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Evaluation of stability and anxiolytic potential of oil-in-water polysaccharide nanoemulsions loaded with chalcone (1E,4E)-1,5-bis(4-methoxyphenyl) penta-1,4-dien-3-one

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Abstract

The desire for products that are healthier, safer, and better for the environment is on the rise in society. Chalcones are aromatic ketones with anxiolytic and antimicrobial properties. The limited solubility, bioavailability, and long-term stability of chalcones hinder their application. Nanoemulsions can be used as a drug transport system to address this problem. The primary objective of our research was to design nanoemulsions through ANOVA (NE) with nanoscale droplets that would maintain exceptional stability, optimizing the anxiolytic capacity of chalcones. Sodium alginate and chitosan were assessed as the continuous phase material, while commercial soybean oil and mineral oil were used as adjuvants, besides surfactant, for the oil phase composition of the droplets. Results showed that the addition of soybean oil improved significantly the stability of the formulations, as did the use of the alginate matrix. The optimal NE showed a nanometer-sized droplet (126 nm) and negative ζ-potential (− 42 mV), showing good stability under different conditions—it synergistically enhances the anxiolytic potential. The mode of operation is associated with the receptors of the serotonergic system (5-HT). Toxicity, locomotion and anxiety tests performed using the zebrafish animal model showed a promising dose (0.0325 mg/mL) for the development of compounds with anxiolytic properties.

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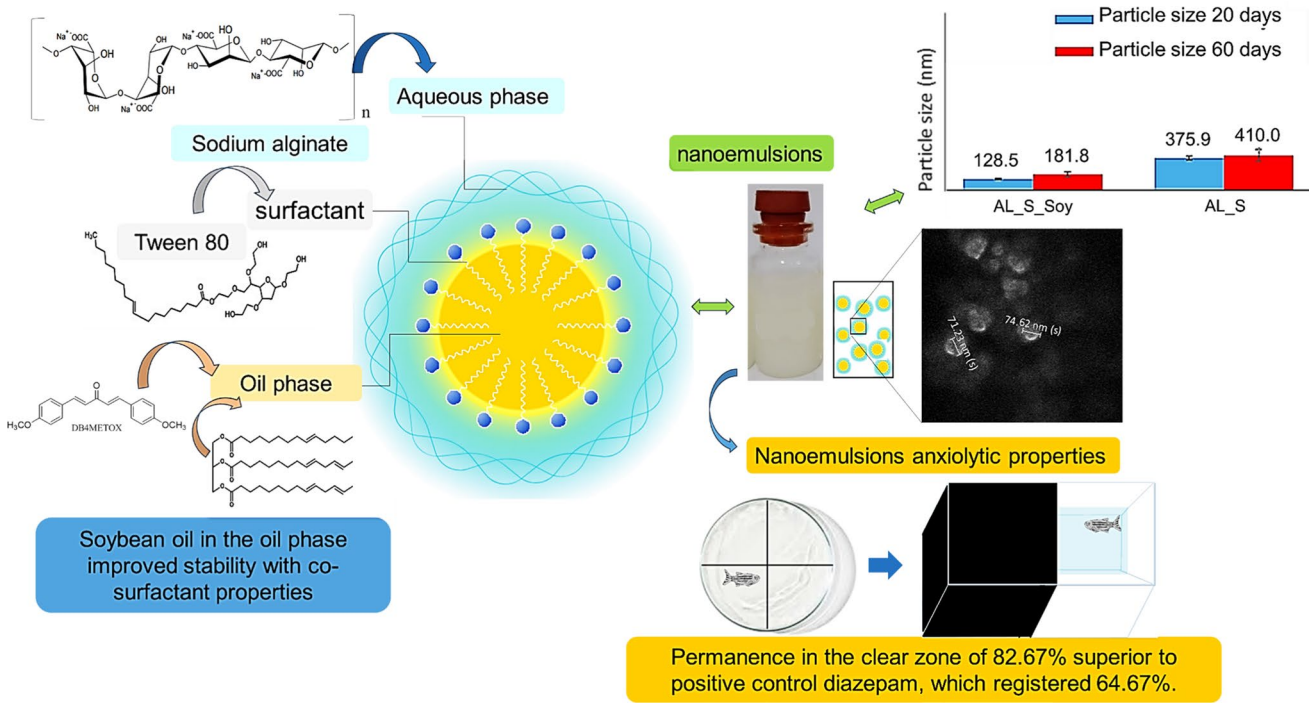
Graphical abstract



Keywords Nanodroplets · Chitosan · Alginate · Anxiety · Zebrafish

Introduction

Anxiety has become a major problem in the modern world, intensified after the coronavirus pandemic (Cunha et al. 2021). Anxiety disorders affect people significantly worldwide, resulting in depression and pain, which are widespread health problems (Gamonal-Limcaoco et al. 2022; Hur et al. 2020). Although efficient, commercial anxiolytic drugs have several undesirable effects, reducing the quality of life (Coldwell et al. 1997; Panes et al. 2020). In this sense, pharmacological therapies with fewer side effects than conventional ones, such as therapies with natural drug or their derivatives, need to be developed (Kupats et al. 2020; Xavier et al. 2021).

Chalcones are aromatic ketones, with two aromatic rings and the electrophilic α, β-unsaturated carbonyl system in continuous conjugation. This feature may explain their low redox potential, stability, ability to participate in electron transfer reactions and, most importantly, their promising biological activities (Rammohan et al. 2020). These compounds of natural origin widely distributed in plants, can be synthesized and have various biological activities. Studies have shown that some chalcones have anxiolytic properties, which prompted this chemical prospection (Higgs et al. 2019; Guedes et al. 2022). In fact, studies have showed that most anxiolytic compounds have substituents such as dimethyl-amine, methoxy, nitro, hydroxyl, methyl and halogens. The chalcone (1E,4E)-1,5-bis(4-methoxyphenyl)penta-1,4-dien-3-one ($DB4OCH_3$) has two methoxy groups (Fig. 1a), which may be related to its anxiolytic effect (Higgs et al. 2019; Tan et al. 2021; Ferreira et al. 2020).

In a study by Aarthi, the chalcone $DB4OCH_3$ revealed potential activity through computational calculations. The molecular coupling between the chalcone and the 1HEI protein was evaluated, confirming a binding energy value of − 7.29 kcal/mol, proving its potential as a drug for resistance to the Hepatitis C virus (Aarthi et al. 2022). However, there are no reports so far in the literature revealing the anxiolytic activity of this compound.

Chalcones have remarkable biological properties, but their clinical use has been hampered by their low bioavailability. Nanoemulsions (NEs) are suitable vehicles for nanoencapsulation of bioactive ingredients, once they can overcome these solubility limitations and preserve their properties, avoiding oxidation and facilitating the handling of the drug (Adiwidjaja et al. 2017; Goyal et al. 2021; Banaee et al. 2022). Such a system may improve the efficacy of bioactive compounds by increasing the uniformity and stability of the distribution (Milkova and Goycoolea 2020; Maurya et al. 2021). In emulsions systems,

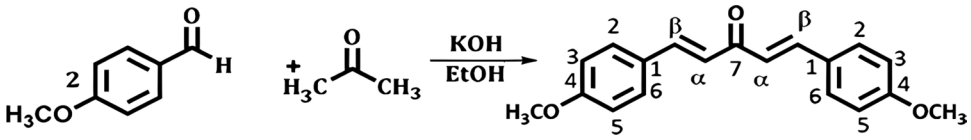


Fig. 1 Representation of the Claisen–Schmidt synthesis for chalcone DB4OCH$_3$

surfactants, phospholipids, proteins, polysaccharides, or combinations can be applied as nano-emulsifiers to protect the active compound from adverse conditions while ensuring its controlled release. (Wijekoon et al. 2023).

The main limitation in the development of therapies in the central nervous system (CNS) is the difficulty of drugs encounter in crossing the blood–brain barrier (BBB) and reaching the brain. Because of its complex structure and the presence of various enzymes, the BBB represents a significant challenge for effective drug delivery. One very promising strategy that has been developed to overcome this barrier is the encapsulation of a drug in NEs (Correia et al. 2022).

