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Original article

Development of a larvicidal nanoemulsion with Copaiba (*Copaifera duckei*) oleoresin

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Copaiba (*Copaifera duckei* Dwyer, Fabaceae) oleoresin is an important Amazonian raw material. Despite its insecticidal potential, poor water solubility remains a challenge for the development of effective and viable products. Nanotechnology has emerged as a promising area to solve this problem, especially oil-in-water nanoemulsions. On this context, the aim of the present study was to develop oil-in-water nanoemulsions using copaiba oleoresin dispersed through a high internal phase; and evaluate its potential insecticidal action against *Aedes aegypti* larvae. Overall, 31 formulations were prepared, ranging from 11.5 ± 0.2 to 257.3 ± 4.1 nm after one day of manipulation. Some of them reached small mean droplet sizes (< 200 nm) and allowed achievement of a nanoemulsion region. The formulation consisted of 5% (w/w) of copaiba oil, 5% (w/w) of surfactant and 90% (w/w) of water, which presented mean droplet size of 145.2 ±0.9 nm and polidispersity of 0.378 ± 0.009 after one day of manipulation, and these were evaluated for larvicidal potential. According to mortality level (250 ppm - 93.3 after 48 h), this nanoemulsion was classified as a promising insecticidal agent against *Aedes aegypti* larvae. The present study allowed the development of low-cost ecofriendly green natural-based nanoformulations with potential larvicidal activity, using a nanobiotechnology approach.

Introduction

Species from the genus *Copaifera* are distributed throughout the Amazon and Midwestern regions of Brazil and yield a transparent yellow-brownish oleoresin, which is obtained by an incision of the tree stem (Biavatti et al., 2006; Custódio and Veiga-Júnior, 2012). This oleoresin is also referred as copaiba oil and has been used since ancient times by native Indians from Brazil, one of most important folk medicines in the Amazonian region (Santos et al., 2008). Several biological properties have

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been attributed to copaiba oleoresin, including antinoceptive (Gomes et al., 2007), anti-inflammatory (Carvalho et al., 2005; Veiga-Júnior et al., 2007) and antileishmanial (Santos et al, 2008) activities. Moreover, copaiba oil is used as raw material for several products, being exported to many countries, including France, Germany and the United States (Gomes et al., 2007).

Oleoresin from *Copaifera* sp. and its isolated substances have been successfully tested against *Aedes aegypti* (Geris et al., 2008; Leandro et al., 2012; Trindade et al., 2013), which is the biological vector responsible for the transmission of the tropical disease known as "dengue" (Rawani et al., 2013). However, an intrinsic characteristic of the oleoresin, the presence of poor water-soluble substances remains a challenge for the development of a stable product for this purpose.

Nanotechnology has been considered a promising area regarding ecofriendly pesticides, including those with natural products incorporated as active ingredients (Rawani et al., 2013; Angajala et al., 2014). These natural product-based nanoformulations have been developed in order to evaluate their larvicidal potential against several insects, such as *Aegle marmelos, Aedes aegypti, Culex quinquefasciatus, Anopheles stephensi* (Rawani et al., 2013; Angajala et al., 2014; Suganya et al., 2014; Veerakumar et al., 2014). Strategies in the development of pesticides using this approach have been associated to repellent or water-soluble formulations, including nanoemulsions (Nuchuchua et al., 2009). This special type of kinetically stable formulation is constituted by two immiscible liquids and one or more surfactants, with small droplet size (20-200 nm) (Solans et al., 2005; Solè et al., 2012), translucent or transparent appearance and bluish aspect (Forgiarini et al., 2000; Solè et al., 2006). Nanoemulsions have a wide range of applications, including pharmaceutical, cosmetic and for food uses (Solans et al., 2005), also a promising tool to control insects (Wang et al., 2007). The aim of the present study was to develop oil-in-water nanoemulsions with copaiba oleoresin dispersed through the internal phase, and to evaluate its potential insecticidal action against *Aedes aegypti* larvae.

