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2^3 central composite rotatable design for the production of neem oil nanoemulsion for antifungal and antiparasitic applications

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Abstract

BACKGROUND: The present study describes the design and production of an oil-in-water nanoemulsion composed of neem oil as the inner phase for use as an antifungal and antiparasitic control in aquaculture. A 2^3 central composite rotatable design adopting surface response was used as the experimental design, setting the neem oil concentration (4–15%), surfactant concentration (0.5–10%) and sonication power (20–90%) as the independent variables, while the droplet size, polydispersity index (PDI) and zeta potential were set as the response (dependent) variables.

RESULTS: Droplet size and PDI showed the best results using the sonication method with 5 min of process. The coefficients of determination were greater than 0.900 for all response. The best formulation was obtained with 9% of neem oil and 5.25% of polysorbate 20.

CONCLUSION: Antifungal activity of the optimized nanoemulsion against *Saprolegnia parasitica* was demonstrated, also attributed to the presence of the surfactant. Concentrations of 200 mg L^{-1} of nanoemulsion resulted in 100% parasitic mortality.

Keywords: emulsions; neem oil; ultrasonic; antifungal; antiprotozoal; experimental design

INTRODUCTION

Natural products derived from plants are valuable sources of new bioactive substances, being increasingly used in the food and pharmaceutical industries, as a result of growing consumer demand for non-synthetic products.[1–4] The natural oils and extracts of medicinal plants have been evaluated for the treatment of various human diseases.[5–8] The prevention and treatment of disease outbreaks in fish culture using natural products has also been considered as an alternative to chemical treatments, to avoid negative impacts on the environment and human health (resistant bacterial strains and residual accumulation in tissue).[9]

Parasitic and microbial infections have been the main problem in aquaculture because of the negative impact on the safety of aquatic foods, generating significant economic losses for this industry.[10] Because of its phytochemical composition in limonoids (e.g. nimbidin, azadirachtin, salannin, meliantriol, and nimbin), neem oil may be an alternative for the treatment of fish diseases. Limonoids are known for their ability to block insect growth, useful in the control of pests in agriculture and contributing to improve human health.[11–13]

Neem (*Azadirachta indica A.* Juss., Meliaceae) is a fast-growing and evergreen tree,[13,14] found in the Southern Hemisphere, that has been used for more than 4000 years as folk medicine. Neem oil fatty acids comprise oleic, stearic, palmitic and linoleic acids, and are mainly used by the pesticide and pharmaceutical industries. Neem oil components show several biological activities, such as anti-inflammatory,[15,16] antipyretic,[17] hypoglycaemic,[17] antiulcer,[18] antitumor,[13] antifungal,[19] antibacterial,[13] antimalarial[20] and as a bioinsecticide.[21]

However, neem oil has low aqueous solubility, limiting its application in aquaculture management. Oil-in-water (o/w) emulsions

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can be used as a strategy to improve the dispersibility of hydrophobic compounds in an aqueous environment.[22,23] Nanoemulsions have been studied to improve the solubility and bioavailability of many lipophilic active compounds.[24–28] Nanoemulsions are isotropic, thermodynamically unstable systems, appearing to have transparency or low opacity (optically or to the naked eye).[23,28] Nanoemulsions are obtained from the combination of oil, water and surfactant, with or without a co-surfactant. As several variables interfere with the formation and stabilization of the system, factorial experimental design is usually recommended for the optimization of the ratios.

In our work, an antifungal and antiprotozoal nanoemulsion composed of neem oil was optimized using a central composite rotatable design (CCRD) setting the oil concentration, surfactant concentration and sonication power as the independent variables, and the droplet size, polydispersity index (PDI) and zeta potential as the dependent variables.

MATERIALS AND METHODS

Materials

Neem oil obtained from Vitaplan (Cascavel, PR, Brazil) was used as a dispersed oil phase. Polysorbate 20 was obtained from BASF (Ludwigshafen, Germany) and used as non-ionic emulsifying agent. Double-distilled water was used upon filtration in a Milli-Q® Plus system, home supplied (Millipore, Darmstadt, Germany).

Methods

Preparation of nanoemulsions

Neem oil was gently dispersed in an aqueous surfactant solution at distinct concentrations. Two methods (magnetic stirring and sonication) were tested to prepare the formulations. Preliminary studies were carried out to identify the ranges of influence of the variables on the formation of nanoemulsions and to obtain a mono-dispersed formulation. A stirring rate of 650 rpm was used to produce nanoemulsions by magnetic stirring at room temperature. For the sonication method, the formulation was prepared using a QR 750 W Ultronique (13 mm tip) with 55% power amplitude, at low temperature (ice bath). For both dispersion techniques, different times (2.5, 5.0, 7.5, 15 and 30 min) have been applied and, after predetermined times of storage (0, 3 7, 14 and 60 days), the droplet size and polydispersity index of each sample were recorded.