NEs have been described in studies evaluating their activity in the CNS in biological animal tests. The ability of NEs to provide more consistent bioavailability and plasma concentration of the drugs used has been demonstrated in all cases, resulting in a more controlled response in terms of distribution and transport across biological barriers. In addition, these systems have demonstrated other advantages, such as the ability to increase drug loading, reduce inter-individual variability and protect the drug from enzymatic degradation (Nehal et al. 2021). In the study by Muresan et al. (2023), the influence of two different formulations, an oil-in-water (O/W) NEs and polymer-coated nanoparticles (PCNPs), on the bio-distribution of cannabidiol (CBD) within the CNS was investigated, revealing that the NEs provided a slower release of CBD.

In Borges' work (Borges et al. 2018), the anti-inflammatory potential of NEs containing the essential oil of *Rosmarinus officinalis L.* was evaluated. This study showed that all the NEs (NECHA, NECULT and NECOM) showed no toxicity to macrophages, besides exhibiting antioxidant activity and potentiating the effect of essential oil on the proliferation of viable fibroblasts. The NEs also demonstrated the ability to potentiate the anti-inflammatory action of the essential oils, exerting immunomodulatory activity by inhibiting the production of the pro-inflammatory mediator nitric oxide.

In the study by Campelo et al.(2023), NEs were prepared and characterized containing clove volatile oil and its main compound, eugenol, using polysaccharides from Agaricus blazei Murill as a stabilizing agent to evaluate their anxiolytic effect. The NEs showed low acute toxicity, reduced locomotor activity and anxious behavior in zebrafish at concentrations of 4 to 20 mg/kg. NEs reduced the anxious behavior of adult zebrafish without affecting their locomotor activity. Furthermore, it was shown that the anxiolytic activity of is linked to the involvement of the GABAergic pathway.

NEs designed with biodegradable polysaccharides offer a promising route to controlled release systems. The inherent stability of these NEs, because of the natural polysaccharides used, can be attributed to their substantial viscosity. This innovative approach has the potential to surpass traditional administration methods, as it can increase efficiency, minimize adverse effects and optimize dosage accuracy through gradual release (Richa and Roy Choudhury 2020). In an emulsion-based delivery system, the bioavailability of hydrophobic bioactive compounds encapsulated in lipid droplets is prominently affected by the structure and composition of the matrix (McClements 2020). Polysaccharides can act as emulsifiers, where the chain can absorb to the surface of freshly formed droplets of an oil–water interface during homogenization, forming a protective membrane that prevents the droplets from aggregating, leading to an increase of the emulsion's stability (Maphosa and Jideani 2018). Among the polysaccharides, alginate (ALG), which is a natural anionic polysaccharide extracted from brown algae, has been widely used as a wall matrix for encapsulation of lipophilic compounds (Odriozola-Serrano et al. 2014; Sun and Xia 2019). Chitosan (CHI) is a widely used a polycationic biopolymer, with emulsifier and stabilizer ability through the adsorption of the protective layer at the oil–water interfaces (Klinkesorn 2013).

The stability of the NEs involving organic agents with low solubility may be improved with a second component in the oily phase. The pharmaceutical grade of mineral oil is composed of highly refined saturated hydrocarbons, straight and branched open-chain alkanes (paraffins) and largely alkylated cycloalkane (naphthene's) (Biedermann et al. 2009; EFSA 2012). Vegetable oils can act as an adjuvant in the stabilization of NEs and potentiate the actions because of their chemical structure, including the control of droplet size and ability to dissolve and stabilize lipophilic drugs (Bajerski et al. 2016).

Some works have already addressed the use of sodium alginate and chitosan as matrices. Branco et al. (2020) conducted a study where NEs containing sodium alginate and various oils were created to assess the impact of the polysaccharide on formulation stability. Xin et al. (2023) used selenium-enriched chitosan NEs in chicken sausage processing as a food additive to reduce sausage spoilage. Thus, with the use of vegetable and mineral oil in its composition, according to the works of Katzer et al. (2014), they produced NEs with mineral oil to

assess their compatibility in contact lenses. Shevalkar et al. (2019) developed a nanostructured lipid carrier (NLC) of propofol from commercialized soybean oil-based NE, for parenteral delivery, reducing injection pain and the risk of microbial contamination.

In this sense, a better understanding of the polymer used as a stabilizer and the type of oil used as an adjuvant could lead to an improvement in the NE stability and their efficacy as an anxiolytic agent. In a previous study, NE produced with only Tween 80 (T80) and chalcone showed flocculation and sedimentation after 7 days. The addition of soybean oil minimized flocculation, but creaming occurred. Finally, the addition of alginate as a co-emulsifier minimized creaming (Abreu, et al. 2023).

In this study, CHI and ALG are polysaccharides chosen as co-emulsifiers. The effect of soybean or mineral oil in combination with T80 is an interesting study, since the influence of the components and their interactions can provide a greater extension of the emulsification properties. Because of the advantages of NEs already mentioned and the use of chalcones with anxiolytic properties already reported (Mathew et al. 2019; Mendes et al. 2023), the design of NEs carrying chalcones could lead to a significant improvement in stability and anxiolytic properties and, to our knowledge, no NEs containing chalcone-based compounds for anxiolytic applications have been reported in the literature. In this work, the influence of the polymer used as a co-emulsifier and the addition or not of mineral oil or soybean oil in combination with T80 used as an adjuvant was evaluated on the stability and related properties of NEs enriched with the synthetic chalcone $DB4OCH_3$, for anxiolytic application.

Experimental

Materials

Sodium alginate (ALG) (MW = 23,759 KDa, Dynamic®), chitosan (CHI) (Degree of deacetylation = 90.0%, MW = 21,4 KDa, Exodo, Brazil), surfactant Tween 80® (Vetec®), Commercial soybean oil from (*Glycine max*) seeds and mineral oil pharmaceutical grade (Natulab®, Brazil). All the chemicals were used as received with no further purification. For the zebrafish test, Diazepam (DZP, Neo Química®), Flumazenil (Fmz; Sandoz®), Dimethyl sulfoxide (3% DMSO v/v; Dynamic®), Granisetron hydrochloride (GRA; Corepharma, Middlesex, NJ, USA) and fluoxetine (FLX; Eli Lilly/EUA-IN).

Methods

Synthesis of chalcone

Chalcone $DB4OCH_3$ was obtained through Claisen–Schmidt aldol condensation reactions in a basic medium. In an Erlenmeyer flask containing 50 mL of 10% KOH (w/v) and 40 mL of ethanol, 7 mL of a mixture of aldehyde:acetone (5:2) was added slowly under stirring and the temperature was maintained at 0 °C. After 30 min, the mixture was filtered, and $DB4OCH_3$ chalcone obtained as a precipitate was washed three times with small portions of distilled water. The chalcone was purified using the recrystallization technique, $DB4OCH_3$ was dissolved in hot ethanol, the solution slowly returns to room temperature, and $DB4OCH_3$ crystals are formed and filtered. The crystals were washed with cold ethanol and dried at room temperature (Oliveira et al. 2021). Figure 1a depicts the synthesis of the chalcone.

Preparation of chalcone-Based Nanoemulsions

Six different oil-in-water (O/W) NEs were produced varying the oil phase and the aqueous phase. In the oily phase, it was tested only T80, T80 plus mineral oil and T80 plus soybean oil. In the aqueous phase, it was added sodium alginate or chitosan. The NEs were prepared by a high-speed homogenization method. The oily phase was formed by adding 20 mg of DB4OCH3, 2 mL of DMSO, 2 mL of T80, and 1 mL of oil to a 10 mL beaker. The components were homogenized for 1 min in ultrasonic tip sonicator at a frequency of 20 kHz at a power of 440 W. Subsequently, the oily phase was slowly poured with the aid of a syringe into the 150 ml of CHI (1% w/v in acetic acid) or ALG (1% w/v in water). The formulation was mechanically homogenized with the aid of an Ultrastirrer homogenizer model ULTRA380 at 22.000 rpm for 3 min to produce the NEs.

