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Short communication

Evaluation of larvicidal activity of a nanoemulsion of *Rosmarinus officinalis* essential oil

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ABSTRACT

Nanotechnology has emerged as a promising area for innovative products, including insecticides. Dengue is a tropical disease which is considered a critical health problem in developing countries, due to negative impacts to the environment caused by synthetic chemicals used for vector control (*Aedes aegypti*). Thus, developing of natural products based insecticidal are considered very promising. On this context, the aim of the present study was to obtain an O/W nanoemulsion containing *Rosmarinus officinalis* L., Lamiaceae, essential oil and evaluate its larvicidal activity against *A. aegypti*. Low energy method was employed, allowing achievement of small droplets. The nanoemulsion also presented low polydispersity and mean droplet below 200 nm, even after 30 days of storage. Potential mortality levels were observed after 24 h (80 ± 10%) and 48 h (90 ± 10%) in *A. aegypti* larvae at final concentration of 250 ppm, related to *R. officinalis* essential oil. This study contributes to nanobiotechnology of natural products, presenting a potential larvicidal nanoemulsion prepared with *R. officinalis* essential oil. Moreover, nanoemulsion production involved a non-heating procedure, describing easy technique which may be useful for integrative control programs.

Introduction

Nanotechnology is a multidisciplinary approach which involves creation and utilization of different systems on a nanometric scale (De Villiers et al., 2009). Several types of nanoformulations have been reported, including nanoemulsions, which are dispersed systems constituted by immiscible liquids and one or more stabilizers (McClements, 2012). Nanoemulsions are characterized by their thermodynamically stability and small droplets, ranging from 20 to 200 nm (Ostertag et al., 2012).

Dengue is an endemic illness on South America and other countries. Recently, it was observed an increase in the morbidity of this pathology, being considered a critical health problem (WHO, 2014). Many substances have been tested to control the vector *Aedes aegypti*. Several substances have been tested to control de vector *Aedes aegypti* (Hirata et al., 2014). However, many of them are synthetic chemicals, including the organophosphate temephos and the pyrethroid deltamethrin, which may lead resistance in the mosquitoes and even negative impacts to the environment (Marcombe et al., 2009).

On this context, ecofriendly alternative integrated control programs have emerged as promising alternatives (Sugumar et al., 2014) and essential oil based nanoemulsions have been recognized as valuable products for mosquito control (Ghosh et al., 2013). *Rosmarinus officinalis* L., Lamiaceae, essential oil has demonstrated larvicidal properties (Prajapati et al., 2005; Freitas et al., 2010; Amer and Mehlhorn, 2006a) and repellent activity (Prajapati et al., 2005; Amer and Mehlhorn, 2006b). However, intrinsic poor water solubility of essential oils is a technological challenge. The aim of the present study was to obtain an O/W nanoemulsion containing *R. officinalis* essential oil and evaluate its larvicidal activity against *A. aegypti*.

Materials and methods

Chemicals

Polysorbate 20 was purchased from Praid Produtos Químicos Ltda (SP, Brazil).

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Essential oil

Essential oil extraction from leaves of *R. officinalis* L., Lamiaceae, was performed by hydrodistillation using a Clevenger apparatus. Experimental protocol for extraction and chemical characterization of the essential oil used in this work were previously described (Fernandes et al., 2013).

Nanoemulsion preparation

Nanoemulsion was obtained by a low energy method (Ostertag et al., 2012) using 90% (w/w) of water, 5% (w/w) of essential oil and 5% (w/w) of polysorbate 20 at a total mass of 50 g. The essential oil and polysorbate 20 were stirred at 800 rpm using magnetic stirrer (Fisatom, Brazil) for 30 min. Then, water was added drop wise at a flow rate of 3.5 ml/min. The mixture was stirred at 800 rpm for 60 min. Nanoemulsion was stored under room temperature (20 ± 2 °C) and evaluated *after* 1, 7, 21 and 30 days of preparation.

Droplet size analysis

Droplet size and polydispersity of the nanoemulsion was determined by photon correlation spectroscopy (Zetasizer ZS, Malvern, UK). Nanoemulsion was diluted with water for injection (1:25) (Fernandes et al., 2013). Measurements were made in triplicate. The average droplet size was expressed as the mean diameter.

Larvicidal assay

A. aegypti larvae were obtained from the Arthropoda Laboratory (Universidade Federal do Amapá, Brazil). Biological assay was performed under controlled conditions, being fourth-instar larvae kept at 25 ± 2 °C, relative humidity of 75 ± 5% and a 12 h light:dark cycle. Experimental protocol was performed according to WHO (2005) with some modifications. All experiments were performed in triplicate with 10 forth-instar larvae in each sample, using the nanoemulsion diluted in distilled water at 250 ppm (related to *R. officinalis* essential oil). Negative control was performed with surfactant at same concentration of tested samples. Mortality levels were recorded after 24 and 48 h of exposure.

Statistical analysis

Analysis of variance (ANOVA) followed by Duncan's test was conducted using StatGraphics Plus software v.5.1 (Stat Easy Co., Minneapolis, USA). Difference was considered significant when $p \leq 0.05$.