Materials and methods

Chemicals

Copaiba oleoresin (*Copaifera duckei* Dwyer, Fabaceae) was obtained from Beraca Ltda. Sorbitan oleate (HLB: 4.3) and Polysorbate 80 (HLB: 15) were purchased from Praid Produtos Químicos Ltda (São Paulo, Brazil).

Required HLB determination

Each formulation was prepared at a final mass of 25 g, containing 90 % (w/w) of distilled water, 5% (w/w) of copaiba oil and 5% (w/w) of a mixture of emulsifiers (Fernandes et al., 2013, Costa et al., 2014). A series of formulations were prepared using sorbitan monooleate as the most hydrophobic emulsifier, and polysorbate 80 as the most hydrophilic emulsifier. HLB values ranging from 4.3 (5% w/w of sorbitan oleate) to 15 (5% w/w of polysorbate 80) were prepared by blending together these emulsifiers. Sorbitan monooleate:polysorbate 80 ratios were used as follows: 100:0 (HLB 4.3), 93.5:6.5 (HLB 5), 84.1:15.9 (HLB 6), 74.8:25.2 (HLB 7), 65.4:34.6 (HLB 8), 56.9:43.9 (HLB 9), 46.7:53.3 (HLB 10), 37.4: 62.6 (HLB 11), 28.0:72.0 (HLB 12), 18.7:81.3 (HLB 13), 9.3:90.7 (HLB 14), 0:100 (HLB 15). Analysis of creaming and phase separation after one day of manipulation supported the identification of the most stable formulation required for HLB determination.

Emulsification method

Required amounts of both emulsifiers were dissolved in the oil phase (copaiba oil) and heated at 65 ± 5°C. Aqueous phase was separately heated at 65 ± 5°C, gently added to and mixed with the oil phase, furnishing a primary formulation. Final homogenization was achieved using a T25 Ultra-Turrax homogenizer (Ika-Werke, Staufen, Germany) equipped with a 25 N-18 G disperser for 5 min (8000 rpm) (Costa et al., 2014).

Pseudo-ternary phase diagram

Nanoemulsion region was determined using pseudo-ternary phase diagram. Each corner corresponded to 100% of water, surfactant and copaiba oil. Composition (w/w), which allowed required HLB value determination was used as starting point (90% of distilled water, 5% of oil and 5% of surfactants blend) and mean droplet size of each prepared composition was performed in order to determine nanoemulsion region (Fernandes et al., 2014).

Macroscopical analysis

Stability of all nanoemulsions was evaluated immediately and after 1 and 30 days of manipulation by macroscopic analysis, such as color, visual aspect, phase separation, creaming and sedimentation. During this period all nanoemulsions were maintained under room temperature (25 ± 2°C) in screw capped glass test tubes (Fernandes et al., 2013). Nanoemulsion type (oil-in-water or water-in-oil) was characterized by dilution of each formulation in deionized water.

Droplet size analysis

Droplet size and polydispersity of nanoemulsions were determined by photon correlation spectroscopy using a Zetasizer 5000 (Malvern Instruments, Malvern, UK). Each nanoemulsion was diluted using ultra-pure Milli-Q water (1:25). Measures were performed in triplicate and the average droplet size was expressed as the mean diameter.

Larvicidal assay

Aedes aegypti larvae were obtained from the Arthropoda Laboratory of Amapá Federal University (Macapá, Brazil). Biological assay was performed under controlled conditions, where fourth-instar larvae were kept at 25 ± 2°C, relative humidity of 75 ± 5% and a 12 h light:dark cycle. The experimental protocol was performed according to WHO (1970, 1980 and 1984) with some modifications. Different concentrations of copaiba oil were prepared diluting the nanoemulsion with distilled water, ranging from 500 to 200 ppm. One gram of each dilution was added to 99 g of distilled water in a plastic pot. All experiments were performed in triplicate with 10 fourth-instar larvae in each sample replicate. Mean temperature of aqueous media was 25°C. Negative controls were performed using surfactant at the same concentration of the tested samples. Mortality levels were recorded after 24 and 48 h of exposure. Larvae were considered dead when they were not able to reach the water surface.

