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Formulation of Larvicide from *Capsicum frutescens* (Linn.) Fruit Extract Against *Aedes aegypti* (L.)

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The crude ethanolic extract of *Capsicum frutescens* fruit was used in the formulation of larvicidal granules. The extract was first screened for phytochemical content and larvicidal activity against *Aedes aegypti*. Five trial formulations were developed using different combinations of excipients identified to be compatible with the active extract. These formulations were evaluated using Quality by Testing (QbT) Method (organoleptic evaluation, moisture content, powder flow property, particle size distribution, dissolution in water and larvicidal bioactivity). The *C. frutescens* extract (500 ppm) killed 100% of the test larvae after 24 and 48 h. Phytochemical analysis revealed the presence of steroids, indoles, alkaloids, flavonoids, sugars, coumarins and anthraquinones. Among the formulations tested, formulation # 5 (25% extract, 59% lactose, 1% 5% PVP-ethanol solution, and 15% microcrystalline cellulose (MCC) was the most active, with LC_{50}-24 h=108.68 ppm, LC_{90}-24 h=233.44 ppm, LC_{50}-48 h=102.68 ppm, and LC_{90}-48 h=182.50 ppm. Larvicidal granules (Formulation #5) against *A. aegypti* were developed. The granules were able to kill 100% of the test larvae after 24 and 48 h of exposure. In addition, the formulation passed all the quality control measurements (low moisture content, excellent powder flow, soluble in water, and 90% cumulative frequency within 0.420–0.841 mm).

Key Words: *Aedes aegypti*, *Capsicum frutescens*, phytochemistry, larvicide, formulation

Abbreviations: DMSO – dimethyl sulfoxide, DCM – dichloromethane, LC_{50}/LC_{90} – lethal concentration (50% or 90%), NF – National Formulary, OL – ovicidal-larvicidal, PVP – polyvinylpyrrolidone, ppm – parts per million, TLC – thin layer chromatography, USP – United States Pharmacopoiea

INTRODUCTION

Vector-borne diseases affect many parts of the world, especially the subtropical and tropical regions. Specifically, the mosquito species *Aedes aegypti* (Linn.) spreads some of the most prevalent mosquito-borne diseases in the Philippines: dengue fever, Chikungunya and zika. Dengue fever is among the top prevalent mosquito-borne diseases in the Philippines, where 15,599 cases and 74 deaths were reported from January to February 24, 2018 alone (Alvarez et al. 2016). Chikungunya is another disease spread by *A. aegypti*. Cases of chikungunya were reported in several areas of the Philippines (Pagudpud, Ilocos Norte and Butuan City) and Cavite City was declared under a state of calamity following an outbreak (Bonolas 2016). The Zika virus has just started to penetrate the Philippines, with the first reports in the country starting September 2016.

To combat the dangers of mosquito-borne diseases, different methods of vector control are being implemented: fumigation, adulticides and bug zappers (WHO 2009). However, these can be inefficient since they target only the adults and not the eggs nor larvae. Fumigants and adulticides can also cause vector resistance, in addition to toxicity to the environment and non-target organisms (WHO 2005). It is for these reasons that researchers turn their focus on biological pesticides. Unlike conventional pesticides, plant-derived pesticides

are composed of several chemical compounds the combined action of which can decrease the chance of pest survival and resistance development (Madhumathy et al. 2007).

Capsicum annuum Linn. (Synonym: Capsicum *frutescens* Linn.) [English name: chili pepper], locally known as siling labuyo, is a small shrub that produces bright red, hot chili fruits (Quisumbing 2009). It is used for stomach upset, intestinal gas, stomach pain, diarrhea, cramps, poor blood circulation, and high cholesterol (USPC 2012). It is also useful for its pesticidal activity against *Rhipicephalus microplus* (Vasconcelos et al. 2014), *Musca domestica* larvae (Mosquito.org 2019), *Anopheles stephensi* and *Culex quinquefasciatus* larvae, and recently, against *A. aegypti* and *A. albopictus* (WHO 2005). Thus, the aim of this study was to develop a larvicidal formulation against *A. aegypti* that makes use of *C. frutescens* as its active ingredient.