Results and discussion

Essential oils are volatile complex mixtures with a wide range of biological activities, including repellent, insecticidal and larvicidal properties (Conti et al., 2010). *R. officinalis* essential oil used in this study has 1,8-cineole (44.0%), camphor (16.1%), β-myrcene (11.1), α-pinene (9.4%); verbenone (4.1%), borneol (3.5%) and camphene (3.3%) as major substances (Fernandes et al., 2013), being essential oils with these substances are described as larvicidal agents (Conti et al., 2010).

However, essential oils have poor water solubility and this is a technological problem for their application as larvicidal products. *A. aegypti* development occurs in water, thus, active substances must be dispersed or solubilized in this medium. On this context, an O/W nanoemulsion of *R. officinalis* essential oil could solve the problem of water solubility.

Nanoemulsion containing 5% (w/w) of essential oil from *R. officinalis*, 5% (w/w) of polysorbate 20 presented a fine appearance and

Fig. 1. O/W nanoemulsion of *Rosmarinus officinalis*.

bluish aspect, which is in accordance with this type of formulation (Fig. 1). It was not observed any signal of instability, including creaming or phase separation.

Fig. 2 shows results concerning mean droplet size and polydispersity, during 4 weeks. Low mean diameter (<200 nm), which is in accordance with the concept of nanoemulsions (Solans et al., 2005; Solè et al., 2012) were observed in all measurements. Particle size distribution after one day presented a polimodal profile, indicating the presence of different particle size populations.

After seven days of preparation it was observed an increase in mean droplet size. However, it was maintained below 200 nm (Fig. 2) and polydispersity was reduced (Fig. 3B). Fig. 3C and D show that mean droplet size after 21 and 30 days also remained under 200 nm. Moreover, no significant difference was observed between polydispersity ($t = -0.6351$; $p = 0.5599$) in the interval of 21–30 days. Micelles are continuously disintegrating and reassembling, being in dynamic equilibrium with individual surfactant molecules

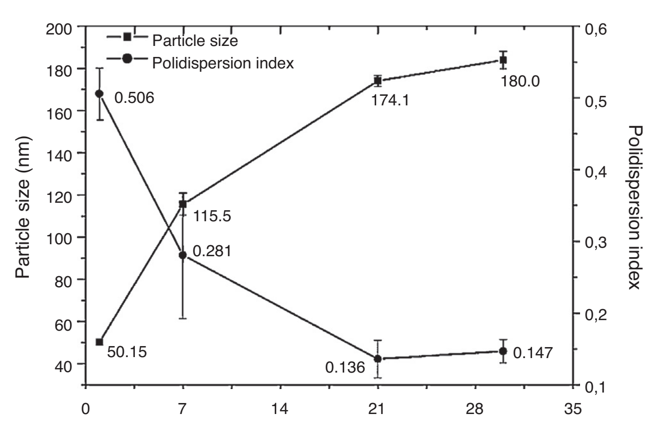

Fig. 2. Mean droplet and polydispersity variation of *R. officinalis* essential oil nanoemulsion during storage.