Statistical Analysis

Significance of the insecticidal data was performed using a G test with 95% confidence interval, using R 3.02 software (R Core Team, 2013). Differences between treated groups and untreated groups were considered significant when $p \leq 0.05$.

Results and discussion

Several formulations were prepared by blending a hydrophobic surfactant (sorbitan monooleate, HLB=4.3) with a hydrophilic surfactant (polysorbate 80, HLB = 15). Most of them (HLB 4.3, 5, 6, 7, 8, 9, 10, 11, 12, 13 and 14) had an unstable behavior after one day of manipulation, such as phase separation or different degrees of creaming, and therefore were discarded. The formulation prepared with 5% (w/w) of polysorbate 80 (HLB = 15) was characterized as an oil-in-water nanoemulsion, and was consider the most stable formulation since it was not observed any creaming, phase separation or macroscopical changes after one day of manipulation. Thus, mean droplet size of this formulation was monitored (Fig. 1), indicating small droplet size and low polidispersity after one day (145.2 ± 0.9 nm) (0.378 ± 0.009) and 30 days (156.5 ± 0.7 nm) (0.286 ± 0.027) of manipulation. Creaming, phase separation or other signals of unstable behavior were not observed even after 30 days, also observing that this formulation presented a bluish reflection, characteristic for nanoemulsions (Forgiarini et al., 2000). A prospective study with copaiba oleoresin performed by Xavier-Júnior et al. (2012) using different blends of surfactants indicated a required HLB value of 14.8 for the oil phase, which is in accordance with our results. Required HLB value of the oil phase is one of most important parameters that should be considered during the development of nanoemulsions (Schmidts et al., 2010; Fernandes et al 2013). It can be determined by calculating the HLB of the surfactant or mixture of surfactants, which allows achieving an emulsion with minimum droplet size, among a set of prepared emulsions (Rodríguez-Rojo 2012, Fernandes et al., 2013; 2014; Costa et al., 2014). Thus, HLB value of copaiba oleoresin used in the present study could be considered around 15.

According to mean droplet size (< 200 nm after 1 and 30 days of manipulation), the nanoemulsion constituted by 5% (w/w) of copaiba oil, 5% (w/w) of polysorbate 80 and 90% (w/w) of water was considered a nanoemulsion. It was used to perform the larvicidal bioassay and as a starting point for the pseudo-ternary phase diagram. Nanoemulsions are also referred as miniemulsions or ultrafine emulsions (Forgiarini et al., 2000; Solè et al., 2006) and have mean droplet size ranging from 20 to 200 nm (Solè et al., 2012). Despite the fact that nanoemulsions are not thermodynamically stable (Forgiarini et al, 2000; Ostertag et al., 2002), they are kinetically stable (Bruxel et al.,

