RESEARCH ARTICLE

Nanoemulsions Based on Linseed Oil Containing Encapsulated Ethyl Butylacetylaminopropionate for Repellent Application

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Abstract

Introduction: Mosquito-borne diseases are a global problem that causes deaths every year. Using synthetic repellents as a form of protection against diseases caused by mosquitoes can pose a risk to both the environment and human health due to their toxicity and low biodegradability. The present work aims to develop nanoemulsion formulations for the co-encapsulation of clove essential oil and ethyl butylacetylaminopropionate (IR3535®).

Methods: The formulations were prepared by the high energy method, thermodynamic stability tests of the nanoemulsions were carried out, the most stable formulation was characterized in terms of diameter, zeta potential, polydispersity index, encapsulation efficiency, morphology and rheology.

Results: The most stable nanoemulsion had a diameter of 188 ± 2 nm, -39 ± 1 mV zeta potential and 0.118–1.120 polydispersity index. The encapsulation efficiency was 61.33 ± 0.84%. In terms of morphology, the nanodroplets were spherical and without aggregates, typical of stable nanoemulsions. The rheological study identified the formulation as a pseudoplastic non-Newtonian fluid.

Conclusion: Therefore, the nanoemulsion containing clove essential oil and ethyl butylacetylaminopropionate was successfully obtained with stability characteristics, being promising as a topical nanorepellent with smaller amounts of synthetic active ingredient.

Keywords: Nanoemulsion, Repellent, Ethyl butylacetylaminopropionate, Clove oil, Stability.

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Introduction

Mosquito-transmitted diseases have seen steady growth in recent years and have caused epidemics across the world. The important diseases transmitted by mosquitoes are malaria, yellow fever, dengue, chikungunya and zika.[1] To control and prevent diseases transmitted by mosquitoes, personal protection measures against mosquito bites are suggested, such as the use of topical repellent cosmetics.[1]

The most used insect repellents applied to the skin registered with ANVISA (National Health Surveillance Agency) generally contain the following synthetic active ingredients: N, N-diethyl-m-toluamide (DEET), ethyl butylacetylaminopropionate (IR3535®) and picaridin or icaridina.[2] However, synthetic repellents can pose a risk to both the environment and human health due to their toxicity and low biodegradability.

There is a growing interest in repellents based on natural active ingredients such as essential oils.[3,4] There are also registered natural products containing plant extract or oil from plants of the genus *Cymbopogon* (citronella) as active substances; however, they do not have the same duration of action as synthetic active ingredients.[2]

Clove oil, from *Syzygium aromatum, Eugenia caryophyllata,* or *Eugenia aromatum*, has been widely used in foods, cosmetics, medicines, and insect repellents. The main components of clove oil are eugenol, eugenol acetate and caryophyllene. Clove oil has been approved for use in dentistry as an analgesic, cement, fragrance in personal care, and transdermal drug delivery systems by the Food and Drug Administration (FDA).[5]

Repellent essential oils associated with synthetic active ingredients in repellent cosmetic formulations can be presented as a strategy for reducing the amount of synthetic active ingredients in the formulation. Furthermore, researchers have tried to nanoencapsulate synthetic repellent active ingredients to use a smaller amount of active ingredients due to controlled release, such as nanocapsules and nanoemulsions.[6,7]

The preparation of a nanoemulsion involves mixing two immiscible liquids, such as oil and water, stabilized by a surfactant to produce spherical droplets with sizes ranging from 20 to 500 nm.[8] The stability of a nanoemulsion depends on the preparation method and the type and amount of surfactant. The preparation methods are high and low energy. The high-energy method provides intense, disruptive forces to promote the mixing oil, water and surfactant into small droplets.[9] The low energy method involves the phase inversion method.[10]

As the oily phase of the nanoemulsion in this work, linseed oil (*Linum usitatissimum*) was used, which has a high content of di- and tri-unsaturated esters and is susceptible to polymerization reactions when exposed to oxygen in the air. This autooxidation polymerization results in increased viscosity and hardening of the oil.[11] And it is used in nanoemulsions to encapsulate organic active ingredients.[8]

Several nanostructured strategies are reported for the encapsulation of repellent active ingredients. Gomes and collaborators (2018) encapsulated DEET via miniemulsion polymerization, successfully promoting the controlled release of this active ingredient.[12] Abrantes and collaborators (2022) synthesized polymeric nanoparticles based on IR3535® and geraniol with polycaprolactone, managing to reduce the quantity in relation to the quantity in commercial products, reducing toxicity and increasing the time of action.[6] Shehabeldine and collaborators (2023) prepared a clove essential oil nanoemulsion using Tween 80 as a surfactant.[13]

IR3535® (ethyl butylacetylaminopropionate) is a synthetic insect repellent whose structure is similar to that of the natural substance β-alanine (Figure 1).