MATERIALS AND METHODS

Reagents

Ovicidal-larvicidal (OL) trap (positive control for larvicidal assay) was bought from the Department of Science and Technology, Gen. Santos Ave., Bicutan, Taguig City, Philippines. Reagents were purchased from Harnwell Chemicals Corporation, Interlab Analytics and Scientific Supplies, and Theo-Pam Trading Corporation.

Aedes aegypti Larvae

F-26 third instar *A. aegypti* larvae were provided by the Department of Entomology – Medical Entomology Laboratory, College of Public Health, University of the Philippines Manila, Philippines.

Plant Material

Approximately 20 kg of green and red *C. annuum* fruits were collected from Paraciles, Isabela, Philippines. Samples were sent to the Botany Division, National Museum of the Philippines, Manila, Philippines for authentication (CN: 16-11-1608) and safekeeping.

Plant Extraction

The *C. frutescens* fruits were air-dried under shade for 4 wk and then milled to obtain a fine powder. The powdered plant material was extracted with 95% ethanol (10 mL solvent/1 g sample) for 48 h with occasional shaking. The extract was then filtered and concentrated in vacuo (40°C) and in a water bath (45°C).

Larvicidal Activity

Prior to the assay, F-26 third instar *A. aegypti* larvae were transferred to distilled water in cups and acclimatized for 2–3 h. The water was ensured to have a depth of 5–10 cm, since deeper water levels may cause larval mortality. The larvae were then put in cups containing 100 mL each of the treatment (500 ppm crude extract in 1% DMSO), negative control (1% DMSO), and positive control (500 ppm OL trap in distilled water). Three replicates were performed for each treatment (15 larvae per trial). Throughout the assay, the temperature was kept at 26 ± 2°C and relative humidity at 60 ± 5% RH. Twenty-four (24)- and 48-h post-exposure, larvae mortality (dead and moribund) was recorded (Madhumathy et al. 2007).

Phytochemical Analysis

Standard TLC spray tests were used to screen the extract for the presence of flavonoids, alkaloids, anthraquinones, indoles, steroids, sugars and coumarins (Guevara 2005).

Preformulation

Air stability testing

Triplicates of two vials containing 200 mg of the lyophilized extract were tested for air stability. One vial was exposed to air while the other was covered with rubber stopper and dipped in molten beeswax. Both vials were stored at room temperature (27°C) for 5 wk, after which, organoleptic evaluation and TLC analysis were done.

Thermal stability testing

Stability of the lyophilized extract at cold, normal and worst-case storage conditions was assessed. Triplicates of vials containing 200 mg of the lyophilized extract were stored at 8°C, 27°C and 40°C for 5 wk after which, TLC analysis was done.

Photostability testing

Two vials containing 200 mg of the lyophilized extract were tested for photostability. One vial was exposed to light while the other was covered with aluminum foil. Both vials were stored at room temperature (27°C) for 6 wk, after which, organoleptic evaluation and TLC analysis were done.

Compatibility testing (Organoleptic inspection and TLC analysis)

Two hundred milligrams (200 mg) each of the lyophilized extract was triturated with the excipients (Table 1) at a ratio of 1:1. The mixture was stored in a vial at 8°C, 25°C and 40°C for 6 wk. Organoleptic inspection and TLC analysis of the mixtures was done to assess possible incompatibilities.

Organoleptic evaluation and TLC analysis

Stability of the extract (air stability, thermal stability and photostability) and its compatibility with the excipients

Table 1. List of excipients used in the study.

Excipient	Function	Incompatibilities	Allowable Amount for Use
Polyvinyl pyrrolidone (PVP)	Binder	Thimerosal, sufathiazole, sodium salicylate, tannic acid	0.5-5%
Starch	Binder/Disintegrant/ Diluent	Strong oxidizing agents, iodine	3-20% (binder), 3-25% (disintegrant)
Talc	Diluent	Quarternary ammonium salts	5-30%
Lactose	Diluent	Amino acids, primary and secondary amines	None specified
Kollidon	Disintegrant	Thimerosal, sulfathiazole, sodium salicylate, phenobar-bital, tannic acid	2-5%
Microcrystalline cellulose (MCC)	Disintegrant	Strong oxidizing agents	5-15%

were assessed by taking note of changes in the physical appearance of the mixtures (organoleptic evaluation, Fig. 1) and by noting the disappearance and appearance of spots in the TLC profile of the extract, in comparison with the crude extract. The formulations were dissolved in methanol (15 mg/mL) and spotted on silica gel 60 F254 aluminum sheet and developed in a DCM: Methanol: Glacial Acetic Acid (9.5:0.1:1.0) solvent system. The spots were visualized using TLC Visualizer equipment (366 nm).

