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Piper ovatum (Piperaceae) extract/starch-cellulose films to control *Aedes aegypti* (Diptera: Culicidae) larvae

Luiz Alberto Kanis[a,*], Bruna Daniel Rabelo[a], Diego Moterle[a], Kauê Muller Custódio[a], Joice Guilherme de Oliveira[b], Alessandra Bittencourt de Lemos[c], Onilda Santos da Silva[c], Karine Modolon Zepon[a], Rachel Faverzani Magnago[d], Josiane Somariva Prophiro[b]

[a] Research Group in Pharmaceutical Technology, Postgraduate Program in Health Sciences University of Southern Santa Catarina, Brazil
[b] Research Group in Immunoparasitology, University of Southern Santa Catarina, Brazil
[c] Department of Microbiology, Immunology and Parasitology – ICBS – Federal University of Rio Grande do Sul, Brazil
[d] Postgraduate Program in Environmental Sciences University of Southern Santa Catarina, Brazil

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ABSTRACT

Most of the botanical extract which showed larvicidal properties against *Aedes aegypti* present low residual activity. Thus, we included *Piper ovatum* extract in a hydrophilic, biodegradable polymeric base to increase the time of action of these larvicides. Standardized extract of *P. ovatum* roots was tested against *Ae. aegypti* larvae in order to investigate some external morphological modification in the larvae body and histological effects in their midgut. Two films called FSEPO5 and FSEPO12 containing 5 and 12% of *P. ovatum* extract were incorporated into biodegradable starch-cellulose films. Loss of weight and mechanical properties were evaluated. The larvae mortality was investigated through immersion of different pieces with known width into the plastic flask containing 200 mL of water and *Ae. aegypti* larvae. After 48 h of exposure, the mortality was checked and new larvae were added. The experiment was conducted until no more larvae mortality was observed. The effect of the surface area-to-volume ratio over larval mortality was assessed as well. The LC_{50} and LC_{99} of *P. ovatum* extracts were 2.57 ppm and 3.80 ppm, respectively. The inclusion of the extract in to the film decrease the elastic modulus, increase the elongation at break and promote higher erosion. The residual activity of the films was achieved for more than 700 h (29 days). The increase in surface-to-volume ratio of the film increased the larvicidal activity. Starch-cellulose films containing standardized extract of *P. ovatum* is a suitable alternative to prevent mosquito breeding in places with potential water accumulation. They can provide industrial larvicide in the future.

1. Introduction

Aedes (Stegomyia) aegypti (Linnaeus, 1762) is considered the main vector of the Dengue, Zika and Chikungunya virus. Over the last years, the incidence of infections caused by this arbovirus has risen because of the anthropophilic behavior of this mosquito species and change in climate conditions in all regions around the world (Campbell et al., 2015; Benelli and Mehlhorn, 2016; Shragai et al., 2017). The intense exposure of mosquitoes to synthetic insecticides in agriculture, livestock, and in public areas allowed for the selection of the most resistant mosquitoes (Prophiro et al., 2011; Bellinato et al., 2016; Goindin et al., 2017).

Previous studies have highlighted the insecticide potential of a wide variety of plants (Komalamisra et al., 2005; Pimenta et al., 2006; Kanis et al., 2009; Dias et al., 2015), including those belonging to the Piperaceae family (Chaithong et al., 2006; Scott et al., 2008; Autran et al., 2009; da Silva et al., 2016; Samuel et al., 2016). Nonetheless, most of the natural products show low residual activity because of the biodegradability of the main components (Amer and Mehlhorn, 2006; Kotte et al., 2014). Therefore, one alternative to increase the residual activity lies on incorporating these natural products into controlled-release systems (Roy et al., 2009; Badawy et al., 2015; Vangelie et al., 2015). These systems include, micro- and nanoparticles, films, beads, and hydrogels based on polymeric materials and loaded with insecticides and pesticides. As a result, the use of this strategy can reduce the degradation rate of active ingredients, prolong the release time, and hence increase the residual activity (Kanis et al., 2012; Custódio et al., 2016; Chaudhary et al., 2017). Moreover, these controlled-release systems can

* Corresponding author.
E-mail addresses: luiz.kanis@unisul.br (L.A. Kanis), onilda.silva@ufrgs.br (O.S. da Silva), rachelfaverzanimagnago@gmail.com (R.F. Magnago), josiane.prophiro@unisul.br (J.S. Prophiro).

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reduce the environmental impact by reducing the total amount of the active ingredient available at the application site (Roy et al., 2014). Therefore, in order to address these requirements, the polymeric material used for producing these controlled-release systems should be biodegradable and obtained from renewable resources. Polysaccharides and cellulose derivatives, amongst the other polymers, have found use for this purpose (Roy et al., 2014; Campos et al., 2015). Among the many alternatives, the blending of starch and cellulose provide polymeric materials with good mechanical properties to produce films. Moreover, these materials are biodegradable (Puglia et al., 2003; Laycock et al., 2017) and has been utilized in polymers designed for controlled drug delivery (Zepon et al., 2014)

Previously, we published a study showing the process of production and standardization of a larvicidal extract against *Ae. aegypti* from *Piper ovatum* (Piperaceae) (Kanis et al., 2013). The extract showed effectiveness against this mosquito with residual activity up to 400 h (16 days). However, we did not verify if the larvae died by ingesting the chemical compounds of this extracts or by other contact ways (e.g., disrupting cuticle, changing in midgut cell organization). As the results were very promising, we incorporated the extract in the biodegradable polymeric starch-cellulose film to prolong the time of residual activity. Thus, the objective of this study was to understand the activity of *P. ovatum* extract against the *Ae. aegypti* larvae, verify some morphological histological modification of the body and midgut of larvae. Moreover, evaluate the residual effect of starch-cellulose polymeric films containing a *P. ovatum* extract and how the extract change the mechanical properties and morphological structure of the film.