Experimental design and statistical analysis

ANOVA was employed to evaluate the properties of the hydrogels selecting as dependent variables the particle size, viscosity, and zeta potential. The independent variables were polysaccharide matrix and composition of the oily phase. The factors related to the independent variables and theirs high (HL), Medium (ML) and low (LL) levels were defined:

- Factor A: Matrix type: HL ALG (+); LL CHI (-);
- Factor B: Composition of the oily phase: LL T80 plus mineral oil (-); ML T80 (0); HL: T80 plus soy oil (+).

Table 1 shows the matrix variables of the designed experiments. 6 runs in triplicate were performed for each dependent variable, and they were performed in groups of 3 experiments randomly chosen to nullify the effect of nuisance variables. (Supplementary materials provide all the data and statistical results from viscosity (Tables S1 and S2), particle size (Tables S3 and S4), polydispersity index (PDI) (Tables S5 and S6) and Zeta potential (Tables S7 and S8).

Table 1 Experimental design of NEs production with $DB4OCH_3$chalcone

Code	Matrix[a]	Oily phase[b]
CHI_S_Min	−1	−1
CHI_S	−1	0
CHI_S_Soy	−1	+1
AL_S_Min	+1	−1
AL_S	+1	0
AL_S_Soy	+1	+1

[a]Polymer matrix (mat), low level (-) = chitosan (CHI); high level (+) = alginate (ALG); b oily phase composition: low level (-) = T80 + mineral oil (min); intermediate level (0) = surfactant T80 (S); high level (+) = T80 + soybean oil (soy)

Nanoemulsions Characterization

Physicochemical assessment was conducted for NEs regarding their stability. About 10 mL of the NEs are placed in closed test tubes where it will be observed periodically for 63 days, the samples were observed visually, checking for signs of instability, such as creaming and/or sedimentation, the volume was measured with a ruler every 7 days (Dickinson 2009). Optical microscopy also was employed to evaluate the presence of coalescence and agglomeration between droplets after 60 days (Supplementary Material, Fig S1). The creaming index is calculated according to Eq. 1 (Mwangi et al. 2016):

$$CI(\%) = \frac{H_c}{H_t} \text{x } 100 \quad (1)$$

where CI = creaming index, H_c = creaming height, H_t = total height of an emulsion.

An Ostwald viscometer was used to measure the viscosity by diluting a sample of the formulation in distilled water and timing the flow time. The analysis was done in triplicate, following the adapted methodology of Almeida et al. (1995). Equation 2 was used to calculate the specific viscosity: 99,

$$\eta_{sp} = \frac{t - t_0}{t_0} \quad (2)$$

where η_{sp} = specific viscosity, t = flow time of the solution in the viscometer; t_0 = flow time of the pure solvent in the viscometer.

Measurements of particle size, polydispersity index, and zeta potential were conducted using the Malvern Zetasizer/Nanoseries Z590. Prior to reading, the diluted samples were stirred continuously for 24 h, and readings were taken in triplicate. The images were processed in ImageJ software. Scanning electron microscopy was performed (Quanta 450-FEG (FEI)), using an accelerating voltage of 20 kV and a magnification of 20.000x. Infrared (FTIR) spectra were obtained using a Thermo Scientific Nicolet iS5 model spectrophotometer. The samples were prepared on KBr tablets at a 1:20 (m/m) ratio (sample:KBr) and the spectra recorded between 4000 and 800 cm^{-1}, using 32 scans and resolution of 4 cm^{-1}. One- and two-dimensional Hydrogen-1 Nuclear Magnetic Resonance (^{1}H NMR) and Carbon-13 Nuclear Magnetic Resonance (^{13}C NMR) were obtained on a Bruker DRX-300 and DPX-500 spectrometer (^{1}H: 300 and 500 MHz; ^{13}C: 75 and 125 MHz), using deuterated solvents.

Encapsulation Efficiency Assessment

The $DB4OCH_3$ content in the NE was determined by measuring the encapsulation efficiency (EE%) at the maximum wavelength of 350 nm. Beforehand, the emulsions were diluted in a 1:4 (v/v) ratio of the NE in 96% ethanol and kept at rest for 24 h. Their absorbances were measured on a KASUAKI UV–VIS spectrophotometer. The concentration of chalcone ($DB4OCH_3$) present in the NEs was determined using the calibration curve of the sample from a 500-ppm stock solution in 96% ethanol. From this, further dilutions generated a calibration curve, according to Eq. 3, relating the concentration to the correspondent absorbance values:

$$y = 0.0876x + 0.0001R^2 = 0.9975 \quad (3)$$

The EE% of the NEs was calculated using Eq. 4:

$$EE(\%) = \frac{C_{total} - C_{free}}{C_{total}} \text{ x } 100 \quad (4)$$

where C_{total} = the initial concentration of the chalcone added to the NE, C_{free} = the calculated concentration of chalcone $DB4OCH_3$ in the formulation.

Zebrafish as a model for anxiety assessment

Ethics statement

We certify that the Project entitled "Use of Zebrafish (*Danio rerio*) as an alternative model for Investigation of the pharmacological potential of natural and synthetic products" in agreement with Ethical Principles in Animal Experimentation, adopted by the Ethics Committee in Animal Experimentation of Ceará State University (CEUA–UECE; nº 04983945/2021).

Zebrafish

Wild zebrafish of all sexes were purchased from a local store in the city of Fortaleza, Ceará State, being 90 to 120 days old, weighing 0.4 ± 0.1 g, with a size of 3.5 ± 0.5 cm. The fish were kept in a glass aquarium (30 × 15 × 20 cm) of 10 L (n = 3/L), at a temperature of 25 ± 2 °C, with chlorinated

water (ProtecPlus®) and air pump with filters, under a temperature of 25 °C and pH 7.0, circadian cycle of 10–14 h (light/dark). The animals are fed with feed (Spirulina®) 24 h before the tests. Fish are anaesthetized prior to drug dosing. At the end of the tests, the animals are immersed in ice water for 1 min to be sacrificed (2 and 4 °C) until the loss of opercular movements.

General Procedure

Zebra fish were randomly selected for the experiments, anesthetized in ice water and treated using 20 µL of the 0.13 mg/mL concentration samples (pure); each sample was diluted 1:1 (0.065 mg/mL) and 1:2 (0.0325 mg/mL) in DMSO 3%. For the control group, we used Diazepam (4 mg/kg) and DMSO 3% (control group—drug diluent). Each set of fish was individually positioned in beakers (250 mL) containing 150 mL of the water in the tank and kept at rest. The experiments were performed orally (*v.o.*).

Statistical analysis

Groups of 6 animals were used; data were analyzed by mean ± standard error of the results obtained. Analysis of variance was performed to investigate differences between groups. Data were processed with GraphPad Prism v. 8.0 software using statistical significance of 95% ($p < 0.05$).

Analysis of locomotor activity

Zebra fish (Zfa) ($n = 6$/group) were orally administered 20 µL of the samples diluted in 3% DMSO. The 3% DMSO was used as a negative control. Locomotor activity was performed according to the method of Ahmad and Richardson (Ahmad and Richardson 2013) with adaptations. Adult zebrafish locomotion was analyzed after exposure to substances with possible anxiolytic effects; the number of crossings made by the fish from one side to the other of the Petri dish for 300 s is evaluated (Kysil et al. 2017).

Anxiolytic evaluation

An animal's anxiety behavior can be observed through the light and dark test. The analysis was performed in an aquarium (30 cm × 15 cm × 20 cm) where half is light, and half is dark. It was filled 3 cm with chlorine-free tap water, simulating a new environment which induces anxiety-like behaviors. The animals were given 20 µL of the sample orally ($n = 6$/group). The negative and positive control groups comprised DMSO at 3% and Diazepam solution at 4 mg/kg, respectively. After 1 h, the animals were observed individually for 300 s. The anxiolytic action was assessed based on how long the individuals remained in the clear zone (Gebauer et al. 2011).

