# ADVANCED LARVAL CONTROL STRATEGY FOR *AEDES AEGYPTI*: REGIONAL CLAY NANOCOMPOSITES FOR CONTROLLED RELEASE OF *CITRUS SINENSIS* ESSENTIAL OIL

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**Abstract:** The objective was to develop nanocomposites with formulations based on clay/PVP and *Citrus sinensis* essential oil for larvicidal application against *Aedes aegypti* larvae. The oil was extracted through hydro distillation and incorporated into the clay/PVP mixture, defined at a 2:1 ratio in an aqueous-ethyl medium. Based on the selected ratio, the clay and the nanocomposite with oil were characterized by X-ray diffraction and Fourier transform infrared spectroscopy. Larvicidal tests were conducted with L3/L4 larvae at concentrations of 5–100 ppm, and the $LC_{50}$ was determined. XRD and FTIR characterizations demonstrated effective intercalation of the clay/PVP/oil in the synthesis of the nanomaterial. The nanocomposite presented a $LC_{50}$ of 8.22 ppm, significantly lower than that of the pure oil (22.34 ppm). The nanocomposite as a carrier for *Citrus sinensis* essential oil has proven to be a promising alternative to conventional chemical treatments, which have faced challenges due to growing mosquito resistance, promoting a lower environmental impact and offering a more sustainable strategy.

**Abbreviations: FTIR, Fourier-Transform Infrared Spectroscopy. XDR, X-ray diffraction, PVP, Polyvinylpyrrolidone.**

## 1. Introduction

*Aedes aegypti* is a mosquito species endemic to more than 128 tropical and subtropical countries, being the vector responsible for transmitting arboviruses such as dengue, yellow fever, Zika virus, and chikungunya. These high-incidence viral diseases have caused great concern for society and public health authorities, as the number of reported cases has increased in recent decades. According to the World Health Organization (WHO), it was found in 2020 that more than 2.5 billion people live in endemic areas for dengue cases[1].

Arboviruses have no specific treatment; management focuses on symptom relief, in addition to vaccination, which is available only for yellow fever and dengue[2]. Due to the lack of effective vaccines for all arboviruses, certain preventive measures are adopted to control mosquito proliferation, such as the use of chemical larvicidal pesticides like carbamates, organophosphorus compounds, and pyrethroids[3].

Despite the effectiveness of pesticides in controlling *Aedes aegypti* populations, synthetic compounds are not selective only for the mosquito; they are also toxic to the environment and to humans, causing tolerance and genetic resistance in *Aedes aegypti* larvae[4]. Due to the toxicity caused by the indiscriminate use of synthetic compounds, the use of natural products, such as plant essential oils with larvicidal properties associated with nanotechnology, emerges as a less toxic and more sustainable alternative.

Thus, research on natural products has been developed to find alternatives for controlling

*Ae. aegypti*, with emphasis on the essential oils (EO) of *Citrus sinensis* due to their various properties, including proven larvicidal efficacy against *Ae. aegypti*[5][6]. *Citrus sinensis* is native to Asia, representing 70% of the annual production of all *Citrus* species. It emerges as an alternative to conventional pesticides; however, its low solubility in aqueous media, photosensitivity, and chemical instability hinder its use[7]. In this regard, the use of controlled release systems enables the application of these oils in the environment.

Among these, nanocomposites obtained from clay/polymer interactions emerge as a strategy to make the use and application of essential oils feasible. The choice of clay and polyvinylpyrrolidone (PVP) is justified by their widespread use in the pharmaceutical industry as efficient carriers. Clay is valued for its ability to adsorb active compounds and its stability, while PVP is a polymer known for its biocompatibility and its role as a suspension and stabilizing agent, improving the solubility and release of substances. The combination of these materials creates a controlled-release system, protecting the essential oil and optimizing its effectiveness[8].