IR3535® has been shown to be effective against several types of insects and is considered an alternative repellent to DEET and icaridin thanks to its low toxicity and equal effectiveness in humans.[14] In repellent cosmetic lotions this active ingredient is used in concentrations of 10%.[15] However, studies have shown that there are traces of IR3535® in wastewater and the chlorination product of this active ingredient can generate mutagenic products.[14]

Therefore, studies that develop formulations with smaller amounts of synthetic-repellent active ingredients are relevant.

In view of the above, the present work presents the development of nanoemulsion formulations for the co-encapsulation of clove essential oil and IR3535® as a proposal for use as a topical repellent cosmetic with a lower synthetic active ingredient content compared to commercial repellents.

Materials and Methods

Syzygium aromaticum (clove) essential oil and *L. usitatissimum* (linseed) seed oil were acquired from previous studies and previously characterized. The surfactant triblock copolymer polyethylene oxide-polypropylene oxide (Pluronic® F127, molecular mass 12.5 kDa, 99% purity) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT reagent) were purchased from Sigma-Aldrich® (St. Louis, USA). Acetonitrile (HPLC grade) were purchased from Tedia® (Ohio, USA). Deionized water was obtained from the Milli-Q Millipore® Corporation (Watford, UK) purifier. Cosmetic-grade ethyl butylacetylaminopropionate (IR3535®) was purchased from Xi'an Quanao Biotech Co. Ltd. (Xian, China). Other reagents used in the analyses were of analytical grade.

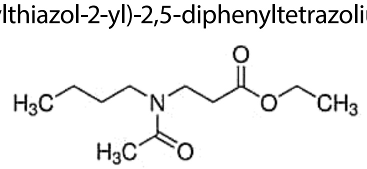

Figure 1: Molecular structure of ethyl butylacetylaminopropionate

Synthesis of nanoemulsion

Formulations were prepared varying the amount of surfactant and IR3535®. The amounts of linseed oil and clove essential oil are kept constant, as shown in Table 1.

Initially, IR3535® was added to the linseed oil while stirring. Then it was left for 2 days under airflow to favor the polymerization of the linseed oil and facilitate the incorporation of IR3535®, then the essential oil was added under stirring in the Ultra-Turrax at 10,000 rpm s min. Then, the aqueous phase already containing the solubilized Pluronic® F127 was added under stirring. Next, the dispersion was subjected to ultrasonication in a Branson Sonifier W-450D Ultra Sonicator (Hielsher, Germany) at 70% amplitude for 2 min under an ice bath in cycles of 5 s on and 5 s off, finally obtaining the nanoemulsion.

Thermodynamic stability test of nanoemulsions

The nanoemulsions were subjected to different time and temperature conditions to evaluate stability, according to Rebouças and collaborators (2022). In the thermodynamic stability study, the nanoemulsions were evaluated by three heating-cooling cycles at 45 and 4°C for 48 hours and monitored for any changes in appearance, including color change, precipitation and phase separation. The nanoemulsions were centrifuged at 3500 rpm for 30 minutes to identify phase separation or precipitation. Finally, the freeze-thaw cycle was performed six times with alternating freeze-thaw cycles at -20 and 25°C for 24 hours at freezer and room temperature, respectively, to check for any changes in appearance, including phase separation. , precipitation and color change.[8]

Characterization of nanoemulsions

The size of the nanoparticles, estimated by the hydrodynamic mean diameter, the polydispersity index (PdI) and the zeta potential (ζ), were determined by dynamic light scattering (ELD) and Doppler microelectrophoresis using a NanoZS® (Malvern Instruments, Worcestershire, UK).