Formulation

Five trial formulations were developed using different combinations and proportions of the excipients (Table 2) identified from the compatibility testing. Wet granulation method was employed in producing the larvicidal granules. The ingredients were passed through sieve #80 (0.177 mm) and mixed together with the granulating solution (5% PVP-ethanol solution or starch paste). The resulting mass was passed through mesh #4 (4.76 mm) and dried at 60°C until the moisture content was less than 3%. The granules were passed through sieve #20 (0.841 mm) and then sieve #40 (0.429 mm), retaining the granules for quality control.

Quality Control

Organoleptic evaluation

The appearance of clumping, microbial growth, change in consistency, gas formation and color of the formulated granules were noted.

Moisture content measurement

The moisture content of the granules prior to sieving was measured using an analytical moisture balance. A measurement of not more than 3% moisture content is considered dried and subjected to particle size reduction.

Powder flow property evaluation

The angle of repose of the different formulations was measured using a procedure from the United States Pharmacopeia (USP 35 – NF 30 2012) to determine its flow property. The funnel height was maintained approximately 2–4 cm from the top of the powder pile as it is being formed in order to minimize the impact of falling powder on the tip of the cone. The angle of repose was determined by measuring the height of the cone of powder and calculating the angle of repose, α, based on the equation:

$$\tan(\alpha) = \text{height}/(0.5*\text{base})$$

(Eq. 1. Formula for calculating the angle of repose, α)

The powder flow property of the formulation was based on the angle of repose and Table 3.

Particle size distribution measurement by sieve analysis

The sieve set was tared to the nearest 0.1 g. An accurately weighed quantity of the formulated granules was placed on the top sieve. The nest of sieves was agitated for 5 min and reweighed. The nest was reassembled and agitated for another 5 min. This procedure was repeated until reweighing of test sieves showed a change in weight of not more than 5%.

Dissolution in water

The formulated granules were placed in 100 mL water to test its ease of dissolution. The test was performed in triplicates. Granules should be easily dissolved upon addition to water with minimal stirring or agitation.

Fig. 1. (Left to right) Crude extract for stability testing and triturated kollidon, starch, PVP, microcrystalline cellulose (MCC), talc, and lactose for compatibility testing.

Table 2. Formulations used in the study.

Excipient	% w/w Used in Formulation				
	Formula #1	Formula #2	Formula #3	Formula #4	Formula #5
Crude Extract	25	25	25	25	25
Starch	25	25	1	15	0
Talc	49	30	0	0	0
Lactose	0	14	69	44	59
5% PVP-ethanol solution	1	1	0	1	1
Kollidon	0	5	5	0	0
Microcrystal-line cellulose (MCC)	0	0	0	15	15

Larvicidal Screening of Trial Formulations

The formulations were screened for larvicidal activity against *A. aegypti*. Three replicates of each formulation (500 ppm), the positive control (commercial herbal granule larvicide), and the negative control (distilled water) were prepared to test the 24- and 48-h mortalities against the larvae.

Dose Response Larvicidal Assay

The formulation with the highest activity, satisfactory quality control and excipient compatibility results was subjected to dose-response assay. The assay protocol was similar to the larvicidal screening assay, however, three replicates of the formulation at concentrations of 100 ppm, 200 ppm, 300 pm, 400 ppm and 500 ppm, and negative control (distilled water) were made. Twenty-four hour (24-h) and 48-h mortalities were determined to calculate the LC_{50}-24 h, LC_{90}-24 h, LC_{90}-24 h and LC_{90}-48 h.

Data Analysis

All assays were performed in triplicates. Percent mortality was calculated as follows:

$$\% \text{ mortality } (\%) = (〚\% \text{ Survival}〛\_\text{control} - 〚\% \text{ Survival}〛\_\text{treatment}) / 〚\% \text{ Survival}〛\_\text{control} \times 100\%$$

(Eq. 2. Formula for percent mortality)

The mean percent mortality and standard deviation were calculated for each treatment. One-way ANOVA with Tukey post-hoc test (GraphPad Prism 6) was used to determine the significance between mean percent mortalities in the treatments (α=0.05). The LC_{50} and LC_{90} values were calculated using probit analysis (LDP Line 1.0).