In view of the above, the objective of this study was to develop nanocomposites from clay/polyvinylpyrrolidone formulations and Citrus sinensis essential oil to evaluate their larvicidal activity against Aedes aegypti.

## 2. Materials and Methods

### 2.1 Extraction and Characterization of *Citrus sinensis* Essential Oil

#### 2.1.1 Essential Oil Extraction

The essential oil was extracted from Citrus sinensis peels in a single batch, with the oranges purchased at the municipal market of Aracaju – SE. Initially, the peels of Citrus sinensis were cleaned, peeled, and weighed on semi-analytical scales following the methodology of Galvão *et al.,* (2015). The peels were then dried in a circulating air oven for 72 hours at 50 °C (Galvão *et al.*, 2015). Subsequently, the dried peels were ground and weighed using semi-analytical scales. The powder obtained was subjected to hydrodistillation in a Clevenger-type extraction apparatus for 4 hours, using 1.5 L of distilled water per 200 g of ground peel. Residual water was removed by incorporating anhydrous sodium sulfate, yielding the essential oil. The hydrolate was discarded, and the essential oil was stored at -4 °C in an amber vial, protected from light. The extraction yield was calculated according to Equation 1:

Equation 1: Extraction Yield Equation:

$$R = (m_o / m_{ct}) \times 100$$

Where:

R= yield, in percentage;

$m_o$= mass of extracted oil, in grams;

$m_{ct}$ = mass of ground peel, in grams.

### 2.1.2 Determination of the Density of *Citrus sinensis* Essential Oil

The density of the oil was determined using the pycnometry method. Calibration of a 5 mL pycnometer was performed with distilled water at 20 ºC. After filling the pycnometer with the oil and adjusting the temperature, its mass was measured. The density result was determined according to the guidelines established by the Brazilian Pharmacopeia (2010).

### 2.1.3 Identification of Chemical Constituents of *Citrus sinensis* Essential Oil by Gas Chromatography-Mass Spectrometry (GC-MS)

The chemical composition characterization analysis was performed using a Gas Chromatograph coupled to a Mass Spectrometer (GC/MS, Shimadzu QP2010 plus, Tokyo, Japan) equipped with an auto-injector (split/splitless). A 5% phenyl - 95% dimethylpolysiloxane column, SPB-5 (60 m × 0.25 mm × 0.25 µm) was used for compound separation, using helium as the carrier gas (White Martins S.A.) at a flow rate of 1 $mL \cdot min^{-1}$. The temperature ramp program was as follows: 80 °C (2 min), increasing at 2 $°C \cdot min^{-1}$ to 100 °C (2 min), then increasing at 10 $°C \cdot min^{-1}$ up to 250 °C. Injection mode was splitless, and the total analysis time was 29 minutes. For the MS conditions: injector temperature at 200 °C, detector temperature at 250 °C, solvent cut time of 6 min, electron ionization mode (EI) at 70 eV, scanning mass range from 40 to 350 Dalton. Chemical compounds were qualitatively identified by comparison with mass spectra from the NIST 107, 21, and Wiley 8 databases. Analyses were conducted at the Multiuser Chemical Laboratory Center (CLQM), Department of Chemistry – UFS.

## 2.2 Physicochemical Characterization of the Regional Clay

### 2.2.1 X-Ray Diffraction (XRD)

X-Ray Diffraction (XRD) was used to evaluate changes in the percentage and crystalline structure of the studied materials. All samples were analyzed by powder X-ray diffraction using a Rigaku Ultima+ RINT 2000/PC, employing $CuK\alpha_1$ radiation ($\lambda$ = 1.5418 Å) in the range of 1–60° 2θ, with a scanning speed of 2°/min.