Acute toxicity 96 h

After the tests (open field, light/dark), the animals were kept under observation for 24, 48, 72 and 96 h. The lethal concentration for killing 50% (LD_{50}) of the ZFa was evaluated and estimated by the Trimmed Spearman–Karber mathematical method with 95% confidence intervals, according to the amount of dead animals after the test period (Arellano-Aguilar et al. 2015).

Evaluation of serotoninergic (5-HT) neuromodulation

In our tests, we used the serotonin receptor antagonist (Granisetron) instead of the GABAa receptor antagonist (flumazenil) since the effect of $DB4OCH_3$ was not blocked by flumazenil, suggesting that the GABAa pathway is not involved. Therefore, the methodology adopted was: The neuromodulation involved in the anxiolytic action of the samples was identified by pretreating the zebrafish ($n = 6$/group) with the antagonist (4 mg/kg; 20 µL; *v.o.*) (Granisetron, serotonin (5-HT) antagonist) before the light and dark test (Benneh et al. 2017). After 15 min, 20 µL; *v.o.* of the concentration with the highest anxiolytic efficacy found in the anxiolytic evaluation was administered. For the negative control, 3% DMSO (vehicle; 20 µL; *v.o.*) was administered and fluoxetine was administered as a positive control. After 1 h, the fish were evaluated in the light/dark test, where they were observed for 300 s and their permanence in the light zone was assessed.

Results and discussion

NEs were developed for the encapsulation of chalcone $DB4OCH_3$, to explore its potential anxiolytic effect. Our research focused on identifying the NE with higher stability. We studied the effect of two polysaccharides, sodium alginate and chitosan, as well as including mineral oil and soybean oil in the oily phase. The chalcone synthesized was evaluated by NMR and FTIR. Figure 1 shows the representation of the Claisen–Schmidt synthesis for the chalcone $DB4OCH_3$. NEs were characterized by SEM, particle size, zeta potential, viscosity, and stability over 60 days, to determine the most promising formulation which was then submitted to biological tests for validation. The tests performed proved that the use of soybean oil and alginate was more effective in the NEs stabilization.

Physicochemical assessment

NE instability is undesirable and creates several problems in prolonged storage (Sharma et al. 2010). Samples AL_S and CHI_S did not show any sign of instability. Figure 2a shows the creaming results for the samples analyzed from 7 to 63 days. The chitosan emulsions showed the highest creaming index, with values between 2.5 and 3.0%. This effect may be partly because larger droplets were produced under these conditions, and the creaming rate is proportional to the square of the droplet diameter by Stokes law (Dickinson and Woskett 1988), which will be evaluated later. The alginate emulsions showed a creaming index of 2.3%. The negative charge of sodium alginate is a result of its carboxylic groups. Mirhosseini et al. (2008) found that negatively charged polysaccharides can enhance electrostatic repulsion between emulsion droplets, thus improving the long-term stability of colloidal dispersions. All samples showed low creaming volume, below 2.9%, and did not increase significantly during the test period, therefore, all meet the stability criterion since the average creaming volume per day was 0.0476%, the samples reached this creaming volume in 21 days, during the rest of the observation period they became constant.

Figure 2b shows the viscosity of the NEs as a function of the concentration. The effect of the matrix type and the type of oil adjuvant on the NEs viscosity was evaluated by analysis of variance (ANOVA). Results showed that NEs with CHI matrix presented significantly lower viscosity than those with ALG. CHI-based NEs presented a shrinking of the polymer network, caused possibly by electrostatic forces from the protonated amine groups, promoting an expansion in polymer conformations, decreasing the degree of entanglement of the polymer chains, resulting in lower viscosity. However, CHI_S_Soy NE presented a slight increase in the viscosity at 20% concentration in comparison with the other CHI-based NEs. The interactions between soybean oil (ester groups), surfactant and amino groups from chitosan chains increased the solution viscosity, probably because of the formation of hydrogen bonds between the carbonyl group of the ester and the protonated amino group of the chitosan chain amino acid (Horikawa et al. 2018). This interaction can lead to the formation of a complex network of hydrogen bonds, which can increase the viscosity of the solution. However, at higher concentrations, there was no significant difference among CHI-based NEs, probably because of the increase in the degree of entanglement between the components. Conversely, ALG-based NEs presented higher viscosity, which may be related to the higher degree of entanglement in the polymer matrix, because of the interactions between the hydrophilic groups from soybean oil and T80 with acid carboxylic groups from ALG. However, AL_S_Soy NE presented reduced viscosity at 30% concentration in comparison with other AL-based NEs. This reduction may be related to the widening of the polymer network, caused by interaction forces from ALG with Soybean oil. These forces promote an expansion in polymer conformations and decreasing the degree of entanglement of the polymer chains (Peng et al. 2021; Lopez et al. 2018).

All formulations contain polysaccharides in the continuous phase of the NEs, so they increase the viscosity of the aqueous phase, improving the stability of the NEs. The mechanisms involve reducing the movement of the droplets, covering with a thin film around the droplets formed, decreasing coalescence and increasing the density of the droplets, bringing them as close as possible to the aqueous phase, thus reducing the rate of cream formation (Shao

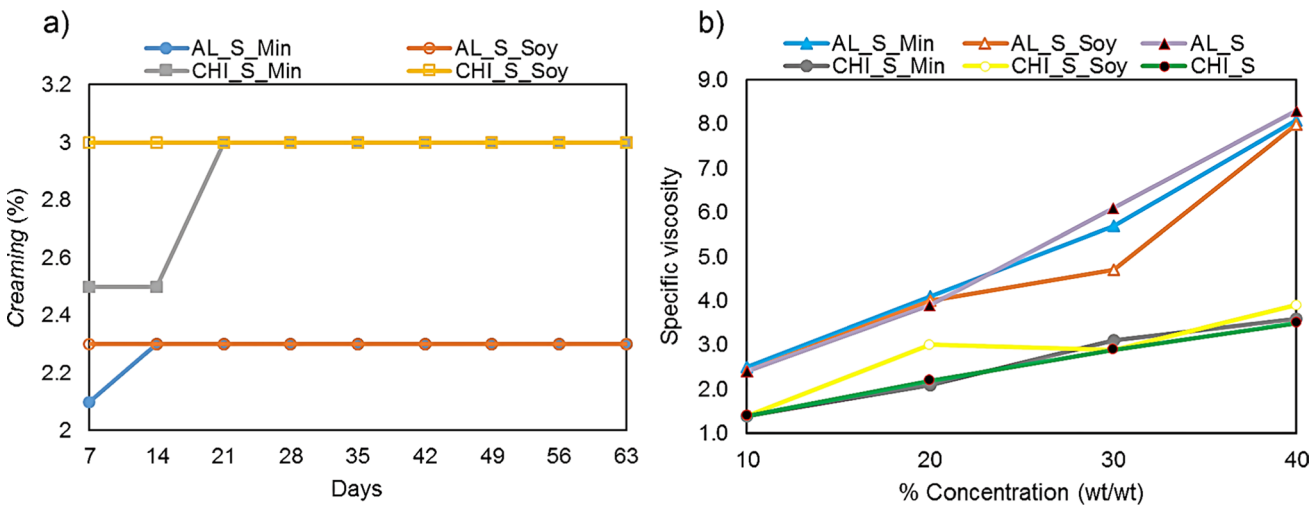


Fig. 2 **a** Creaming index for NEs with ALG and CHI loaded with DB4OCH$_3$ chalcone with different compositions in the oil phase and **b** Specific viscosity as a function of NE concentration ($N=3$)

et al. 2020). The specific viscosity as a function of concentration of ALG and CHI NEs (Fig. 2b) showed that chitosan NEs presented a very similar viscosity profile, with no apparent influence of the oily phase composition in the stability. However, the combination of the ALG, surfactant plus soybean oil reduced the surface tension between the phases, lowering the viscosity and promoting a single-phase system. To enhance the preparation of NEs, ultrasound can provide superior control over emulsion properties (Branco et al. 2020; Abreu, et al. 2023). Another approach is to combine multiple emulsification techniques, resulting in longer-lasting stable NEs without creaming (Chen et al. 2022).

