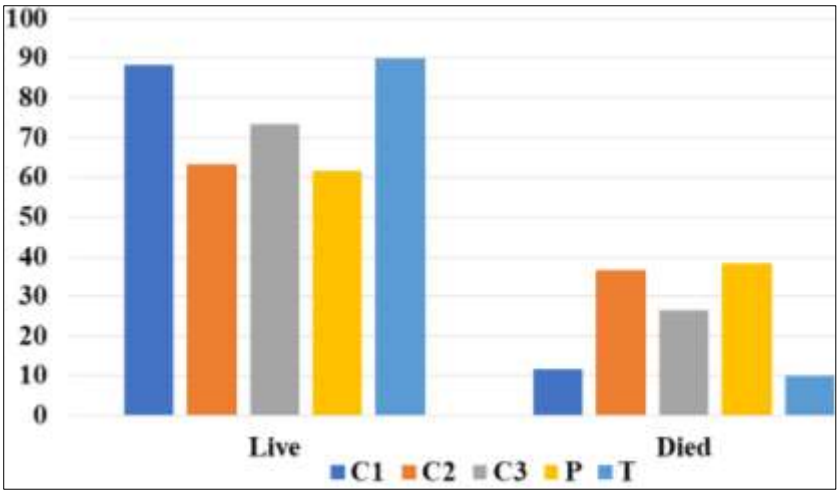

Fig 6: Percentage of mortality vs viability of *Aedes aegypti* larvae.

Table 2: Analysis of Variance for the mortality of *Aedes aegypti* larvae between treatments

Inter-subject effects tests					
Dependent variable: Number of Dead Larvae					
Source	**Type III of the sum of squares**	**Degrees of freedom**	**Quadratic mean**	**F**	**Sig.**
Corrected model	220.279[a]	55.1	4.079	9.220	0
Inter section	63.06	1.0	63.055	142.521	0
Hours	81.35	10.2	8.135	18.386	0
Concentrations	28.89	4.1	7.221	16.322	0
Hours * concentrations	110.05	40.8	2.751	6.218	0
Error	48.67	112.2	0.442		
Total	332.00	168.3			
Total corrected	268.95	167.3			
[a]R^2 =.819 (R^2 adjusted =.730)					

Table 3: Tukey statistical test comparing the mortality of *Aedes aegypti* larvae between treatments

Multiple comparisons						
Dependent variable: Number of dead larvae						
HSD Tukey						
		Mean difference (I-J)			**95% confidence interval**	
(I) Concentrations	**(J) Concentrations**		**Standard error**	**Sig.**	**Lower limit**	**Upper limit**
C_1	C_2	-.45*	0.164	0.05	-0.91	0
	C_3	-1.12*	0.164	0	-1.58	-0.67
	P	-.48*	0.164	0.03	-0.94	-0.03
	T	0.03	0.164	1000	-0.42	0.48
C_2	C_1	.45*	0.164	0.05	0	0.91
	C_3	-.67*	0.164	0.001	-1.12	-0.21
	P	-0.03	0.164	1000	-0.48	0.42
	T	.48*	0.164	0.03	0.03	0.94

C_3	C_1	1.12*	0.164	0	0.67	1.58
	C_2	.67*	0.164	0.001	0.21	1.12
	P	.64*	0.164	0.002	0.18	1.09
	T	1.15*	0.164	0	0.7	1.61
P	C_1	.48*	0.164	0.03	0.03	0.94
	C_2	0.03	0.164	1000	-0.42	0.48
	C_3	-.64*	0.164	0.002	-1.09	-0.18
	T	.52*	0.164	0.018	0.06	0.97
T	C_1	-0.03	0.164	1000	-0.48	0.42
	C_2	-.48*	0.164	0.03	-0.94	-0.03
	C_3	-1.15*	0.164	0	-1.61	-0.7
	P	-.52*	0.164	0.018	-0.97	-0.06
It is based on observed means.						
The error term is the mean square (Error) = 0.442.						
*. The mean difference is significant at the 0.05 level.						

4. Discussion

P. ovata is a species widely evaluated in different insects; its insecticidal or growth-regulating effect stands out in species of agricultural importance; its effect was evaluated and a 60% mortality in nymphs was recorded. Different products and parts of plants like neem have been shown to have wide use as a mosquito insecticide, such as the case of the repellent effect of the oil derived from the seed with 80% in *Culex quinquefasciatus* (Culicidae)[20], or the insecticidal effect generated in the immature *Aedes aegypti* (Culicidae) by the ethanolic extract. In the present work, the insecticidal effect of *P. ovata* extract on the viability of *Ae. Aegypti* larvae was verified in the laboratory.