Droplet size, polydispersity index (PDI) and zeta potential

Analyses of droplet size and PDI of nanoemulsions were performed in a Zetasizer Nano ZS® (Malvern Zetasizer Nano-ZS-4800, Malvern, UK). The measurements were performed in triplicate at a scattering angle of 173° at 25 °C. The data were computed using the software (DLS, Nano, Version 5.0) provided with the instrument. The droplet size represents the mean droplet diameter of the formulations, whereas the PDI represents the accumulated analysis of width measurements for droplet size distribution. The same equipment was used to measure the zeta potential, which was determined from electrophoretic mobility based on Smoluchowski's equation.

Experimental design

For the experimental design step, the formulations were prepared using 5 min of sonication based on the kinetics studies data results. A central composite rotatable design (CCRD) was used, composed of five levels within each variable, following a symmetric distribution around a central point (Table 1).

Table 1 depicts the independent variables and their analyzed coded levels, while Table 2 shows the different weight percentages of neem oil (X1), Tween 20 (X2) and power ultrasonic amplitude (X3) that were used to obtain the nanoemulsions, as determined by experimental design (CCRD 2^3).

From the analysis of planning data we obtained 17 trials, including three replicates at the central point. The full factorial matrix of CCRD design consisted of 17 runs, comprising eight linear points (runs 1–8, Table 2), six axial points (runs 9–14, Table 2) and three replicates at the center point (runs 15–17, Table 2). Response surface methodology (RSM) was used to investigate the effects of the independent variables on the responses: droplet size (Y1), polydispersity index (Y2) and zeta potential (Y3).

Statistical analysis

The data were analyzed using the software Statistica 7.0 (Statsoft, Inc., Tulsa, OK, USA) according to the significance level established to obtain the mathematical model. The significance of the regression coefficients and the associated probabilities, $p(t)$, were determined by Student's *t*-test; the model equation significance was determined by Fisher's *F*-test. The variance explained by the model is given by the multiple determination coefficient, R^2.

In vitro antifungal activity

The antifungal activity was determined for the formulation selected based on experimental design assays. *Saprolegnia* sp. was isolated and identified as previously described.[29] The fungus was cultivated in potato dextrose agar (PDA) and incubated at 25 °C. Antifungal assays on solid PDA were carried out using a randomized design, with three concentrations, one negative control (without formulation) and in triplicate. The formulations were dissolved in the PDA (final formulation concentration of 2.5%, 5.0% and 10.0%), placed in Petri dishes. A disk of medium with the isolated fungus *Saprolegnia parasitica* (9 mm) was inoculated into the center of each Petri dish, and then the progress of mycelial growth was observed each 24 h over a period of 96 h.

Table 1. Independent variables and their investigated coded levels of a 2^3 central composite rotatable design

	Coded levels				
Independent variable	−1.68	−1	0	1	+1.68
Neem oil (%)	4	6.2	9.5	12.8	15
Surfactant (Tween 20) (%)	0.5	2.4	5.25	8.1	10
Power ultrasonic amplitude (%)	20	34.2	55	75.8	90

+, higher level; 0, intermediate level; −, lower level.

Table 2. Experimental and predicted values of the variables for central composite rotatable design (CCRD 2^3) and obtained mean droplet size, polydispersity index (PDI) and zeta potential (ZP)

Run	Neem oil (%)	Surfactant (%)	Sonication power (%)	Droplet size (nm)	PDI	ZP (mV)
1	−1 (6.2)	−1 (2.4)	−1 (34.2)	199.10	0.267	−6.02
2	−1 (6.2)	−1 (2.4)	1 (75.8)	264.86	0.395	−2.21
3	−1 (6.2)	1 (8.1)	−1 (34.2)	195.53	0.301	−13.03
4	−1 (6.2)	1 (8.1)	1 (75.8)	225.80	0.320	−8.75
5	1 (12.8)	−1 (2.4)	−1 (34.2)	315.00	0.389	−0.251
6	1 (12.8)	−1 (2.4)	1 (75.8)	408.30	0.470	−1.04
7	1 (12.8)	1 (8.1)	−1 (34.2)	408.10	0.417	−0.888
8	1 (12.8)	1 (8.1)	1 (75.8)	438.20	0.399	−1.78
9	−1.68 (4)	0 (5.25)	0 (55)	151.93	0.299	−22.96
10	1.68 (15)	0 (5.25)	0 (55)	581.10	0.502	−3.97
11	0 (9.5)	−1.68 (0.5)	0 (55)	365.76	0.461	−1.55
12	0 (9.5)	1.68 (10)	0 (55)	312.90	0.408	−4.25
13	0 (9.5)	0 (5.25)	−1.68 (20)	259.53	0.394	−3.35
14	0 (9.5)	0 (5.25)	1.68 (90)	294.90	0.400	−1.78
15	0 (9.5)	0 (5.25)	0 (55)	172.66	0.313	−16.6
16	0 (9.5)	0 (5.25)	0 (55)	185.40	0.306	−18.9
17	0 (9.5)	0 (5.25)	0 (55)	188.13	0.305	−19.7

Mycelial growth was determined by means of the perpendicular diameters.