### 2.2.2 Fourier Transform Infrared Spectroscopy (FTIR)

Absorption spectra in the infrared region of the samples were obtained using potassium bromide (KBr) pellets with a solid support. For pellet preparation, 1 mg of sample was mixed with 100 mg of KBr, then ground and homogenized into a fine powder. The mixture was pressed under 78.5 N using a Shimadzu hydraulic press for 10 minutes. After pellet preparation, it was analyzed with a Shimadzu IRTracer-100 spectrometer in the region between 4000 and 400 $cm^{-1}$, with 45 scans and a resolution of 4 $cm^{-1}$.

### 2.3 Synthesis of the Nanocomposite Combined with Clay and Essential Oil

The polymer/regional clay nanocomposite was obtained by the intercalation method using a water/ethanol mixture. Subsequently, the solvent was removed by centrifugation at 4000 rpm for 30 minutes. The same procedure was repeated for nanocomposite preparations, in which 1.5 mL of essential oil, whose selection was based on preliminary studies by the research group, was added during agitation.

### 2.4 Evaluation of Larvicidal Activity of *Citrus sinensis* Essential Oil and Components of Solid Nanocomposite Forms Against *Ae. aegypti* Larvae

Aedes aegypti eggs from the Rockefeller strain were obtained from the insectary of the Tropical Entomology and Parasitology Laboratory (LePat) at the Federal University of Sergipe. For assays, paper strips containing the eggs were submerged in a rectangular container with water and approximately 200 mg of fish feed. The container was maintained at the Bioprocess Engineering Laboratory at the Institute of Technology and Research/UNIT, under controlled temperature (26–28 °C) to allow hatching and larval development for four days, until reaching L3/L4 stage.

Larvicidal activity was evaluated using aqueous solutions of *Citrus sinensis* essential oil. Initially, 100 mg of oil was separately dispersed in 0.25 mL of Tween 80 in a 10 mL beaker, to which 4.75 mL of distilled water was added to obtain stock solutions of 20,000 ppm. These solutions were then diluted to prepare 100 mL aqueous solutions with concentrations ranging from 5 to 70 ppm. For each assay, 20 *Aedes aegypti* larvae (Rockefeller strain L3/L4) were exposed to these solutions in disposable cups containing 100 mL of mineral water. Solid forms were directly added to 100 mL of water in disposable cups. An aqueous solution of Tween 80 (0.1 mL) and water (19.9 mL) was used as control. All assays were performed in triplicate. After 24 hours of exposure, the number of dead larvae was recorded. Probit analysis was used to determine the lethal concentration for 50% mortality (LC50).

## 3. Results and Discussion

### 3.1 Extraction and Characterization of *Citrus sinensis* Essential Oil

#### 3.1.1 Yield and Density of CS Essential Oil (OECS)

The yield of *Citrus sinensis* essential oil (OECS) was evaluated starting from 3,450 g of crushed peel, obtaining 154.33 g of essential oil, resulting in an approximate yield of 11% w/w. The oil obtained in this study exhibited homogeneity, presenting as a translucent oily liquid with a characteristic citrus aroma, in accordance with the Brazilian Pharmacopoeia (1988) specifications.

Using the same extraction methodology with dried and crushed peels, similar results have been reported in the literature, such as Santos *et al.* (2024), who reported a yield of 9.65%, and Araujo *et al.* (2016), who achieved 9.10%. These findings demonstrate the consistency and effectiveness of the methodology employed in this study, aligning with previously documented results and highlighting the feasibility of the adopted extraction process.

The relative density of the obtained oil also showed close agreement with values reported in the literature, with no significantly lower values found. The average density of the OECS in this work was approximately 0.850 g/mL, which is consistent with previously reported data of 0.850 g/mL[13]. This coherence reinforces the accuracy and reliability of the results.

#### 3.1.2 Identification of Chemical Constituents of *Citrus sinensis* Essential Oil by Gas Chromatography-Mass Spectrometry (GC-MS)

The chromatographic analysis identified the compounds listed in Table 1. Figure 1 shows the chromatogram of the *Citrus sinensis* essential oil. The results revealed limonene as the major component, along with other compounds commonly found in this oil, in quantities consistent with those reported in the literature [14].