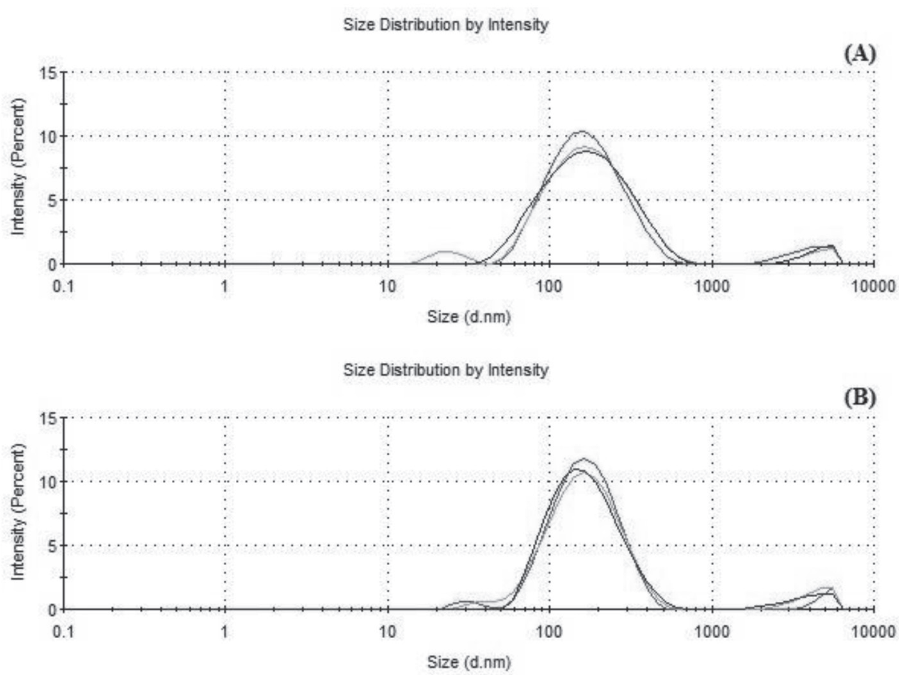

Figure 1 – Particle size distribution of a nanoemulsion constituted by 5% (w/w) of copaiba oil, 5% (w/w) of polysorbate 80 and 90% (w/w) of water. Mean droplet size and polidispersity: (A) 145.2 ±0.9 nm, 0.378 ± 0.009 (after 1 day of manipulation); (B) 156.5 ± 0.7 nm, 0.286 ± 0.027 (after 30 days of manipulation).

2012) and have a long-term physical stability (Solè et al., 2006). Moreover, small droplets can be achieved even at relative low surfactant concentrations (Solè et al., 2012). They have a wide range of advantages, such as solubility enhancement of poor water-soluble substances (Bruxel et al., 2012; Wang et al., 2009; Zhang et al., 2011), including insecticidal natural products (Wang et al., 2007).

The surfactant used during the pseudo-ternary phase diagram construction was polysorbate 80 (HLB - 15), since it coincides with the required HLB value of copaiba oil used in the present study. Overall, the 31 nanoemulsions prepared, ranged from 11.5 ± 0.2 nm to 257.3 ± 4.107 nm after one day of preparation (Table 1). According to the concept that nanoemulsions have a mean droplet size below 200 nm (Solè et al., 2012), it was possible to delineate a nanoemulsion region (Fig. 2), constituted by 25 formulations. The high percentage of nanoemulsions obtained (approximately 80%) may be attributed to heating and mechanical energy achieved by the T25 Ultra-Turrax, which have been associated to an extended oil-in-water miniemulsion region (Mahdi et al., 2011). It was also observed that some formulations within nanoemulsion region were transparent and reached mean droplet size below 100 nm. Considering that microemulsions are isotropic, thermodynamically systems with very small droplet size, usually around 5-100 nm (Pakpayat et al., 2009), further investigations with these formulations (12.5 % (w/w) of surfactant/5% (w/w) of oil/82.5 (w/w) of water; 17.5 % (w/w) of surfactant/2.5% (w/w) of oil/80 (w/w) of water; 20 % (w/w) of surfactant / 5% (w/w) of oil/75 (w/w) of water; 20 % (w/w) of surfactant/2.5% (w/w) of oil/77.5 (w/w) of water) should be carried out to confirm whether they can be classified as microemulsions or nanoemulsions.

It is well recognized that not every composition allows achievement of nanoemulsions (Shakeel et al., 2010; Mahdi et al., 2011). Thus, nanoemulsion region obtained in the present study may be important for further studies with copaiba oleoresin, since these types of formulations could be obtained with compositions within this zone. Mean droplet size and polidispersity after 30 days of manipulation of the nanoemulsions prepared during pseudo-ternary phase diagram are presented in Table 1.