Table 1: Percentage composition of nanoemulsion formulations

Composition	*Percentage*					
	NE-1	*NE-2*	*NE-3*	*NE-4*	*NE-5*	*NE-6*
Clove essential oil	2	2	2	2	2	2
Linseed oil	5	5	5	5	5	5
IR3535®	3	2	1	3	2	1
Pluronic® F127	2	2	2	3	3	3
Deionized water	88	89	90	87	88	89

NE- Nanoemulsion

The effective charge density of droplets dispersed in the aqueous phase can be inferred from electrophoretic mobility measurements, that is, obtained through laser Doppler velocimetry. The electrophoretic mobility is directly proportional to the zeta potential, ζ, which gives a measure of the effective charge of the droplets according to the Henry equation[16]:

$$ue = \frac{2\varepsilon\zeta\, f(ka)}{3\eta} \quad \text{(Equation 1)}$$

Where, ue is the electrophoretic mobility, ε is the dielectric permittivity, η is the viscosity of the continuous phase, ζ is the zeta potential and f(ka) is the Henry function.

ELD measurements were performed at 25°C at scattering angle θ = 90° after dispersing 50 µL of the nanoemulsions in 5.0 mL of Milli-Q water.[17] The analysis of the intensity autocorrelation functions allows estimating the apparent diffusion coefficient (Dapp) which for spherical scatterers diffusing in a continuous Newtonian medium allows estimating the size of the droplets as the apparent hydrodynamic diameter (dh) by the Stoke-Einstein relation[16]:

$$dh = \frac{Kb\, T}{3\pi\, \eta\, Dapp} \quad \text{(Equation 2)}$$

Measurements were performed in triplicate at 25°C and with comparable conductivity to determine the zeta potential.

Encapsulation Efficiency

Encapsulation efficiency (EE) was obtained from a Turbo 15 Vivaspin ultrafiltration tube (Sartorius, Gottingen, Germany) at 4000 rpm for 30 min, with a cutoff of 3000 Daltons. 1 mL of nanoemulsion was added to the tube. After centrifugation, the liquid collected from the bottom was analyzed by high-performance liquid chromatography (HPLC).[8]

The analysis of the encapsulation efficiency of IR3535® was quantified by HPLC on a Shimadzu CTO-20 chromatograph (Shimadzu Corporation, Kyoto, Japan) under isocratic elution, using a C18 column, 5 µm x 4.6 mm x 150 mm (Phenomenex, USA) according to Pinto and collaborators (2017).[18] The mobile phase consisted of water and acetonitrile in a ratio of 50:50 v/v. The analysis was carried out at 40°C. Detection was set at 218 nm with a flow rate of 1.0 mL.min-1 and an injection volume of 20 µL. For the calibration curve, working standard solutions of IR3535® were prepared in the concentration range of 10 to 100 ppm. The observed retention time was 3.9 minutes.

Thus, the encapsulation efficiency of IR3535® was determined by the difference between the initial value of active added to the nanoemulsion formulation and the free amount observed after the filtration process divided by the initial amount determined by HPLC.

Transmission Electron Microscopy

The morphology of the nanoemulsion was observed by transmission electron microscopy TEM JEOL JEM 1101 (Tokyo, Japan, Jeol Corporation) at 40 kV. One drop was placed on a copper grid with Formvar, 200 mesh, and stained with 50 µL of 2% phosphotungstic acid. The stained sample was allowed to dry at room temperature for 10 minutes.

Rheological study

The rheological properties of the nanoemulsion were determined using cone and plate geometries (40 mm inch diameter; 1° cone angle and 27 µm range at 25°C) on AR Rheometer 2000 (TA Instruments, New Castle, USA), the software used was Rheology Advantage Instrument Control AR.[19] The power law was applied to determine the type of fluid using the equation:

$$\tau = K\gamma^{f} \quad \text{(Equation 3)}$$

Where τ is the shear stress, γ is the shear rate, K is the consistency index and f is the flow index.

Results And Discussion

The nanoemulsions were formulated so as not to exceed 10% of the organic phase (IR3535®, clove essential oil, and linseed oil). In previous works, a size between 160 to 240 nm was observed for emulsions with linseed oil formulations and Pluronic® F127 for the encapsulation of betulinic acid with an oil phase content of 10%.[8]

Particle size has a great effect on the transdermal transport of nanoemulsions. Nanodroplets with an average size of 200 nm show moderate transdermal permeation effects, ensuring non-penetration into deeper layers of the skin, which is desirable for a topical repellent, while particles smaller than 80 nm reach deeper layers.[20] The droplet sizes obtained in this work (188 ± 2 nm) were favorable for repellent application, offering no risk of permeation into deeper layers of the skin.