In vitro antiparasitic activity

Tambaqui (*Colossoma macropomum*) fingerlings, naturally infected with *Ichthyophthirius multifiliis*, were obtained from a commercial fish farm. Infected fish (4–5 g) were gently scraped to remove trophonts from their skin for *in vitro* assays. The isolated trophonts were washed several times with dechlorinated tap water to remove the fish mucus and then used to perform the assay. The experimental design was completely randomized, with four concentrations (50, 100, 150 and 200 mg L^{-1}) of nanoemulsions (oil neem 4%, Tween 20 and water sufficient for 100% of the formulation), one control (control water + Tween 20) and in triplicate. This mixture was vigorously stirred in a vortex until complete homogenization. A dispersion containing the nanoemulsions or the isolated compound mixture was added to a 5 mL Petri dish with 15 parasites each, which were exposed for 1 h. After 1 h of exposure to nanoformulations, the parasite mortality was measured using a viability test by staining the cells with SYBR-14 fluorescent and propidium iodide (PI) probes (Molecular Probes®, Eugene, OR, USA). The parasites were transferred to concavity slides and, in each well, 2.5 µL SYBR-14 was added for 5 min, followed by 2.5 µL PI for an additional 5 min. Visualization was performed using an epifluorescence microscope (Eclipse 50i, Nikon, Tokyo, Japan).

RESULTS AND DISCUSSION

The influence of the production time used in both methods was evaluated using droplet size and PDI as variable responses. As shown in Fig. 1(A), the emulsions obtained by magnetic stirring presented high droplet size (>1000 nm), regardless of the production time. When using the sonication method, however, nanoemulsions of droplet size < 600 nm were obtained. The higher the production time, the lower the droplet size ($P < 0.001$) (Fig. 1(A)). Regarding the PDI results (Fig. 1(B)), the formulations prepared applying 5 min for the homogenization presented the lowest polydispersity (0.111 and 0.189 for sonication and magnetic stirring, respectively). For both methods, the PDI increased with increase in production time (≥7.5 min). The use of sonication resulted in formulations of lower PDI than those prepared with magnetic stirring ($P < 0.001$), requiring 15 and 30 min to homogenize. The size of the droplets obtained from sonication results from cavitation, turbulence and shear forces generated in the process. The higher the applied energy, the smaller were the resulting droplets. The applied energy is also dependent on the

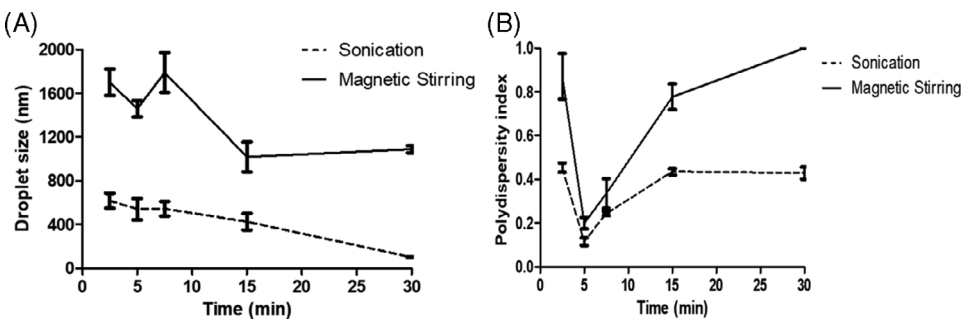

Figure 1. Droplet size (A) and polydispersity index (B) of the emulsions prepared applying sonication *versus* magnetic stirring for dispersing neem oil in an aqueous surfactant solution, at predetermined time intervals, up to 30 min.