Table 3. Powder flow property evaluations based on the angle of repose (USP35 – NF30 2012).

Angle of Repose (°)	Powder Flow Property
25-30	Excellent
31-35	Good
36-40	Fair-air not needed
41-45	Passable-may hang up
46-55	Poor-must agitate/vibrate
56-65	Very poor
>66	Very, very poor

RESULTS AND DISCUSSION

Plant Extraction

A total of 2219.3 g powdered plant material was extracted with 95% ethanol to yield 273.36 g crude extract (12.32% yield).

Larvicidal Screening of Crude Extract against *A. aegypti*

The crude extract (500 ppm) was screened for larvicidal activity against *A. aegypti*. The mean percent mortality of the crude extract treatment after 24 and 48 h were both 100±0%, compared with 0±0% mean mortality (24-h and 48-h post-treatment) of the negative control (1% DMSO) and 8.89±3.14% mean mortality (24-h post-treatment) and 46.67±21.77% mean mortality (48-h post-treatment) of the positive control.

Phytochemical Screening of the Crude Extract

Steroids, indoles, alkaloids, flavonoids, sugars, coumarins and anthraquinones were detected in the crude extract with use of TLC spray tests.

Preformulation

Air stability (Organoleptic evaluation and TLC analysis)

Physical appearance of the unsealed extract did not significantly change over the 5-wk duration compared with the sealed extract. There was no clumping, change in color, change in odor, microbial growth, liquefaction or formation of gaseous matter. TLC analysis also showed no additional spot in the TLC profile of the unsealed extract compared with the sealed extract (Fig. 2-A).

Thermal stability (Organoleptic evaluation and TLC analysis)

There was no significant change between the extracts stored at 4°C, 27°C and 40°C, except that the consistency of the extract stored at 40°C was relatively less viscous. Besides that, there was no clumping, change in color, change in odor, microbial growth, liquefaction or formation of gas. TLC analysis also showed no additional

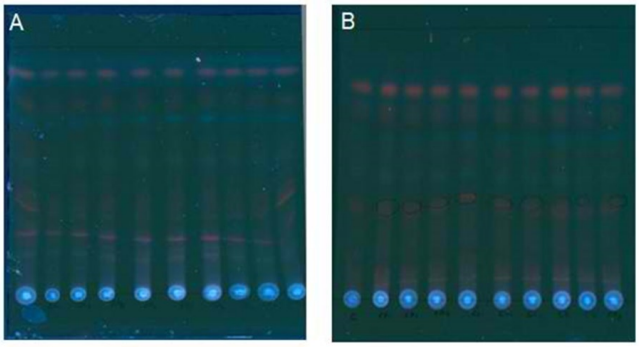

Fig. 2. A: (Left to right) Spots for the crude reference, crude extract exposed to air, crude stored at 40°C and crude stored at 8°C. B: (Left to right) Spots for the crude reference, crude extract stored at 27°C and crude extract stored in a sealed container.

spot in the TLC profile of the extracts stored at different conditions (Fig. 2-A and 2-B).

Photostability (Organoleptic evaluation and TLC analysis)

There was no significant difference between the extract stored in the aluminum foil-covered vial and the uncovered vial. There was no clumping, change in color, change in odor, microbial growth, liquefaction or formation of gaseous matter. TLC analysis also showed that no new spots appeared in the extract in the uncovered vial compared with the extract in the aluminum foil-covered vial (Fig. 2-B).

Excipient compatibility

There was no significant difference between the extracts stored with PVP, starch, talc, lactose, Kollidon, and MCC stored in 4°C, 27°C and 40°C, except that the excipient-extract mixtures stored at 40°C had a softer consistency than the extracts stored at colder temperatures. Besides this, there was no clumping, change in color, change in odor, microbial growth, liquefaction or formation of gaseous matter.

The TLC profiles of the excipient-extract mixture stored at 4°C, 27°C and 40°C also showed no significant differences compared with the crude extract (Fig. 3-A to 3-F).

Formulation

Quality control

Five formulations were developed (Fig. 4). The granules were subjected to quality control testing where all formulations passed for moisture content, powder flow and particle size distribution. However, differences in appearance and solubility and water were observed (Table 4).