Particle size, zeta potential and efficiency of encapsulation

The particle sizes ranged from 128.47 to 661.37 nm, demonstrating an expressive difference in droplet size when varying the matrix type. Alginate as matrix produced NEs of desirable size below 128.47 nm. McClements (McClements 2004) reported that the increase in particle size can increase the speed of cream formation. Chitosan-based NEs presented a larger particle size, exceeding 660 nm, probably due to the high entangled network and presented higher cream formation rate, reaching 3%, confirmed by the stability assessment.

High zeta potential in absolute values confers greater stability to nanodroplets since these values determine the charge on the particle surface. These values influence the behavior of particles in a liquid and the tendency of aggregation and/or flocculation (Larsson et al. 2012). All formulations present values greater than + 30 mV or less than −30 mV, which indicates repulsion of adjacent particles, therefore are electrostatically stabilized (Salvia-Trujillo et al. 2015). The negative charges of the alginate are attributed to their carboxylic groups, while the positive charges of chitosan are attributed to their protonated amine groups; these groups played the role of electrostatic stabilization of the NEs (Artiga-Artigas et al. 2017).

Considering the polydispersity index (PdI) of NEs we can observe the homogeneity of dispersions, where lower PdI values indicate more homogeneous dispersions (Baboota et al. 2007). The NE with the best balance of properties, considering particle size, zeta potential and PdI, is AL_S_Soy, because of their lower size, stable zeta potential values and its average PdI value is 0.430 ± 0.02 indicating homogeneity in the distribution of particles in the aqueous phase. The detailed data are presented in Table 2.

Analysis of variance (ANOVA) was used to assess the impact of matrix type (ALG/CHI) (factor A) and oil type (mineral/vegetable) (factor B) on nanoemulsion properties. We can statistically affirm that for PS, the single factor that was significant was factor A, matrix type, therefore ALG can produce formulations with smaller PSs compared to CHI, regardless of the type of oil, and lower PdI value than CHI. For the ZP, all factors (A and B) were significant, were CHI presented positive ZP values, while ALG presented negative values. There was no correlation between the oil type and CHI. However, the ZP values for ALG were affected by the presence of oil. The formulation with only the surfactant showed the lowest potential value. As a result, the interaction effect is solely present in the ALG matrix samples.

Table 2 Results of particle size (PS), zeta potential (ZP) and polydispersity index (PdI) for NEs produced with ALG and CHI

Code*	PS (nm)	ZP (mV)	PdI
AL_S_Min	185.27 ± 20.07	−42.30 ± 0.20	0.330 ± 0.11
AL_S_Soy	128.47 ± 4.04	−45.30 ± 5.16	0.430 ± 0.02
AL_S	375.90 ± 20.65	−75.50 ± 5.46	0.603 ± 0.35
CHI_S_Min	661.37 ± 474.40	42.00 ± 0.10	0.950 ± 0.09
CHI_S_Soy	493.30 ± 115.00	45.73 ± 1.00	0.727 ± 0.32
CHI_S	410.80 ± 51.15	43.17 ± 3.82	0.697 ± 0.09

*AL_S: Alginate plus surfactant; AL_S_Min: Alginate, surfactant plus mineral oil; AL_S_Soy: Alginate, surfactant plus soybean oil; CHI_S: chitosan plus surfactant; CHI_S_Min: chitosan, surfactant plus mineral oil; CHI_S_Soy: chitosan, surfactant plus soybean oil; (N = 3)

A further investigation on the stability of the ALG NEs was conducted regarding the PS as a function of the storage period. Figure 3 shows the average PS after 20 days and after 60 days for the ALG NEs and their corresponding encapsulation efficiency. The investigation into the stability of the NE over time discovered that the use of soybean oil as an adjuvant to T80 yielded better results in controlling the PS of the droplets. Specifically, the droplets labeled as AL_S_Soy maintained a size of less than 200 nm even after 60 days. Using mineral oil as an adjuvant to T80 seemed to cause the opposite effect, where the PS was visibly larger than the other two formulations. The formulation with only the T80 surfactant (AL_S) showed a larger PS even after 20 days. AL_S_Soy showed the highest encapsulation efficiency (EE%) (Fig. 3) with a value of 99.8%, making it the best formulation.

Soybean oil ester groups provided interaction with ester, ether, and hydroxyl groups from T80 by hydrogen bonding and dipole–dipole interactions, which favored stabilization of the hydrophilic phase with the alginate polymer matrix. Conversely, hydrophobic segments from soybean and T80 favored the compatibilization of the chalcone molecules within the droplets. In this sense, a stabilization between the aqueous and oily interface of droplets occurred, promoting a better encapsulation of the active ingredient, as corroborated by Song et al. (2011). Soybean oil, being a long-chain triglyceride, can form hydrogen bonds mainly with ALG, a high molecular mass polysaccharide (Zhao

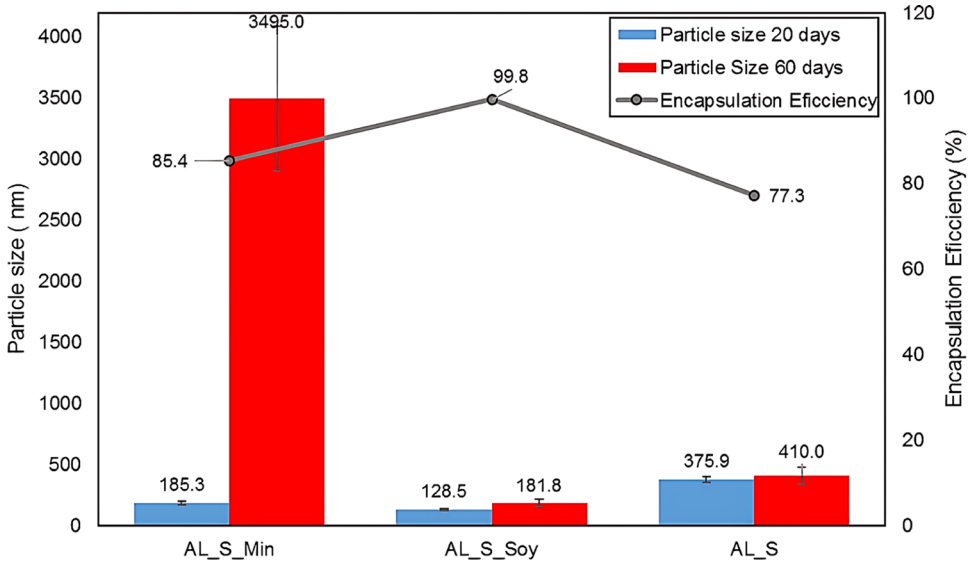


Fig. 3 Results of mean particle size in relation to storage time for ALG NE and encapsulation efficiency

et al. 2015; Driscoll et al. 2001). The study from Su et al. (2018) revealed that ALG can be adsorbed to the surface of soybean oil by electrostatic interaction indicating that the anionic ALG molecules neutralized the positive charge on the surface of the oil bodies and ended up being saturated with ALG and that emulsions coated with ALG were more stable than uncoated emulsions, indicating that ALG can influence the stability of oil compounds.

Other studies describe the production of NEs with particle sizes similar to those proposed in this work, using mineral oil and castor oil in the work by Katzer et al. (2014), which showed PS larger than 234 nm, while in the work by Shevalkar et al. (2019) using soybean oil and propofol showed sizes smaller than 200 nm. Bajerski et al. (2016) report that the use of plant oils in NE systems has demonstrated several advantages for topical and systemic administration of cosmetic and pharmaceutical agents, including droplet size control, protection against photosensitivity, volatilization and thermal degradation, and the increase in solubility of lipophilic drugs. In our study, the droplet size control is easily observed by relating the PS with the EE%, since it was possible to notice the size reduction in the NEs containing soybean oil compared to the other formulations, besides presenting the smallest PS and the highest EE%.