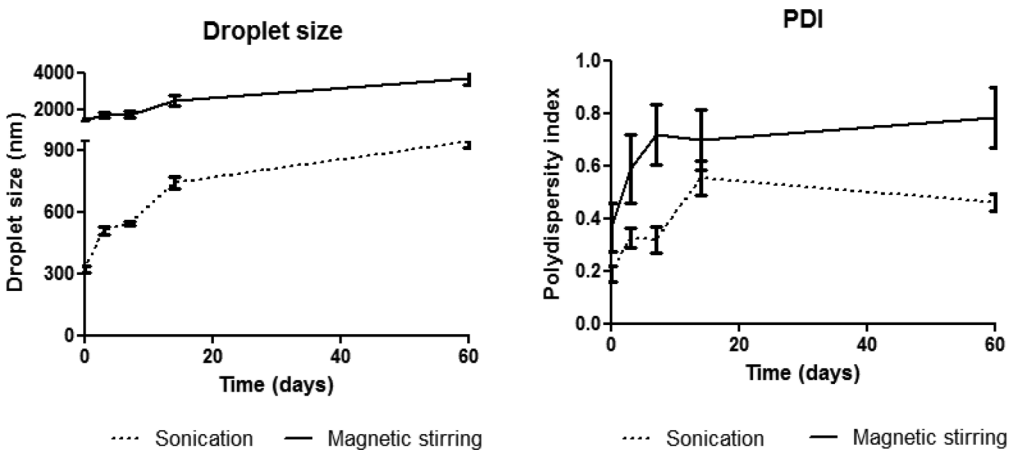

Figure 2. Droplet size (A) and polydispersity index (B) of the emulsions prepared under a magnetic stirring method and sonication at different storage times.

viscosity and interfacial tension of the formulations, requiring higher energy for highly viscous systems, which may also increase the cost of production.

The droplet size of formulations obtained after 5 min of sonication increased over 60 days of storage. The PDI also changed over time (Fig. 2). Although 5 min is sufficient time to obtain nanoemulsions, the formulation can be stabilized by improving other variables, such as surfactant concentration, sonication power intensity and oil concentration.

The values used in the design of experiments (Table 1) were established from preliminary tests conducted by our research group. The most stable and translucent formulation was obtained using an oil:surfactant ratio of 1:3 and 3% oil concentration. For a higher formulation efficiency, the concentration of oil should be as high as possible in the final product, as the biological activity is very much dependent on the amount of neem oil available for an antifungal and antiparasitic effect. The increase in the oil phase will require changes in the surfactant concentration as well, in order to ensure the thermodynamic stability of the system. To determine the optimized concentration of phases, a 2^3 central composite rotatable design (CCRD) was used. Since the kinetic studies showed that magnetic stirring was not suitable to obtain nanoemulsions, the experimental design was done using sonication over a period of 5 min. The central point of the factorial design (run 15–17) was established according to a kinetic study using 9% neem oil, 5.25% Tween 20 and a sonication frequency of 55% (412.5 W). Table 2 lists the required 17 experiments for the development of the formulations, with the corresponding response variables. Runs 1–8 are shown as linear points that are good for mathematical model determination and the statistical parameters related to the 17 experiments. Runs 9–14 are shown as the axial points for the construction of the quadratic model and from 15–17 are the central points (triplicate) to obtain the experimental error.[30–33]

Formulations with a droplet size under 500 nm were obtained for most of the runs, pointing to the formation of nanoemulsions.[34] An increase in oil concentration (run 10–15% oil) or decrease in surfactant concentration (run 11–0.5% surfactant) favored an increase in droplet size. For the same runs (runs 10 and 11) the PDI also increased, showing a lower homogeneity of droplet size.[30]

Table 3. Estimated effects of the 2^3 central composite design rotatable (CCRD) for the nanoemulsion formulation for values of linearity (L) and quadratic (Q)

	Effect			*P*-value		
Variable	Droplet size (nm) (Y1)	Polydispersity index (Y2)	Zeta Potential (mV) (Y3)	Droplet size (nm) (Y1)	Polydispersity index (Y2)	Zeta potential (mV) (Y3)
Mean	184.218	0.311	−18.485	0.000666*	0.000065*	0.002507*
Oil (*O*) L	210.349	0.107	8.492	0.000451*	0.000482*	0.010356*
Oil (*O*) Q	115.578	0.044	4.080	0.001806*	0.003391*	0.051010
Surfactant (*S*) L	−5.682	−0.025	−2.851	0.331406	0.008546*	0.082011
Surfactant (*S*) Q	96.355	0.068	11.550	0.002595*	0.001433*	0.006817*
Frequency power (*F*) L	36.413	0.032	1.325	0.014729*	0.005314*	0.267523
Frequency power (*F*) Q	52.433	0.042	11.787	0.008685*	0.003800*	0.006549*
O by *S*	48.975	−0.000	3.043	0.013917*	0.886039	0.115998
O by *F*	14.410	−0.021	−2.442	0.132350	0.020870*	0.164940
S by *F*	−32.240	−0.052	0.091	0.031267*	0.003495*	0.943082

* Statistically significant at 95% confidence level.