Larvicidal Screening of the Formulations

The formulations were screened for larvicidal activity against *A. aegypti* at 500 ppm (Fig. 5). Formulation #5 showed the highest mortality after 24 and 48 h of exposure.

Dose response assay

Dose-response assay was done on Formula #5 because it has the highest larvicidal activity. The 50% and 90% lethal concentrations after 24-h and 48-h post-exposure (LC_{50}-24 h, LC_{90}-24 h, LC_{50}-48 h, LC_{90}-48 h) are shown in Table 5.

DISCUSSION

In a study by Alvarez et al. (2016), ethanolic and aqueous extracts of different plant samples were screened for their larvicidal activity against *A. aegypti* and *A. albopictus*. *Capsicum frutescens* ethanolic extract (800 ppm) caused 100% mortality on the larvae of both species after 24 and 48 h. Partial purification of the ethanolic extract via column chromatography increased the potency of the active fraction compared with the crude extract. In the present study, *C. frutescens* was used as the active component in a larvicidal formulation against *A. aegypti*.

C. frutescens crude ethanolic extract was first tested for larvicidal activity against *A. aegypti*, in order to confirm the activity found in literature. At 500 ppm, the crude extract caused 100% mortality on *A. aegypti* larvae. Phytochemical analysis of the crude extract was conducted to identify constituents present in the crude extract. Phytochemicals such as alkanes, alkenes, alkynes and simple aromatics, lactones, essential oils and fatty acids, terpenes, alkaloids, steroids, isoflavonoids, pterocarpans and lignans from various plant samples

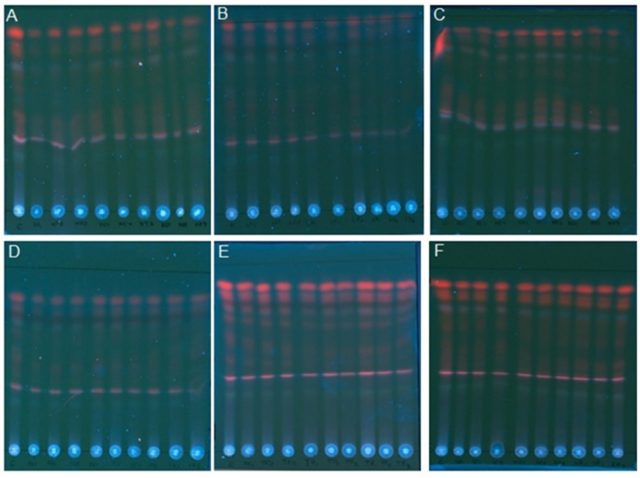

Fig. 3. (Left to right) Spots for the crude reference and extract-excipient mixture (A: kollidon; B: lactose; C: Microcrystalline cellulose (MCC); D: PVP; E: talc; F: starch) stored at 27°C, 8°C and 40°C.

Fig. 4. (Left to right). Larvicidal granule formulations # 1, 2, 3, 4, and 5.

have previously been identified to possess mosquito larvicidal activity (Quiming et al. 2015). Based on the results of the phytochemical analysis, the lyophilized *C. frutescens* ethanolic extract showed positive for flavonoids, alkaloids, coumarin, anthraquinone, indoles, steroids and sugars. These results are consistent with previous literature data on the phytochemicals found in the said extract.

Prior to formulation, the crude extract was subjected to preformulation studies to assess its stability and compatibility with the excipients; these are important in determining the stability and appropriate storage conditions for the larvicide. The crude extract was subjected to various simulated environmental stress conditions (e.g. thermal stress, light exposure, air exposure, excipient compatibility) for 5 wk to determine if slow solid state reactions occurred. These were assessed using organoleptic evaluation and TLC analysis. In the organoleptic evaluation studies, no clumping, change in color, change in odor, microbial growth, liquefaction or formation of gas occurred when the extract was exposed to the environmental stresses. The only notable change in the physical characteristic was the change in consistency of the extracts stored at 40°C for 5 wk; however, these were not considered signs of instability or incompatibility since capsaicin, a major component of *C. frutescens*, is oily in nature and was expected to be in a more liquid state at elevated temperatures. In addition to organoleptic evaluation of the extracts and extract-excipient combination, TLC analysis was done. The appearance of additional spots and apparent disappearance of spots in reference to the crude extract were not observed in all the plates. These results confirm the stability of the crude extract when exposed to air, light, and varying temperatures, and the compatibility of the crude extract with the excipients used.