Annabel *et al*. (2009) [21], using the ethanolic extract of the leaves of *Azadirachta indica* at concentrations of 0.05 g/ml, 0.1 g/ml, 0.15 g/ml and 0.2 g/ml, obtained percentages of mortality in larvae of *Aedes aegypti* of 35%, 60%, 70% and 85% respectively at 3 days (72 hrs). Furthermore, Jung Bong *et al* (2008) [22], using concentrations of 0.001 g/ml, 0.002 g/ml and 0.005 g/ml of ethanolic extract of few plants, observed higher mortality of the larvae (93%) at 3 days (72 hrs) in the extract at 0.005 g/ml concentration, and in the extracts at 0.001 g/ml and 0.002 g/ml 47% and 70% respectively. Although similar percentages are not achieved in control, it is because, in the work of Annabel *et al*. (2009) [21], the concentrations were much higher. However, the concentrations from Wandscheer *et al*. (2013) [23] are almost the same; Thus, 71.65% were obtained after 3 days (72 hrs) with C3 (0.005 g/ml) and 30% with C_2 (0.0034 g/ml), but dying in a shorter time, that is, between 1 day (24 hrs) and 36 hours. h respectively.

Hertlein *et al*. (2010) [24] evaluated the ovicidal and larvicidal effects of the bioinsecticide spinosad on *Aedes aegypti* and *Anopheles stephensi* at concentrations of 0.1 ppm, 5 ppm and 10 ppm for four weeks of exposure under controlled temperature conditions (25±1 ºC), obtaining intermediate ovicidal effect only for Anopheles, in addition to 100% mortality of larvae of both species in the three concentrations; demonstrating that its potential lies in its larvicidal effect rather than in its ovicidal activity. This coincides with the authors' position, with the contrast that *P. ovata* extract does not act as an ovicide but rather presents a slight delay in the hatching time of *Aedes aegypti* concerning the control, although not statistically significant.

The serious cuticle of culicid eggs is composed of chitin and is associated with resistance to desiccation and impermeability [18-19]. We conclude that given the same lipid nature of *P. ovata*, it could be slightly filtered through the cuticle of the *Aedes aegypti* of our experiment, showing consequences in the course of its subsequent evolutionary stages such as those mentioned by Troy (1975) [25], that the natural components of *P. ovata* have a growth regulator function with insecticidal action, causing insects to delay or inhibit their feeding, interfering with the moulting processes and exuvia remains attached to the abdomen and legs, which prevents the correct development of the insect, which dies in the attempt to free itself from its moult. Furthermore, the adult females from eggs subjected to *P. ovata* treatment could not feed on blood since they did not comply with what Canyon, Hii, & Muller (1999) [26] mention when saying that the blood-taking process of the mosquito must comply with four conditions: recognition of the host and rest on it, exploration of the area and sting, suction of blood and removal of the proboscis from the skin; causing his death.

5. Conclusions

The ethanolic extract of Fish berry "*P. ovata"* does not significantly decrease the egg viability of *Aedes aegypti*, presenting hatching percentages greater than 70%. However, hatching times were slower compared to the control. The ethanolic extract of Fish berry "*P. ovata"* significantly decreases the viability of the Ae larva aegypti, with C_3 (0.0051 g/ml) being the one that presented the highest percentage of mortality (73.35%) after 5 days (120 hrs) of exposure. The ethanolic leaf extract of *P. ovata* did not significantly reduce *Aedes aegypti* egg viability, with hatching percentages larger than 70%. However, hatching times were slower than in the control group. The ethanolic extract of the *P. ovata* considerably reduces the viability of the *Aedes aegypti* larva with C3 (0.0051 g/ml), showing the highest percentage of mortality (73.35%) after 5 days (120 hours) of exposure.

5.1 Future study

More research is needed to assess its effect on other mosquito vectors, particularly those collected in the wild, and the prospect of using *P. ovata* plant sources as a botanical pesticide at home.

6. Funding

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7. Conflict of Interest

The authors have no conflicts of interest regarding this investigation**.**

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