A basic characteristic of any surfactant is its ability to interact with polar and non-polar substances. The oldest one is based on the hydrophilic–lipophilic balance (HLB). A high HLB value, greater than 11, indicates a good solubility of the surfactant in water, while a low HLB value, less than 9, indicates a lower aqueous solubility and a higher relative affinity for the organic phase (Katsuta et al. 2002; Suzuki et al. 2010). Among the hydrophilic surfactants, T80 shows excellent solubility for essential oils and miscibility in water, being one of the most used, with an HLB value of 14.9 (Mazarei and Rafati 2019; Zhang et al. 2017; Ghosh et al. 2013).

According to Niculae and collaborators (Niculae et al. 2012), the medium-chain triglyceride (MCT) core deals with imperfections in the lipid network, which favors the accommodation and retention of butyl methoxy-dibenzoylmethane-type structures. This may justify the better efficiency of soybean oil over mineral oil. Although soybean oil is formed by LTC (long-chain triglycerides), composed mainly of linoleic, oleic and palmitic acids (Cheng et al. 2009) carbonyl esters (C = O), there is a greater similarity between them than with mineral oil. In our work, the compound DB4OCH$_3$ is a chalcone-type structure, which has structural similarities with the substance studied by Niculae.

Scanning electron microscopy (SEM)

NE systems have a characteristic spherical shape and relatively uniform domains (Abu Ali et al. 2021; Ghouchi Eskandar et al. 2009; Falsafi et al. 2020). The ALG NEs presented uniform globules, similar to those in the literature, as shown in Fig. 4.

Uniform and spherical domains were found in the alginate sample with only surfactant, though they were larger (Fig. 4a). The addition of soybean oil in the oil phase prevented its agglomeration, and a decrease in the domains is observed (Fig. 4b). NE of chitosan with only surfactant presented an irregular morphology, where the matrix is totally entangled as a continuous phase with high porosity, similar to hydrogel formation (Annabi et al. 2010) (Fig. 4c). Some droplets are visualized embedded in the network. With the addition of soybean oil in the NE formulation, the morphology assumes a spherical shape with a nanometric droplet size, reinforcing the idea that indeed soybean oil improved

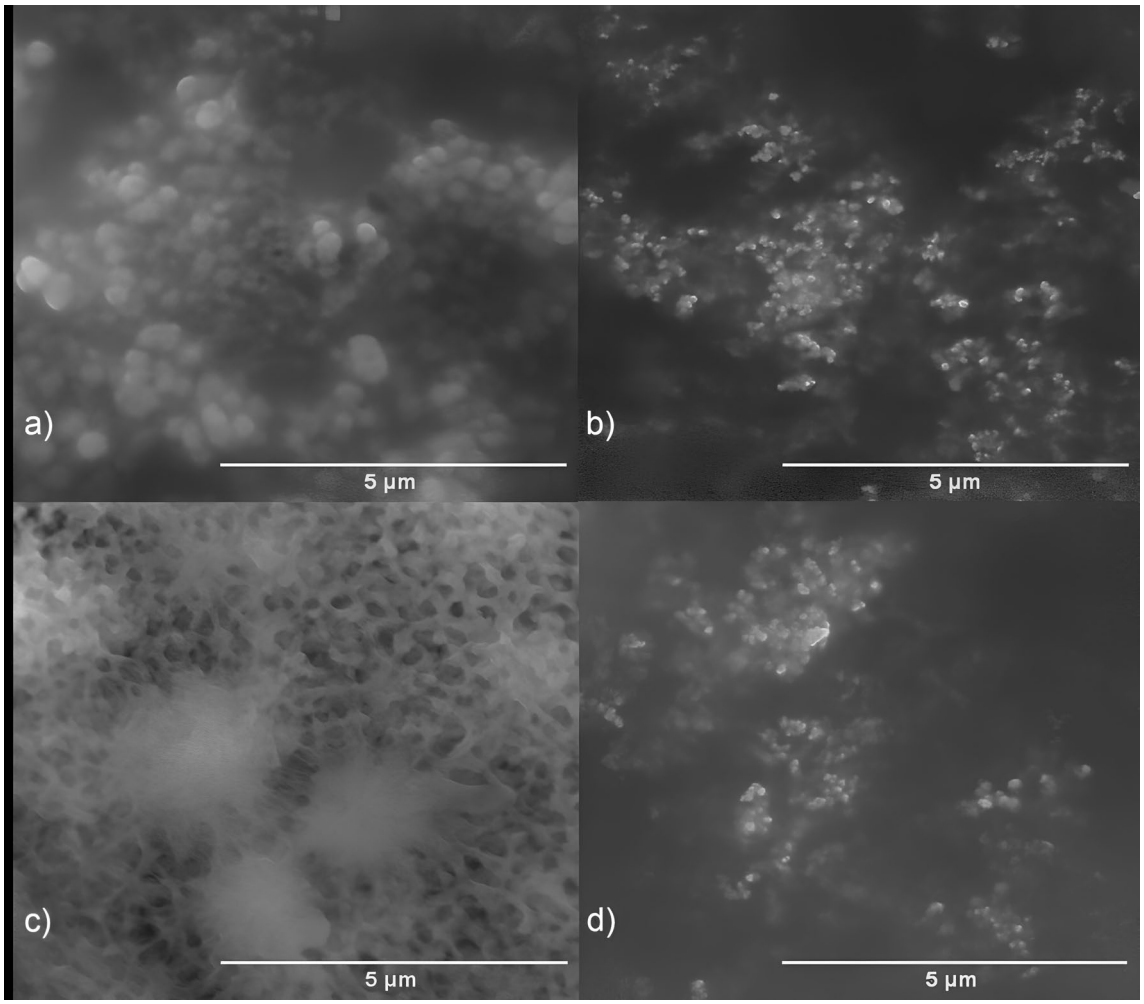


Fig. 4 Scanning electron microscopy surface images of chalcone loaded ALG and CHI NEs prepared with different oil phase compositions: **a** AL_S; **b** AL_S_Soy; **c** CHI_S and **d** CHI_S_Soy

the stability of the NE (Fig. 4d). NEs produced with the sodium alginate matrix presented higher stability and lower particle size, being chosen for the anxiolytic and toxicity evaluation.

Fourier transform infrared spectroscopy–FTIR

Infrared analysis highlighted the main functional groups of soybean oil, sodium alginate, DB4OCH$_3$ and NE AL_S_Soy, as shown in Fig. 5.

In the spectrum of vegetable oil, the main bands were at 3008 cm^{-1} assigned to stretching of the cis double bond of olefins (=CH); 2922 cm^{-1} and 2852 cm^{-1} characteristic of asymmetric and symmetric stretching of the C–H bond of the methylene group, respectively; the intense band at 1743 cm^{-1} assigned to stretching of the C=O group of ester; 1653 cm^{-1} referring to stretching of the olefin double bond (C=C); 1464 cm^{-1} characteristic of symmetric in-plane bending of the methylene group. The bands at 1160 and 1099 cm^{-1} assigned to coupled stretching of the C–O group of ester (Tudorachi and Mustata 2015; Barbosa et al. 2022).

The FTIR of sodium alginate was also analyzed, as all NEs were prepared with this polymeric matrix. Containing the alcohol functional groups (–OH) with the characteristic bands at 3400 cm^{-1}. The bands at 2925 and 2857 cm^{-1} correspond to the stretching (C–H). Most acids when they are dissolved in solvents, their band appears between 1760 and 1730 cm^{-1}, and for this reason the (C=O) points appear at 1741 cm^{-1} in the spectrum. In the range 1100–1030 cm^{-1} is the stretching (–OH) out of the plane, which can be observed in the spectrum (Pavia et al. 2010).