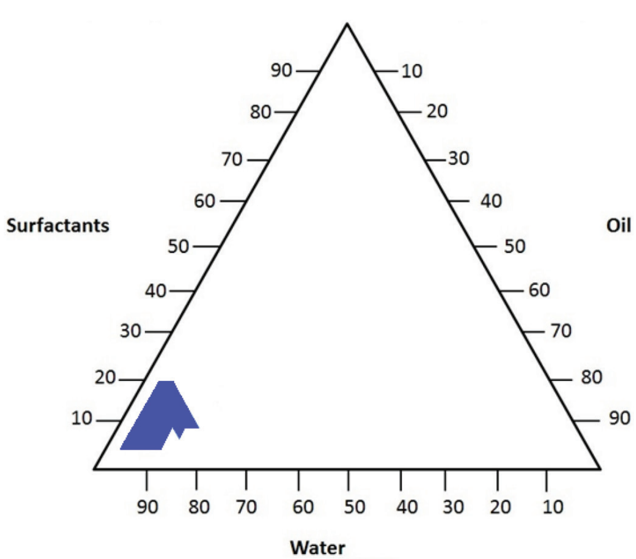

Figure 2 – Pseudo-ternary phase diagram constructed with water, copaiba oleoresin and polysorbate 80 (HLB =15) at different compositions. Nanoemulsion region is delimited in blue.

The oleoresin from *Copaifera multijuga* was also used to prepare nanoemulsions, which were achieved using high-pressure homogenization. Mean droplet analysis revealed droplets around 120 and 140 nm (Dias et al., 2012). Copaiba oleoresins are mainly constituted by sesquiterpene hydrocarbons, which often correspond to almost 90% of the total relative composition of the oil, being beta-caryophyllene considered the main constituent. Previous chemical characterization of the oleoresin used in the present study confirmed this profile (Lima et al., 2011). Diterpenes are also found in smaller quantities in copaiba oleoresin (Sousa et al., 2011). Beta-caryophyllene has also been considered an important substance involved in the pharmacological activities of *Copaifera* spp., and is considered a phytochemical marker and standard for quantification studies (Lima et al., 2011; Dias et al., 2012). The development of a nanoemulsion with copaiba oleoresin is also important for the stability of this sesquiterpene. A reduced degradation of caryophyllene from a nanoemulsion was observed with copaiba oleoresin dispersed through the internal phase after acid hydrolysis, exposure to UV-A irradiation, oxidative (H_2O_2) and thermolytic (60ºC) conditions, when compared to beta-caryophyllene content after exposure to same conditions (Dias et al., 2012).

The natural product-based nanoformulations have been developed in order to evaluate their larvicidal potential, including nickel nanoparticles, silver nanoparticles, nanoemulsions among others (Rawani et al., 2013; Angajala et al., 2014; Suganya et al., 2014; Veerakumar et al., 2014; Sugumar et al., 2014). Larvicidal evaluation of copaiba oleoresin has been related against *Aedes aegypti*, the main vector of dengue, a serious Brazilian health problem (Prophiro et al., 2012). Kanis et al., (2012) achieved microformulations using different polymers which were able to disperse copaiba oleoresin in water. However, despite some of them were able to affect *Aedes aegypti* larvae, mean droplet size ranged from 12100 nm to 578000 nm. To our knowledge, the role of copaiba oleoresin nanoformulations as active agents against *A. aegypti* remains unexplored.

On this context, the present study aimed to evaluate the potential larvicidal activity of a nanoemulsion with copaiba oleoresin, considering that this type of formulation has a mean droplet size below 200 nm. The chosen nanoemulsion within the region obtained in the pseudo-ternary diagram was constituted by 5% (w/w) of copaiba oil, 5% (w/w) of polysorbate 80 and 90% (w/w) of water. The experimental groups treated with concentrations higher than 250 ppm presented mortality levels above 95 % after 24 h and 48 h of observation. It was not possible to estimate LC_{50} of copaiba oleoresin using tested nanoemulsion dilutions, since the lowest concentration (200 ppm) was able to induce 70.0 ± 26.5 % and 90.0 ± 10.0 % of mortality after 24 h and 48 h of experiment, respectively. A mortality of 73.3 ± 11.5% and 93.3 ±11.5% was observed after 24 h and 48 h of , exposure to the nanoemulsion with copaiba

Table 1
Composition, mean droplet size and polydispersity of each formulation prepared during the construction of pseudo-ternary phase diagram for delimitation of nanoemulsion region. All nanoemulsions were maintained under room temperature (25 ± 2ºC) in screw capped glass test tubes.