Pluronic® F127 was selected as a surfactant as it is non-ionic, which reduces the possibility of skin irritation.[21] Flaxseed oil was used in the preparation of the formulations because it is a fixed oil with a high iodine content and drying properties, which can favor the incorporation of active ingredients through polymerization. The drying of linseed oil is a chemical process that occurs spontaneously when exposed to air, due to a process of autooxidation followed by polymerization.[22]

Thermodynamic stability tests are used to diagnose metastable formulations in the development process. Temperature changes (hot and cold cycles) and mechanical stress conditions (centrifugation) accelerate instability phenomena resulting from problems in the composition or preparation method. Visual changes such as precipitation, phase separation, color change and creaming should be observed. The observed results are shown in Table 2.

Table 2: Results of the thermodynamic stability test of nanoemulsions

Test	*Formulations*					
	NE-1	*NE-2*	*NE-3*	*NE-4*	*NE-5*	*NE-6*
Hot and cold cycles	SP	SA	SA	SA	SA	SA
Freezing cycles	SP	SP	SP	CR	CR	SA
Mechanical stress	SP	SA	SA	SA	SA	SA

SA (no change); SP (phase separation); CR (cremation)

In the hot and cold cycles (4 and 45°C), it was observed that only the NE-1 nanoemulsion showed phase separation. This nanoemulsion had a higher IR3535® content and only 2% surfactant.

In the freezing cycles, only the NE-6 formulation did not change, this formulation has 3% surfactant and a smaller amount of IR3535® (1%). Emphasizing that in this test the NE-4 and NE-5 formulations, which had respectively 3% and 2% of IR3535®, showed creaming and the NE-1, NE-2 and NE-3 formulations showed phase separation and these formulations had the smaller amounts of surfactants. The results revealed that the most stable formulation was the one with the lowest amount of IR3535® and the highest amount of surfactant.

The remaining tests were conducted only with the NE-6 formulation as it was the only one stable in all stability tests. This can be explained considering that the stabilization of nanoemulsions requires the formation of a dense layer of surfactant around the oil droplets to prevent coalescence (phase separation) and Ostwald ripening (cremation).[16]

The most stable nanoemulsion was characterized in terms of droplet dimensions evaluated in terms of the average apparent hydrodynamic diameter obtained from ELD measurements and the effective surface charge of the droplets evaluated in terms of electrophoretic mobility. The nanoemulsions obtained hydrodynamic diameter values of 188 ± 2 nm, -39 ± 1 mV of zeta potential and 0.118 to 1.120 of polydispersity index.

Zeta potential provides information about the potential stability of formulations. Thus, high absolute values of mobility, or zeta potential, are indicative of a high surface charge of the droplets, which provides the droplets with high resistance against aggregation. Figure 2 shows the respective intensity and zeta potential distributions.

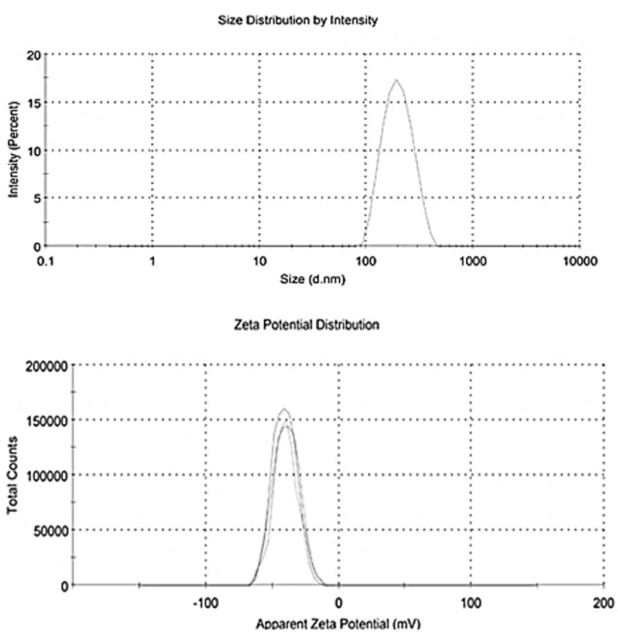

Figure 2: Diameter distribution and zeta potential of the NE-6 nanoemulsion

The results indicate that the droplets are suitable for superficial topical application; furthermore, according to the negative electrophoretic mobility values, the nanoemulsions should be considered relatively stable systems. Considering that Pluronic® F127 is a non-ionic surfactant, it is expected that the negative charge may be due to the composition of linseed oil fatty acids such as linolenic and linoleic in the interfacial layers, these acids having dissociated carboxyls oriented towards the phase aqueous. This results in electrostatic repulsions between the droplets, which provides stability to the formulations.[23]

Therefore, the above results suggest that the studied nanoemulsions may be suitable platforms for the encapsulation of lipophilic active ingredients.