After preformulation, five trial formulations were developed. These formulations were all composed of 25% w/w crude extract and 75% excipient (composed of varying proportions of the excipients used). They also passed TLC analysis (appearance of additional spots and apparent disappearance of spots in reference to the crude extract were not observed), exhibited excellent powder flow (angle of repose between 25 and 30), and had 90% cumulative frequency within the 0.420–0.841 mm particle size range. For solubility in water, only formulations #2 and #5 dissolved in water without residue, formulation #4 dissolved in water with minimal residue, and formulations #1 and #3 dissolved in water with residue. Although excipients are often characterized as inactive or inert, they have a significant role in the stability, manufacturability and ease of delivery of the active pharmaceutical ingredient on the target organism. Excipient effects on bioavailability is not an issue in this regard since the granules developed are not intended to be orally ingested or absorbed.

Among the five formulations tested, formulation #5 resulted in the highest mortality (100%) after 24 and 48 h of exposure at 500 ppm. Thus, the larvicidal activity of this formulation was further assessed using dose-response assay. After 24 h of exposure, the LC_{50} and LC_{90} values were 108.68 ppm and 233.44 ppm, respectively.

Table 4. List of excipients used in the study.

Formulation	Organoleptic Evaluation	Moisture Content (%)	Powder Flow	Particle Size Distribution (mm)	Dissolution in Water
Formulation #1	Color change to dark green	1.71	Excellent (16.3895°)	0.420–0.841	Soluble with residue
Formulation #2	Color change to brown-ish green	2.02	Excellent (21.5014°)	0.420–0.841	Soluble without residue (for uniformity)
Formulation #3	Color change to dark green	1.26	Excellent (22.0872°)	0.420–0.841	Soluble with residue
Formulation #4	Color change to dark green	1.94	Excellent (27.2141°)	0.420–0.841	Soluble with residue
Formulation #5	Color change to dark green	1.22	Excellent (25.9423°)	0.420–0.841	Soluble without residue

Table 5. Lethal concentrations of Formula #5.

Exposure (h)	LC_{50} (ppm)	LC_{90} (ppm)
24	108.68	233.44
48	102.68	182.50

After 48 h of exposure, the LC_{50} and LC_{90} values were 102.68 ppm and 182.50 ppm, respectively. This activity demonstrated by the formulation is also significantly higher compared with that of the positive control, which is a commercially available herbal granule larvicide. Compared with the LC_{50} and LC_{90} values of the crude and purified *C. frutescens* in literature, the activity of the formulation was comparable with that of the *C. frutescens* partially purified ethanolic extract: LC_{50}-24 h=97.225 pm (76.341–113.3585 ppm); LC_{90}-24 h=181.1339 ppm (155.7739 –228.5227 ppm); LC_{50}-48 h=66.0239 ppm (29.0088–86.3972 ppm); LC_{90}-48 h=132.8026 ppm (108.3948–178.3831 ppm) (Vasconcelos et al. 2014). Thus, the formulation was able to increase the potency of the crude extract, possibly by increasing the dissolution of the active components in aqueous solution.

With the satisfactory development of the *C. frutescens* larvicidal granules, we recommend more tests in improving their potency and larvicidal activity by purifying the extract and/or further optimization of the formulation. Afterwards, simulated field trials and actual field trials can be done to assess the efficacy of the larvicide in actual application. Toxicity tests on humans and other non-target organisms, and ecotoxicity tests on plants can also be done.

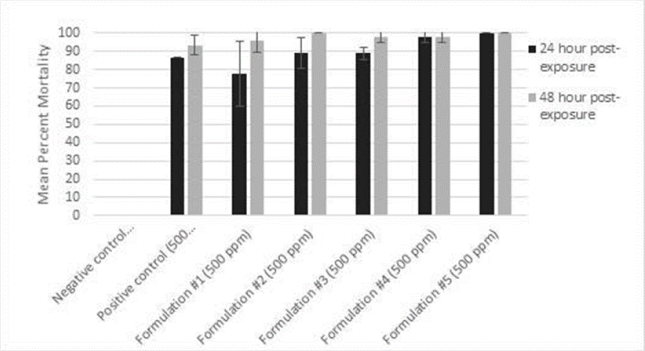

Fig. 5. Larvicidal screening assay of the different formulations.

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