The effects of the significant variables in the nanoemulsion process were analyzed using statistical *P*-values and Student's *t*. Table 3 shows the results obtained. As expected, the size of the droplets is directly proportional to the oil concentration and applied sonication frequency, showing positive values. On the other hand, the surfactant is inversely proportional to the oil (negative value), influencing the droplet size; i.e. the greater the amount of surfactant and the smaller the amount of oil, the smaller the droplet size. The higher oil concentration promoted an increase in droplet size owing to the decrease in interfacial tension.[35] The size of the formed droplets was likely to increase with increasing oil fraction and, owing to the increase in interfacial tension, without influencing the energy of the system.[36] Concerning the surfactant, the results confirm that its low concentration compromises the droplets' stability, thus increasing the risk of coalescence.[33]

Considering the values obtained from the dependent variables for droplet size effect, we can observe a significant interaction ($P < 0.05$) between oil and surfactant and between surfactant and sonication frequency. However, the interaction between oil and sonication frequency was not statistically significant. For the interaction between surfactant and frequency a negative result was recorded, highlighting that this interaction is inversely proportional to the droplet size. This effect has been attributed to the influence of the viscosity and interfacial tension exposed by the increase in surfactant in the process, requiring greater sonication frequency to be able to form the droplets.[37] The lowest droplet size was found for run 9, also showing the lowest zeta potential (−22.96 mV), suggesting that this condition would be an interesting option. With respect to the polydispersity index, all variables showed a significant difference in the dispersion of droplets ($P < 0.05$), except for the oil–surfactant interaction. The interaction of surfactant and oil with the frequency of sonication was shown to be significant during homogenization and formation of droplets. This has been attributed to the energy given to the system that generates a greater shear, and consequently a greater trend for breaking of the droplets, in obtaining nanoemulsions. Larger, more spherical drops will typically flow more easily than smaller or distorted droplets, which tend to stick together.[35,37]

Concerning the zeta potential, most of the formulations presented values close to 0, favoring the risk of coalescence of the droplet and creaming.[38] The best results were found at the central point (zeta potential between −16.6 and −19.7 mV) and on run 9 (−22.96 mV). The ideal value should be lower than −30 mV to