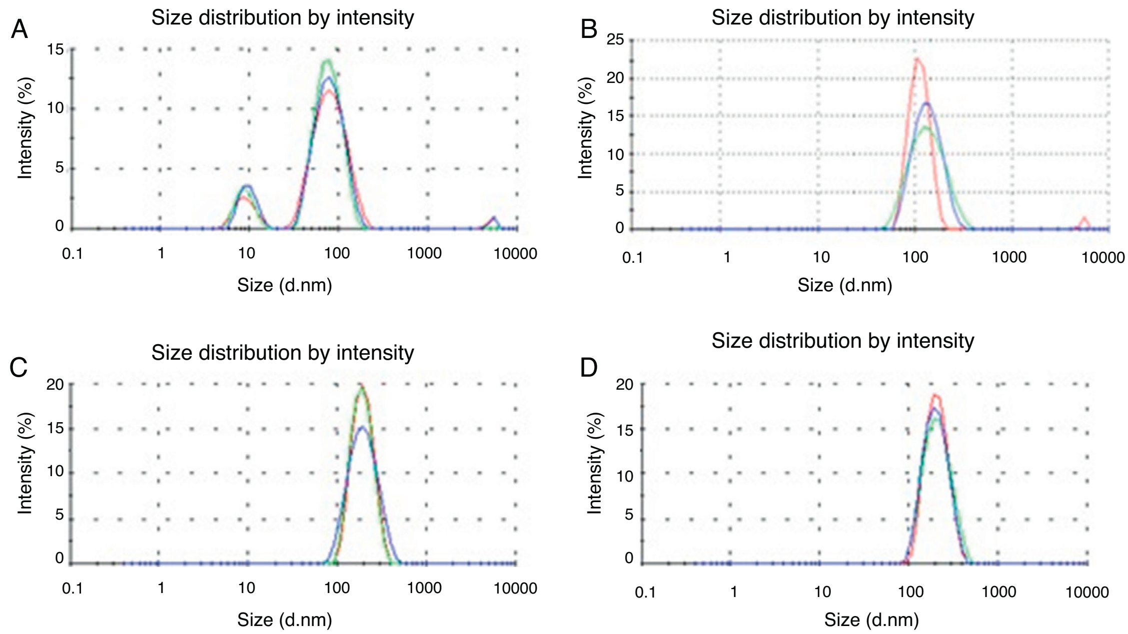

Fig. 3. Particle size distribution of *R. officinalis* nanoemulsion after (A) 1 day: mean droplet – 50.15 ± 1.306 nm; polydispersity – 0.506 ± 0.036 nm. (B) 7 days: mean droplet – 115.5 ± 5.147 nm; polydispersity – 0.281 ± 0.089. (C) 21 days: mean droplet – 174.1 ± 2.536 nm; polydispersity – 0.136 ± 0.026 nm. (D) 30 days: mean droplet – 184.0 ± 4.133 nm; polydispersity – 0.147 ± 0.016 nm.

(Patist et al., 2002). Thus, it could be suggested that micelles reached dynamic equilibrium in this period, showing a kinetic stability of the nanoemulsion.

Previous study with *R. officinalis* essential oil allowed determination of required HLB value and achievement of an O/W nanoemulsion. Emulsification method involved in the procedure used heating of the oil phase, constituted by essential oil and surfactant, in order to obtain small droplets (Fernandes et al., 2013). Essential oils are complex mixtures of volatile substances and heating step would lead loss of substances. As part of our ongoing studies concerning nanobiotechnology of *R. officinalis* essential oil, we decided to test a method without heating, which proved to successfully generate a nanoemulsion. Titration low energy method used in this study is based in a catastrophic phase inversion (Ostertag et al., 2012).

It was observed that the nanoemulsion containing essential oil of *R. officinalis* caused 80 ± 10% of mortality after 24 h and 90 ± 10% of mortality after 48 h (Fig. 4). No mortality was observed for control group. After one day of preparation, it was observed some particles around 10 nm, which were responsible by the polimodal profile. Further characterization revealed that narrow distribution was achieved, probably due to disintegration and regeneration of micelles. Penetration through cuticle is crucial for insecticidal activity and recognized as one of possible mechanisms of insectides (Kasai et al., 2014). Considering that particle size of droplets remained in a nanometric range, penetration and potential larvicidal activity may not be affected. Further investigations would be necessary to confirm these findings.

Potential larvicidal application of natural products can be obtained considering mortality levels of larvae after 48 h of treatment with samples at 250 ppm as follows: promising (>75%), partially promising (>50% and <75%), weakly promising (>25% and <50%) and inactive (<25%) (Montenegro et al., 2006). On this context, our results suggest that the nanoemulsion containing 5% (w/w) of *R. officinalis* essential oil, 5% (w/w) of polysorbate 20 (w/w) and 90% (w/w) of water can be considered a promising larvicidal agent.

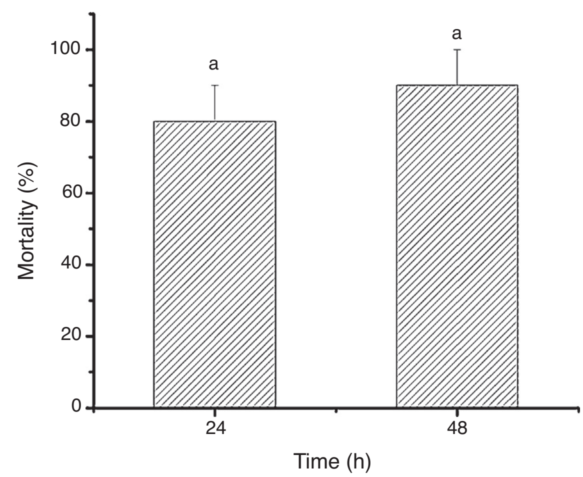

Fig. 4. Mortality levels of *Aedes aegypti* larvae after treatment with nanoemulsion containing *Rosmarinus officinalis* essential oil. Tested concentration – 250 ppm (related to *R. officinalis* essential oil). Columns with the same superscript do not have significant difference.

Conclusion

Natural products have been recognized as valuable sources of insecticidal agents. On this context, many researchers have focused to evaluate these substances as promising tools for integrative control programs. However, studies concerning final formulations remain scarce. Moreover, many natural products have poor water solubility. This fact should be considered, for example, if an effective larvicidal product is desired for *A. aegypti* control, the main vector of dengue, a public health problem in many developing countries. The present study allowed achievement of a nanoemulsion with potential activity against. Moreover, nanoemulsion production involved a non-heating procedure, describing a easy technique which may be useful for integrative control programs.

Authors' contributions

JLD (undergraduate student) and AEMFM (master's student) contributed running the laboratory work, analysis of the data and drafting the paper. AMF contributed in nanoemulsions characterization. RASC and DQF contributed to critical reading of the manuscript. JRRA and RNPS contributed to biological assay and statistical analysis. JCTC and CPF designed the study, supervised the laboratory work and contributed to critical reading of the manuscript. All the authors have read the final manuscript and approved the submission.

Conflicts of interest

The authors declare no conflicts of interest.

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