### 3.2 Synthesis of the Nanocomposite Combined with Clay and Essential Oil

The regional polymer/clay nanocomposite was obtained by the intercalation method using water/ethanol. Stoichiometric amounts of PVP and regional clay were weighed to maintain a 2:1 (w/w) ratio, and the system was left under agitation for 24 hours at room temperature. The solvent removal was performed by centrifugation at 4000 rpm for 30 minutes. The same process was repeated in the preparations of nanocomposites in which 1.5 mL of essential oil was added during the agitation process, followed by drying in a desiccator.

### 3.3 Characterization of the Nanocomposite

#### 3.3.1 Fourier Transform Infrared Spectroscopy (FT-IR)

To characterize polymer/clay nanocomposites, it is important first to analyze the clay to detect clay peaks corresponding to Si-O bonds. The peaks become sharper when an intercalant is added to the clay matrix [15].

The spectrum of bentonite shown in Figure 2 exhibited a strong band at 3624 $cm^{-1}$ and another at 3441 $cm^{-1}$, attributed to the axial vibration of OH groups in the structure and hydroxyl groups of water involved in hydrogen bonding. At 1648 $cm^{-1}$, a band corresponding to the asymmetric stretching of Si-O-Si is observed, along with another low-intensity band at 1461 $cm^{-1}$ corresponding to Si-O stretching. Bands arising from vibrations of the silicate layers can be observed between 1024 and 469 $cm^{-1}$ [16].

### 3.3.2 X-ray Diffraction (XRD)

The X-ray diffraction patterns of the sample can be observed in Figure 3. The presence of montmorillonite was confirmed at an angle of 5.76° (Figure 3a). The notable peaks in the diffractogram indicate the presence of specific crystalline phases in the bentonite. The highest peak around 2θ = 20° is characteristic of smectite clays, which are the main components of bentonite. Other peaks may be associated with different mineral phases present in the sample, such as quartz or feldspar, commonly found in natural clays. The diffraction patterns of the two samples (inert nanocomposites and those containing the oil) show similar clay peaks (Figure 3b), shifted towards lower angles, demonstrating that the crystalline structure of the clay was preserved after polymer intercalation, as shown in Figure 3b.

### 3.4 Evaluation of the larvicidal activity of *Citrus sinensis* essential oil and the solid-form components of the nanocomposites against *Ae. aegypti* larvae

The isolated CS-EO showed an $LC_{50}$ of 22.43 ppm against *Aedes aegypti* larvae, a value consistent with literature data. According to Galvão *et al.* (2015), $LC_{50}$ values of 30 ppm for CS-EO and 50 ppm for D-limonene, its main component, have been reported. These results support the hypothesis of a synergistic effect among the major compounds of the essential oil, contributing to its larvicidal efficacy.

After incorporating CS-EO into nanocomposites and subsequently compressing them into solid forms, a significant improvement in larvicidal efficacy was observed. The final formulation achieved an $LC_{50}$ of 8.22 ppm, demonstrating a substantial increase in biological activity. This enhanced efficacy may be related to the protection and controlled release of the essential oil provided by the nanocomposites, as well as improved stability and bioavailability of the active compound in the aquatic environment. These factors, combined with the encapsulation technology, highlight the potential of solid forms as a promising strategy for vector control.

## 4. Conclusion

The study demonstrated that developing solid formulations with *Citrus sinensis* essential oil resulted in promising larvicidal activity against *Aedes aegypti*. The formulation proved significantly more effective than the pure oil, showing a remarkable reduction in the $LC_{50}$ to 8.22 ppm. This improved efficacy is attributed to nanoencapsulation, which enhanced the oil's stability and protected it from degradation. The use of regional clay as a carrier material was crucial, as it enabled the gradual release of the oil into the aqueous medium, increasing its availability and interaction with the larvae. The clay's structure facilitated the dispersion of the active compound, intensifying its effect and directly contributing to the $LC_{50}$ reduction. Thus, the combination of essential oil with clay proved to be an effective and environmentally safe strategy for vector control.