2. Materials and methods

2.1. General remarks

Samples of *P. ovatum* were collected in March 2011 in Braço do Norte, Santa Catarina, Brazil. A voucher specimen (SRS5134) was deposited in the *Laelia purpurata* herbarium of the University of Southern Santa Catarina. Ethyl alcohol (95%) and NaOH (+97% purity) were obtained from Fmaia Indústria e Comércio Ltda (São Paulo, Brazil). The starch-cellulose polymer (Mater-Bi® YI01U class) was obtained from Novamont SpA (Italy). USP Polysorbate 80 was obtained from Synth® (São Paulo, Brazil). Pure ethyl acetate (+99.5% purity) was obtained from Neon Comercial Ltda (São Paulo, Brazil).

2.2. Mosquitoes

Rockefeller strains of *Ae. aegypti* were continuously maintained in our laboratory under a 14-h light/10-h dark photoperiod. Larvae were fed on powdered pet food (Purina® Cat Chow®, 0.2 g/100 mL, three times per week). Adult males and females were continuously provided with a 5% honey solution, while females were blood-fed on BALB/c mice, twice per week, in order to obtain eggs for colony development. For all tests described below, late third or early fourth instar larvae were used. All bioassays were performed at 25 °C and 80% (± 10%) relative humidity in an ELETROlab® 132FC incubator.

2.3. Standardized extract of Piper ovatum (SEPO)

Piper ovatum roots were dried for 5 days at 40 °C. The material was ground up to obtain particle size smaller than 550 µm. Then, it was subjected to dynamic maceration for 48 h, at a ratio of 1:10 plant/solvent, using 95% ethanol as solvent. Subsequently, the ethanol extract was obtained by evaporation of solvent in a rotary evaporator at 40 °C. The extract was solubilized in 0.05 mol/L (10:1) NaOH/ethanol solution. Next, it was placed in a separator funnel followed by extraction with 3 ethyl acetate fractions, which were combined, and the solvent was evaporated in a rotary evaporator under vacuum at 30 °C temperature to obtain the ethyl-acetate fraction called SEPO, which was then used for the studies (Kanis et al., 2013).

2.4. Determining lethal concentrations of the SEPO extract

The SEPO extract was previously dissolved in 20 mL of water containing polysorbate 80 at a concentration of 0.015% at a temperature of 40 °C under mechanical stirring for 30 min. From this solution, eight dilutions were prepared at concentrations ranging between 1 and 6 ppm. Plastic containers were filled with 100 mL of the dilutions containing the SEPO extract, and 20 larvae were added to each one. The tests were run in triplicate (three groups of test larvae and a control group) for all concentrations. For each experiment, controls with water and water polysorbate 80 were used. Larval mortality was measured after 24 h of exposure to the different concentrations. Larvae were already considered dead if they did not move in response to being probed with a histological needle. All bioassays mentioned above followed the protocol recommended by the World Health Organization (WHO, 1981). The lethal concentration values (LC_{50} and LC_{99}) were estimated using Probit analysis (Finney, 1971). Dead larvae were examined under an optical stereomicroscope (EMZ-5TR Meiji, Meiji Techno Co. Ltd., Tokyo, Japan) at 20 times magnification. Untreated healthy larvae were used as control.

2.5. Morphological and histological studies

After 24 h of exposure to a SEPO solution at LC_{50} concentration, some larvae were separated for morphological and histological studies. Histological procedures were made according to the protocol of Lemos et al. (2018). Larvae that were unable to reach the water surface when probed with a needle, but were still alive, were taken for histological studies. So, 20 larvae (10 treated with SEPO and 10 healthy larvae) were fixed in Bouin solution for 24 h at room temperature. After that, they were dehydrated in 70% ethanol for 10 min, 95% ethanol for 15 min, absolute ethanol for 20 min, and again absolute ethanol for 30 min. The impregnation process was carried out at room temperature in a resin solution (Leica Historesin-Embedding Kit), according to the manufacturer's instructions. The inclusion was held in polyethylene molds (Leica) and each inclusion block contained two larvae, which were positioned lengthwise. The molds were kept at 40 °C for at least 12 h for complete polymerization of the resin. The cuts were made lengthwise, 5 µm thick, using a Rotary microtome (Leica RM2125) with a disposable stainless steel razor (Histoblade). For each block of historesin, 10 blades were mounted with six sequential cuts. Then, they were dried at 40 °C for 24 h. Later, they were colored with Harri's hematoxylin for 15 min and yellowish Eosin 1% for 5 min. The midgut of larvae was analyzed and photographed using a microscope Olympus BX41. The scale bar was added using ImageJ software version 1.50f.

2.6. Preparation of polymeric films for controlled release of SEPO

Two different polymeric films were prepared with a total mass of 500 mg and an area of 38.5 cm^2. The first one was composed of 95% starch-cellulose and 5% SEPO (for FSEPO5), and the second contained 88% starch-cellulose and 12% SEPO (for FSEPO12). The polymer and SEPO were solubilized in 10 mL acetone under constant agitation and heated in a water bath at 50 °C. The solution was poured into a Petri dish (7 cm in diameter), and the solvent was evaporated at room temperature and pressure. A polymeric film was prepared as previously mentioned, without the addition of SEPO, to be used as a control.

2.7. Residual activity

The bioassay was performed in triplicate as mentioned previously to evaluate the residual activity of FSEPOs, by placing a sample of 1 cm^2 FSEPOs in a recipient containing 200 mL of water, followed by the addition of 50 healthy *Ae. aegypti*. They were then removed after 48 h of