The encapsulation efficiency of IR3535® in the nanoemulsion was 61.33 ± 0.84%, which can be explained by the solubility of IR3535® in water (70 g L-1) and the polymerization time in an air atmosphere. No Encapsulation Efficiency results for IR3535® were found in the literature for comparison of results, however, there is a report of a nanoemulsion containing *Cymbopogon pendulus* essential oil encapsulated with encapsulation efficiency between 41 and 60%.[24]

The droplet size, surface morphology and particle structure of the NE-6 oil-in-water nanoemulsion were characterized by transmission electron microscopy (TEM) in which an electron beam is transmitted through a material to create an image. As the beam passes through the sample, an image is created as a result of the electrons' interactions with it.

According to the TEM analysis, the nanoemulsion droplets have a limited size distribution, spherical and without aggregates, with a diameter between 100 and 120 nm, smaller than that obtained by ELD, which a difference in measurement method can explain. Considering that the ELD takes into account the charged layer that depends on the dispersant.[25] A similar morphological appearance was obtained through TEM of *Nigella sativa* essential oil nanoemulsions stabilized with Tween 80.[26] In TEM analysis, the nanodroplets revealed themselves to be spherical, as shown in Figure 3.

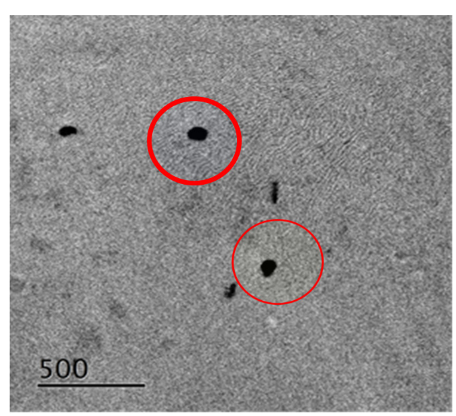

Figure 3: Micrograph obtained by TEM of the NE-6 nanoemulsion.

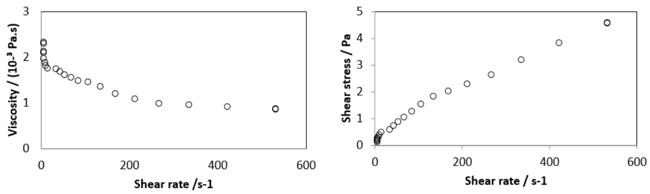

Figure 4: Graphs of the rheological study of the NE-6 nanoemulsion

The rheological evaluation of the nanoemulsion is an important parameter for developing and applying nanoemulsions. In Figure 4 it is observed that the viscosity of NE-6 decreased markedly with increasing shear rate until it stabilized, proving the typical behavior of a non-Newtonian pseudoplastic fluid.

When the shear rate increased to overcome Brownian motion, the drop-drop interaction was disrupted and became more ordered offering less resistance to flow which resulted in a decrease in viscosity. The power law model was used to fit curves of shear stress versus shear rate, where f is the slope of the straight line 0.0035 and ln K = 0.0456 (K = 1.0467), where τ is the shear stress, γ is the shear rate, K is the consistency index and f is the flow index, obtaining the expression $\tau = 1.0467\gamma^{0.0035}$.

Conclusion

This work successfully obtained a nanoemulsion (NE-6) for encapsulating IR3535® and clove oil with Pluronic® F127, as a proposal for an insect repellent product. The NE-6 nanoemulsion showed colloidal stability in thermodynamic stability tests, rheological behavior characteristic of a pseudoplastic fluid and encapsulation efficiency of the active ingredient IR3535® greater than 50%. The morphology of the nanodroplets was typical of stable nanoemulsions with spherical shapes and no agglomerates.

Therefore, we conclude from the results that the NE-6 nanoemulsion is a promising system for the development of topical repellents with lower amounts of IR3535® compared to commercial repellents, associated with clove essential oil. However, future in vivo studies are needed to evaluate the repellent efficacy of the formulation.

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