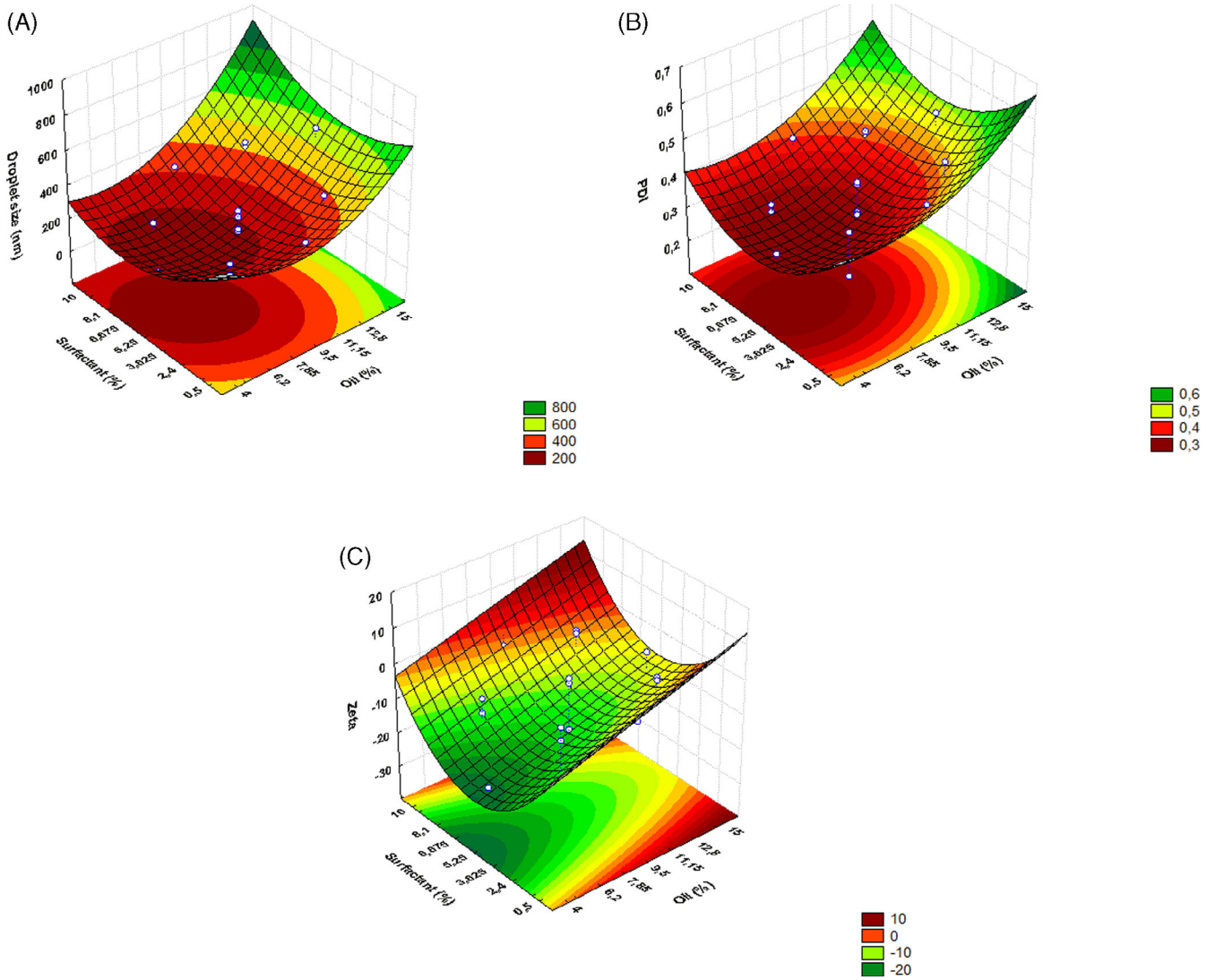

Figure 3. Fitted response surface for the nanoemulsion (55% power amplitude): droplet size (A), PDI (B) and zeta potential (C).

Table 4. Variance droplet size (Y1), polydispersity index (Y2) and zeta potential (Y3) analysis for validation of mathematical models (ANOVA)

	Sum of squares			Degrees of freedom			Mean square			Tabulated *F*			Calculated *F*		
Factor	Y1	Y2	Y3	Y1	Y2	Y3	Y1	Y2	Y3	Y1	Y2	Y3	Y1	Y2	Y3
Regression	212 125.4	0.0673	796.96	7	8	3	30 303.62	0.008	265.65	3.29	3.43	3.41	24.23	6.98	23.81
Residuals	11 252.8	0.009	145.04	9	8	13	1250.31	0.001	11.15					*P*-value	
Lack of fit	11 116.5	0.009	139.86	7	6	11	1588.06	0.001	12.71						
Pure error	136.4	0.000	5.18	2	2	2	68.18	0.000	2.59				Y1	Y2	Y3
Total	223 378.2	0.076	942.00	16	16	16							3.77E−05	0.006297	1.48E−05

obtain the best stability,[39] which was obtained with run 9. Zeta potential expresses the surface electrical charge of droplets in the emulsion. It provides insights into the charge interactions that may occur between the droplets during the formation of an emulsion by electrostatic repulsion or attraction. The electrostatic repulsion delays coalescence and flocculation and, consequently, phase separation. Therefore, zeta potential is identified as an indicator of the stability of emulsified systems.[40] In the analysis of the zeta potential, the oil concentration (linearity), surfactant and frequency power (quadratic) showed statistically significant outcomes ($P < 0.05$). However, no significant interaction ($P > 0.05$) was observed between oil–surfactant, surfactant–sonication frequency or oil–sonication frequency (Table 3). Although no interaction was observed in the range of studied variables, the proportion of oil:surfactant changed from 19:1 (run 11) to 0.76:1 (run 9), directly affecting the results. Both formulations were prepared applying the same sonication frequency and, as expected, the formulation with higher oil proportion (run 11) presented a lower zeta potential (−1.5 mV) compared to lower oil proportion (run 9), which showed −22.96 mV. According to Rinaldi *et al.*,[41] neem oil nanoemulsions presented more negative zeta potential values when the concentration of surfactants was increased. The zeta potential of the nanoemulsion indicates a degree of repulsion between the components with similar charge droplets.[42]

The effect of variables on the production of nanoemulsions under ultrasonic methods in relation to droplet size, PDI and zeta