DB4OCH$_3$ showed bands at 2961 and 2840 cm^{-1} are associated with stretching modes (C–H). Between 1300–100 cm^{-1} it is configured as ether (C–O) and more than one high-intensity band may appear, as occurs at 1242 and 1029 cm^{-1}. The FTIR spectrum shows medium and high

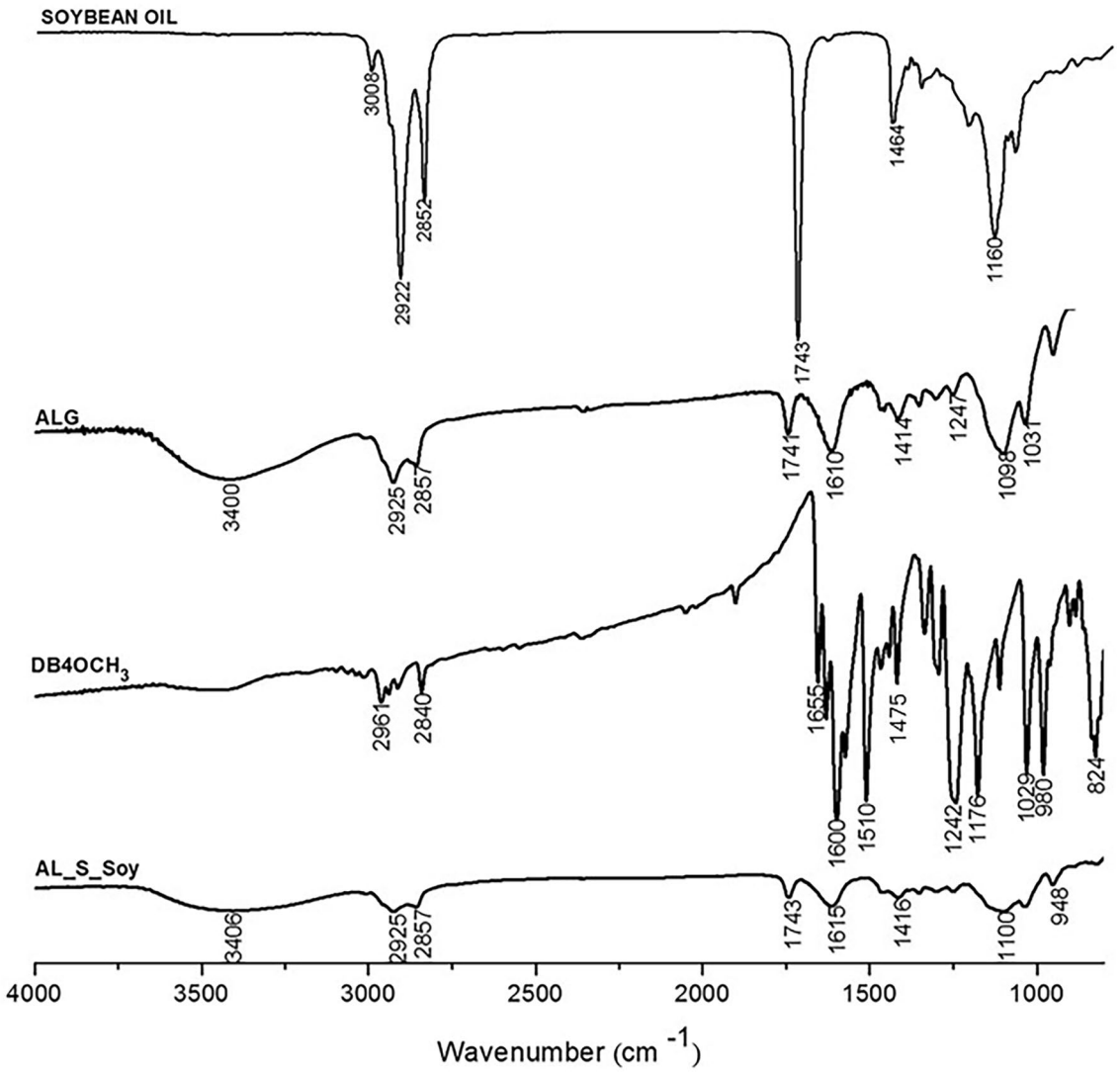


Fig. 5 FTIR bands, showing the main functional groups of soy oil, ALG, DB4OCH$_3$ and NE of AL_S_Soy (Alginate, surfactant plus soybean oil)

intensity infrared bands between 1655–1617 cm^{-1} associated with the presence of the carbonyl (C=O) stretching vibration. The DBOCH$_3$ molecule exhibits strong intensity bands between 1600 and 1475 cm^{-1}, indicating the C'–C' and C''=C'' ring stretching vibrations, commonly referred to as semicircle stretching (Sudha et al. 2012).

Mattos and collaborators (Mattos et al. 2016) performed a study relating the interactions of a chalcone and the components of the NE formulation. By performing an FTIR assay with T80, it was found that chalcone interacts with the methylene groups of MCT, the C–O group, C–C–O and C–H bonds of T80. In addition, the C–H bond of T80 was the group most influenced by the interaction with chalcone. The analysis showed that chalcone increases the mobility of the C=O group because of band broadening. Because of the similarity between the components in this work, the peaks shown are justified. The NE shows the characteristic peaks of sodium alginate, which is the NE's coating material. The other components are encapsulated in the NE, which is why they do not appear in the AL_S_Soy spectra.

Acute toxicity 96 h

The zebrafish is a vertebrate organism that has many biological similarities to humans, such as the CNS, the immune system, and the cardiovascular system. For this reason, adult zebrafish are increasingly being used in toxicity experiments (Levin and Cerutti 2009; Ferreira et al. 2021). It was used as an animal model to analyze the acute toxicity of the DB4OCH$_3$ transporter samples at different concentrations.

Adult *zebrafish* exposed to concentrations of 0.065 mg/mL and 0.0325 mg/mL in the AL_S_Min NE did not

exhibit any toxicity over a 96-h period. However, one live animal showed toxicity at a concentration of 0.13 mg/mL (LD_{50} = 0.13 mg/mL). The AL_S_Soy and AL_S NE samples were not toxic to adult *zebrafish* at any of the concentrations administered (LD_{50} > AL_S_Soy and AL_S).

Evaluation of locomotor activity and anxiolytic evaluation

This is the first record of evidence on the anxiolytic effect of NEs containing the synthetic chalcone DB4OCH_3 stabilized by sodium alginate, using an experimental model in adult zebrafish (*Danio rerio*), as far as we know. Locomotor activity testing is quite common in zebrafish through the open field test, because zebrafish movement and swimming behavior are highly sensitive to external and internal stimuli, including changes in their physical environment, emotional factors, and pharmacological responses (Hamilton et al. 2021; Taylor et al. 2017). The analysis of locomotor activity through the open field test can thus provide relevant results on the effect of different substances and conditions on zebrafish behavior, helping to better understand the mechanisms involved in toxicity and behavioral disturbances (Tao et al. 2020; Guo et al. 2022).

Figure 6 shows the locomotion behavior in the open field (Fig. 6 a, 6 b and 6 c) and the anxiolytic effect (Fig. 6d–f) in the light and dark test after the administration of the samples. The animals' locomotion was altered by all the concentrations, and all of them remained longer in the light zone of the aquarium.

NE AL_S_Min was more effective in reducing their locomotion at the concentration 0.065 mg/mL (Fig. 6a)), since there were only 25 crossings, lower than the control Diazepam, where 28 crossings occurred [$^{****}p < 0.0001$] and Diazepam [$^{****}p < 0.0001$ (4 mg/kg)]. The locomotion of the animals was altered by all concentrations of NE AL_ S_Soy, particularly at the concentration of 0.0325 mg/mL [$^{**}p < 0.01$] with a reduced locomotor activity of 46 crossings, when compared to the control groups (Fig. 6 b)) and Diazepam [$^{**}p < 0.01$ (4 mg/kg)]. The locomotion of the animals was altered by all concentrations of AL_S, but to a lesser extent, the most significant reduction being at concentration 0.0325 mg/mL [$^{*}p < 0.05$] and Diazepam [$^{****}p < 0.0001$ (4 mg/kg)], with 83 crossings occurring being observed in Fig. 6c.