	Composition			After 1 Day		After 30 Days	
	% of Surfactant (w/w)	% of Oil (w/w)	% of Water (w/w)	Mean diameter ± SD (nm)	Polidispersity ± SD	Mean diameter ± SD (nm)	Polidispersity ± SD
1[a]	5	5	90.0	145.2 ±0.9	0.378 ± 0.009	156.5 ± 0.7	0.286 ± 0.027
2	2.5	5	92.5	235.4 ± 16.3	0.613 ± 0.020	201.0 ± 1.6	0.551 ± 0.028
3[a]	5	2.5	92.5	157.0 ± 2.2	0.432 ± 0.004	124.5 ± 1.4	0.506 ± 0.004
4[a]	7.5	2.5	90.0	171.3 ± 1.6	0.300 ± 0.006	192.6 ± 14.8	0.314 ± 0.046
5[a]	7.5	5	87.5	134.5 ± 1.0	0.271 ± 0.010	139.6 ± 2.1	0.238 ± 0.006
6[a]	5	7.5	87.5	175.5 ± 2.1	0.370 ± 0.006	191.3 ± 7.7	0.388 ± 0.025
7	2.5	7.5	90.0	227.2 ± 6.5	0.561 ± 0.029	266.7 ± 7.1	0.623 ± 0.017
8[a]	12.5	2.5	85.0	116.3 ± 1.2	0.216 ± 0.002	127.6 ± 1.3	0.192 ± 0.008
9[a]	12.5	5	82.5	86.9 ± 2.9	0.520 ± 0.035	119.4 ± 2.8	0.304 ± 0.040
10[a]	10	7.5	82.5	144.6 ± 2.1	0.201 ± 0.010	156.0 ± 0.9	0.220 ± 0.006
11	7.5	10	82.5	215.1 ± 6.0	0.569 ± 0.020	218.8 ± 2.9	0.409 ± 0.012
12[a]	5	10	85.0	169.7 ± 1.0	0.292 ± 0.025	292.9 ± 9.0	0.673 ± 0.057
13	5	12.5	82.5	247.1 ± 6.9	0.598 ± 0.008	246.3 ± 5.0	0.619 ± 0.058
14[a]	7.5	12.5	80.0	182.3 ± 2.2	0.376 ± 0.005	186.2 ± 4.7	0.375 ± 0.009
15[a]	10	10	80.0	144.2 ± 1.4	0.251 ± 0.003	141.2 ± 0.7	0.217 ± 0.008
16[a]	12.5	7.5	80.0	171.3 ± 1.5	0.442 ± 0.036	151.9 ± 0.9	0.277 ± 0.005
17[a]	15	5	80.0	123.2 ± 1.1	0.215 ± 0.009	117.0 ± 0.8	0.253 ± 0.012
18[a]	15	2.5	82.5	101.0 ± 0.9	0.227 ± 0.004	94.7 ± 0.89	0.248 ± 0.011
19	5	15	80.0	257.3 ± 4.1	0.532 ± 0.091	284.7 ± 13.7	0.609 ± 0.026
20	7.5	15	77.5	214.9 ± 1.3	0.391 ± 0.019	316.8 ± 27.5	0.597 ± 0.110
21[a]	10	12.5	77.5	187.5 ± 0.8	0.238 ± 0.009	240.5 ± 15.1	0.402 ± 0.012
22[a]	12.5	10	77.5	157.9 ± 1.7	0.209 ± 0.008	228.5 ± 17.9	0.329 ± 0.030
23[a]	15	7.5	77.5	133.2 ± 1.6	0.454 ± 0.011	119.7 ± 11.0	0.731 ± 0.208
24[a]	17.5	5	77.5	109.7 ± 1.7	0.212 ± 0.010	117.1 ± 2.7	0.191 ± 0.007
25[a]	17.5	2.5	80.0	60.28 ± 2.0	0.506 ± 0.035	63.5 ± 1.1	0.488 ± 0.012
26[a]	10	15	75.0	200.0 ± 2.8	0.268 ± 0.003	198.8 ± 1.3	0.265 ± 0.006
27[a]	12.5	12.5	75.0	188.1 ± 1.4	0.212 ± 0.003	195.6 ± 1.2	0.187 ± 0.011
28[a]	15	10	75.0	177.7 ± 0.6	0.218 ± 0.005	200.8 ± 1.3	0.230 ± 0.013
29[a]	17.5	7.5	75.0	151.1 ± 0.5	0.458 ± 0.006	105.4 ± 10.9	0.428 ± 0.034
30[a]	20	5	75.0	93.0 ± 0.6	0.221 ± 0.008	147.8 ± 4.5	0.334 ± 0.024
31[a]	20	2.5	77.5	11.5 ± 0.2	0.318 ± 0.003	13.9 ± 3.6	0.243 ± 0.102