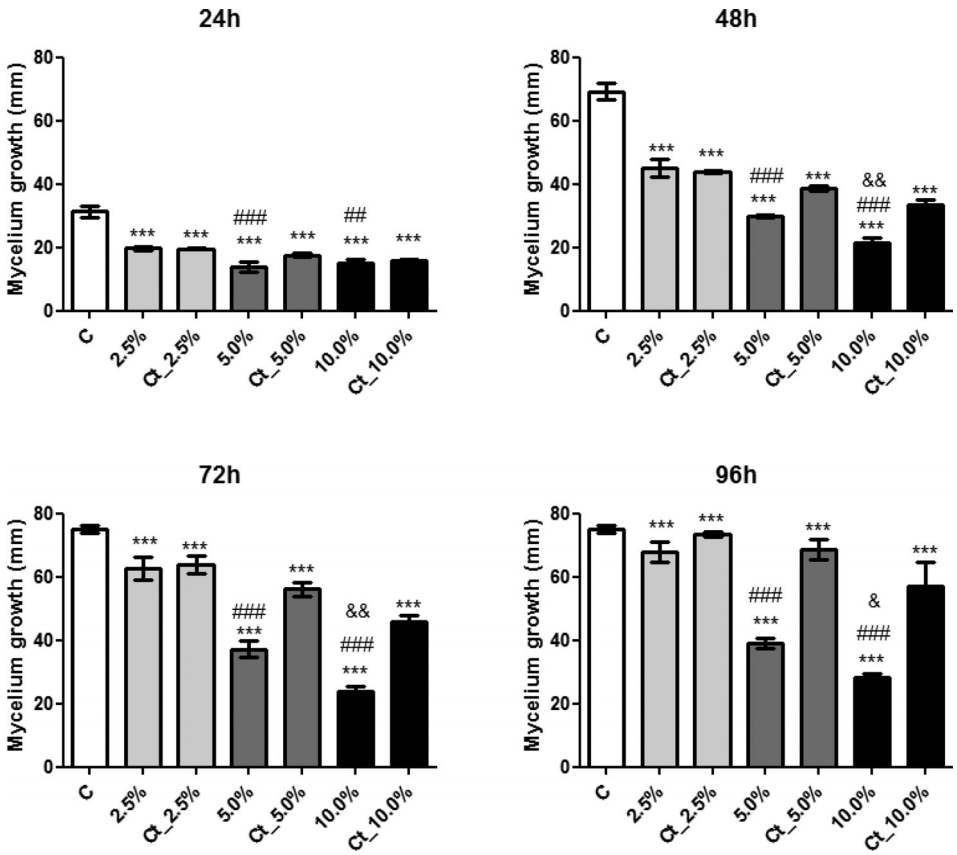

Figure 4. Antifungal activity of nanoemulsions (run 9) on mycelium growth of *Saprolegnia* sp. C, control group (distilled water treatment); 2.5%, 5.0% and 10%: test groups (treatment with 2.5%, 5.0% and 10.0% of nanoformulation); Ct_2.5%, Ct_5.0%, Ct_10.0%, control surfactant groups (treatment with Tween 20 at the same concentration used in 2.5%, 5.0% and 10.0% of nanoformulation). *Significant difference with C; #significantly lower than 2.5%; &significantly lower than 5.0%. ANOVA, Tukey $\alpha = 0.05$.

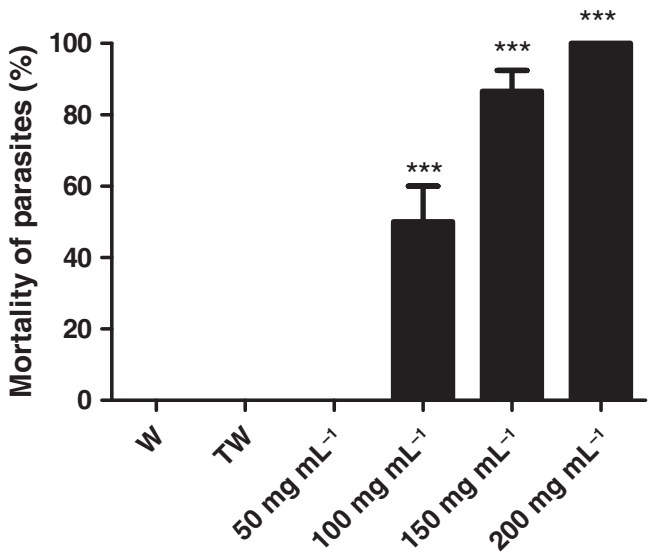

Figure 5. Antiprotozoal activity of nanoemulsions (4% of neem oil; 5,25% Tween 20®) at concentrations of 50, 100, 150 and 200 mg L^{-1} on viability of parasites. W, negative control group (distilled water treatment); TW, vehicle control group (Tween 20® treatment). ***$P < 0.001$, significant difference with W and TW, ANOVA, Tukey $\alpha = 0.05$.

potential can be clearly observed in the surface response plots (Fig. 3(A,B,C) respectively).

Experimental data were fitted to the model and the adequacy to the model was performed by analysis of variance and the parameter R^2 ($Y = ax + b$). The statistical test of models was performed by Fisher's statistical test for analysis of variance (Table 4).