## Tables, figures and graphics

**Table 1:** Volatile components extracted from the essential oil of *Citrus sinensis*.

| Picos | Tempo de retenção (min) | Componente | Área (%) | IR (exp) | IR (lit) |
|---|---|---|---|---|---|
| 1 | 10.665 | α-pineno | 0.27 | 928 | 932 |
| 2 | 12.730 | Mirceno | 1.39 | 984 | 988 |
| 3 | 14.790 | Limoneno | 97,86 | 1034 | 1024 |
| 4 | 16.975 | Linalol | 0.48 | 1095 | 1095 |

**Figure 1**. Volatile components of the extracted *Citrus sinensis* essential oil.

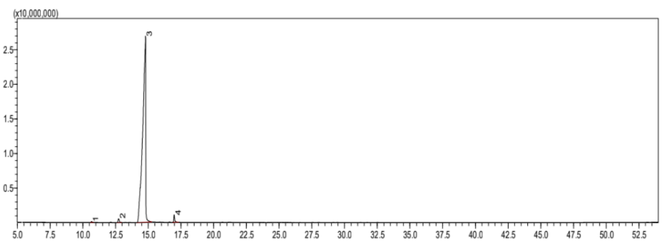


**Figure 2.** Infrared spectrum of Bentonite

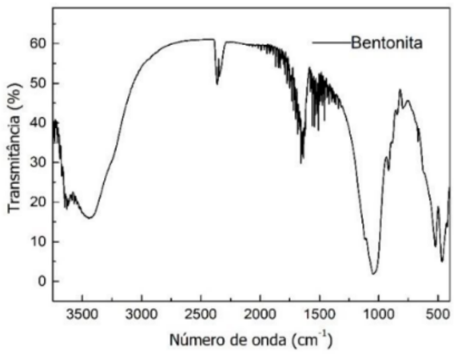
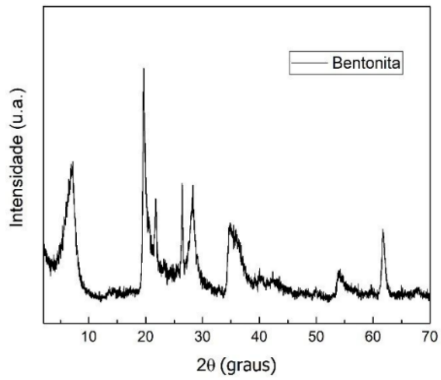



**Figure 3.** Diffraction patterns: (a) regional clay and (b) nanocomposite, inert and with Citrus sinensis oil incorporated at a 2:1 ratio.

(a)




(b)

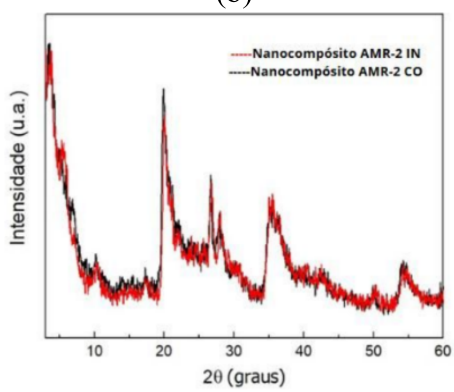



**Table 2.** $LC_{50}$ determination of *Citrus sinensis* essential oil against *Ae. aegypti* larvae in L3/L4 stages after 24 h of exposure.

| Formulation | LC50 (95% IC) ppm |
|---|---|
| OECS | 22,34 (22,02 –22,52) |
| PVP | - |
| Clay | - |
| Nanocompósite | - |
| oil-loaded nanocomposite | 8,22 (8,03 – 8,48) |

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