Oil - *Copaifera duckei* oleoresin.
Surfactant – Polysorbate 80.
[a]Formulation in the nanoemulsion region.

oleoresin concentration of 250 ppm, respectively. Significant differences between treated groups and control groups were detected at all observation periods ($p < 0.05$) (Table 2). Regarding to the mortality levels of larvae after 48 h of treatment with samples at 250 ppm, larvicidal agents can be classified as promising (> 75%), partially promising (> 50% and < 75%), weakly promising (> 25% and < 50%) or inactive (< 25%) (Montenegro et al., 2006). Our results suggest that a nanoemulsion containing copaiba oil may be a promising formulation for *Aedes aegypti* larvae control, which also supports the concept that natural products nanoformulations may be considered an alternative as potential larvicidal agents.

Conclusion

A. aegypti may develop resistance to conventional chemical pesticides, therefore making it important to discover new effective agents against this insect (Suganya et al., 2014). On this context, low cost ecofriendly green natural-based nanoformulations appear as promising insecticidal products (Veerakumar et al, 2014). Oleoresins obtained from several species of the genus *Copaiba* are considered one of most important Amazonian raw materials. Moreover, oleoresin extraction may be considered a sustainable process (Medeiros and Vieira, 2008), contributing to the development of viable

Table 2
Composition, mean droplet size and polydispersity of each formulation prepared during the construction of pseudo-ternary phase diagram for delimitation of nanoemulsion region. All nanoemulsions were maintained under room temperature (25 ± 2ºC) in screw capped glass test tubes.

Group	% Mortality ± SD			
	Treated		Control	
	24 h	48 h	24 h	48 h
200 ppm	70.6 ± 26.5	90.0 ± 10.0	3.3 ± 5.8	6.7 ± 11.5
250 ppm	73.33 ± 11.5	93.3 ± 11.5	3.3 ± 5.8	6.7 ± 11.5
500 ppm	100 ± 0	100 ± 0	0	6.7 ± 5.8

Concentrations are expressed as copaiba oil content (treated groups) or polysorbate 80 (control groups).
Significant differences were observed between treated groups and their respective control groups ($p < 0.05$) at all observation periods.
The percentage of total insects (n = 10) was calculated in each group (in triplicates) at the beginning of the experiment.

products. At the present study, a series of oil-in-water nanoemulsions containing *Copaifera duckei* oleoresin dispersed through internal phase were developed. One of them was considered an effective agent against *A. aegypti*. Moreover, nanoemulsions with increasing amounts of copaiba oleoresin may be obtained from the nanoemulsion region. The present study supports the concept that an oil-in-water nanoemulsions containing copaiba oleoresin may be used as a potential larvicidal.

Authors' contributions

ECRR (undergraduate student) contributed by running the laboratory work, analysis of the data and drafting the paper. ADF, JCEV and FBA contributed in nanoemulsions characterization. RASC contributed to critical reading of the manuscript. ACF 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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