The *F*-values calculated for droplet size (Y1), polydispersity index (Y2) and zeta potential (Y3) were highly significant and higher than the tabulated *F* (Table 4). The reliability of a model can be checked through the coefficients of determination (R^2) and correlation (*R*). Coefficients of determination (R^2 = 0.9496 for droplet size, R^2 = 0.87477 for PDI and R^2 = 0.96402 for zeta potential) imply that the sample variations of 94%, 87% and 96% in the nanoemulsion formulation are attributed to the independent variables and can be accurately explained by the model. The *R* (coefficients of correlation) values (0.97447 for droplet size, 0.93529 for PDI and 0.98184 for zeta) suggest a satisfactory representation of the process by model and a good correlation between experimental and theoretical values provided by the model equation.[43] Equations (1), (2) and (3) represent the mathematical model, depending on the variables obtained from experimental data:

$$\text{Droplet size}: 184.218 + 210.349\times O + 36.413\times F + 115.578\times O^2 + 96.355\times S^2 + 52.433\times F^2 + 48.975\times O\times S - 32.240\times S\times F \quad (1)$$

$$PDI: 0.311 + 0.107\times O - 0.025\times S + 0.032\times F + 0.044\times O^2 + 0.068\times S^2 + 0.042\times F^2 - 0.021\times O\times F - 0.052\times S\times F \quad (2)$$

$$\text{Zeta potential}: -18.485 + 8.492\times O + 11.550\times S^2 + 11.787\times F^2 \quad (3)$$

Saprolegniasis, caused by *Saprolegnia* infection, is one of the most common diseases in freshwater fish,[44] and it has been selected as *in vitro* model. The effects of nanoemulsion administration (run 9) on fungal growth at different concentrations (2.5%, 5.0% and 10.0%) are shown in Fig. 4.

All groups treated with nanoformulation or with the surfactant showed a lower mycelium growth compared to the control group. After 48 h, although the surfactant-treated groups still presented activity compared to control, the nanoemulsion-treated groups at 5.0% and 10.0% showed better performance related to surfactant groups. Commercial use of antibiotics in aquaculture needs to be reduced and replaced by other equally effective and non-resistance-causing natural and bioactive products. Neem nanoemulsions have already demonstrated fungicidal activity against strains of *Rhizoctonia solani* and *Sclerotium rolfsii*, with lethal dose 50 varying from 13.67 to 109.71 mg L^{-1}.[45] Aqueous and ethanolic extracts from neem have already demonstrated efficacy in the treatment against *Aphanomyces invadans* at concentrations of 1% and 0.2%, respectively.[46]

The effects of the nanoemulsion administration (run 9) on antiprotozoal activity at different concentrations (50, 100, 150, 200 mg L^{-1}) are shown in Figs 5 and 6. In the antiprotozoal activity against parasites (*Ichthyophthirius multifiliis*), it can be observed that at a concentration of 200 mg L^{-1} of nanoemulsion there was 100% death of the

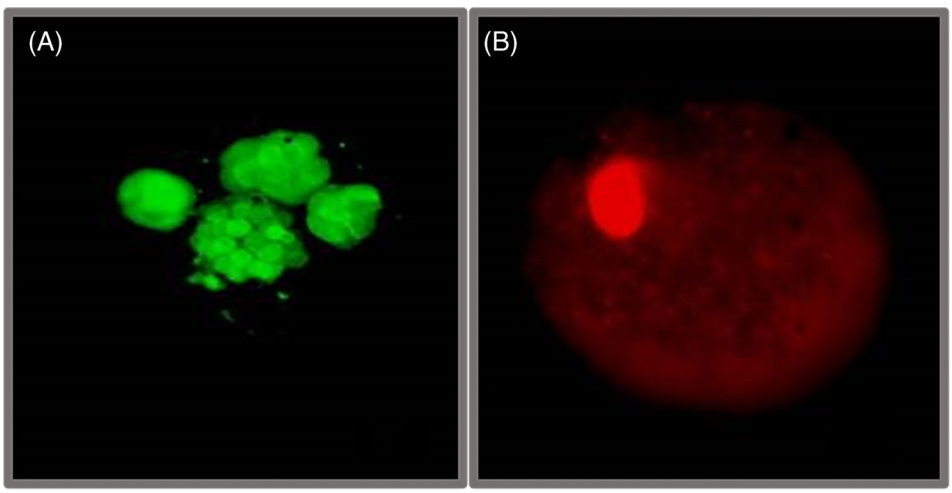

Figure 6. *Ichthyophthirius multifiliis* stained with fluorochromes SYBR-14 and propidium iodide (PI): (A) showing live parasite (B) showing damage in cellular membrane and parasite death.

parasites. Our study also demonstrates that the nanoemulsion content of neem oil at a concentration of 200 mg L^{-1} was active against *I. multifiliis*. Confirmation of the antiprotozoal potential of the nanoemulsion content neem oil qualifies this formulation as a source of promising raw material for commercialization of bioproducts to control parasitic disease in fish.

CONCLUSIONS

An efficient experimental design for nanoemulsion formulation through the ultrasound method, using a shorter process time and higher amount of oil, was developed. The mathematical model suggests a satisfactory representation of the process and good correlation between experimental results and theoretical values provided by the model equation. The nanoemulsion showed a potential antifungal and antiprotozoal activity, and the surfactant Tween 20 also contributed to this activity.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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