NEs AL_S_Min and AL_S_Soy showed a greater reduction in locomotion (20.83% and 38.33%, respectively, compared to the control. These results are similar when compared to other study with the use of hydrazone, where the locomotion was reduced by 38.59% (SOUZA et al., 2022). In this way, the NEs developed in this study can be a drug vehicle to potentiate its effect. The reduction in zebrafish swimming activity is directly related to the sedative effect of the drug, its time of action and intensity of duration and directly linked to drugs acting on the CNS of zebrafish (Benneh et al. 2017; Wang et al. 2020). The presence of such an effect indicates substances with anxiolytic effect; for this reason, light/dark tests were performed.

To identify the anxiolytic potential of compounds, the light/dark test is used in adult zebrafish that resembles the composting of reefers (Gonçalves et al. 2020). When placed in an area with light and dark spaces, the fish normally spend most of their time in the dark area. However, when exposed to substances with anxiolytic properties, the fish spend more time in the light zone, indicating a reduction in anxious behaviors and an increase in exploratory activity (Gebauer et al. 2011).

Anxiolytics results (Fig. 6d–f) are similar to the positive control DZP and statistically different from the controls (naïve and vehicle). Considering that all samples were evaluated for 300 s, the control DZP presented a permanence of 64.67% in the clear zone. Thus, the samples with the greatest anxiolytic effect are AL_S (0.13 mg/mL) (Fig. 6f), as it had a higher effect than the control 82.67%, AL_S_Min (0.065 mg/mL) (Fig. 6d), having an effect of 57.67% and AL_S_Soy (0.0325 mg/mL) (Fig. 6e)) having an effect of 55.00%. It is important to mention that AL_S_Soy presented an anxiolytic effect with the lowest concentration of DB4OCH_3.

The results show the concentrations can be applied in the production of substances with anxiolytic properties and, in comparison with another study, had a better effect, requiring lower concentrations to obtain superior effects. In the work by Xavier and C et al., (2020), a chalcone synthesized from cinnamaldehyde obtained a permanence result in the clear zone of the aquarium of 60.16% (0.5 mg/kg *i.p*), which is lower than the results found in this study.

Assessment of neuromodulation by serotoninergic (5-HT)

The mediation of anxiety is influenced by serotonergic neuromodulation, and there is extensive research on the role of serotonin (5-HT) in this disorder (Artaiz et al. 1998; Boer and Koolhaas 2005). Granisetron is a selective 5-HT $_{3A/3B}$ receptor antagonist, which acts on the serotoninergic system, and is often used to assess the specific role of the serotonergic system in regulating anxiety symptoms (Hannon and Hoyer 2008). This may help to elucidate the mechanisms underlying anxiety and provide information on the functioning of the serotonergic system in this disorder. This information may be useful in developing more effective therapeutic approaches to anxiety care (Gonçalves et al. 2020). Figure 7 shows the assessment of serotoninergic (5-HT) neuromodulation.

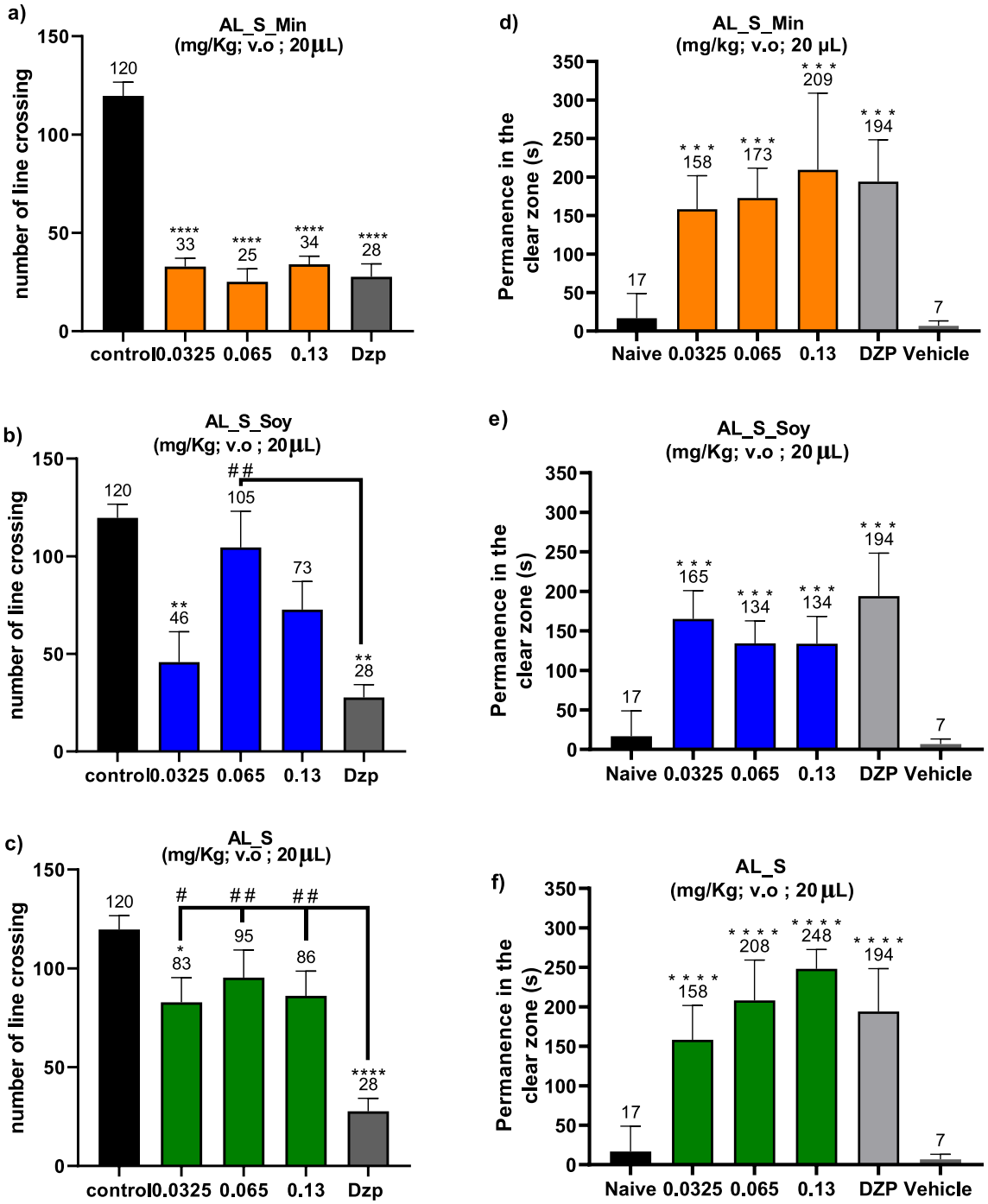


Fig. 6 Effect on zebrafish locomotion behavior of samples **a** AL_S_Min, **b** AL_S_Soy and **c** AL_S and Effect on anxiolytic behavior of samples **d** AL_S_Min, **e** AL_S_Soy and **f** AL_S analyzed for 300 s. The data show the mean ± standard error of the mean for 6 animals per group; ANOVA followed by Tukey's test was used for statistical analysis. Asterisks indicate statistical significance in comparison with the control group (vehicle—DMSO 3%), * $p < 0.05$ versus Control, ** $p < 0.01$ versus Control, *** $p < 0.001$ versus Control, **** $p < 0.0001$ versus. Control; the number symbol indicates statistical significance in comparison with the Diazepam group # $p < 0.05$ versus DZP, ## $p < 0.01$ versus DZP

Granisetron did not reverse the anxiolytic effect of AL_S_Min (Fig. 7a)) and was also considered toxic after the 96-h test (AL_S_Min mg/kg; 20 µL; v.o.), unlike fluoxetine (## $p < 0.01$) (40 mg/kg; 20 µL; v.o.). The results therefore reveal that the mechanism of action is not related to the receptors of the serotonergic system. Unlike the AL_S_Soy

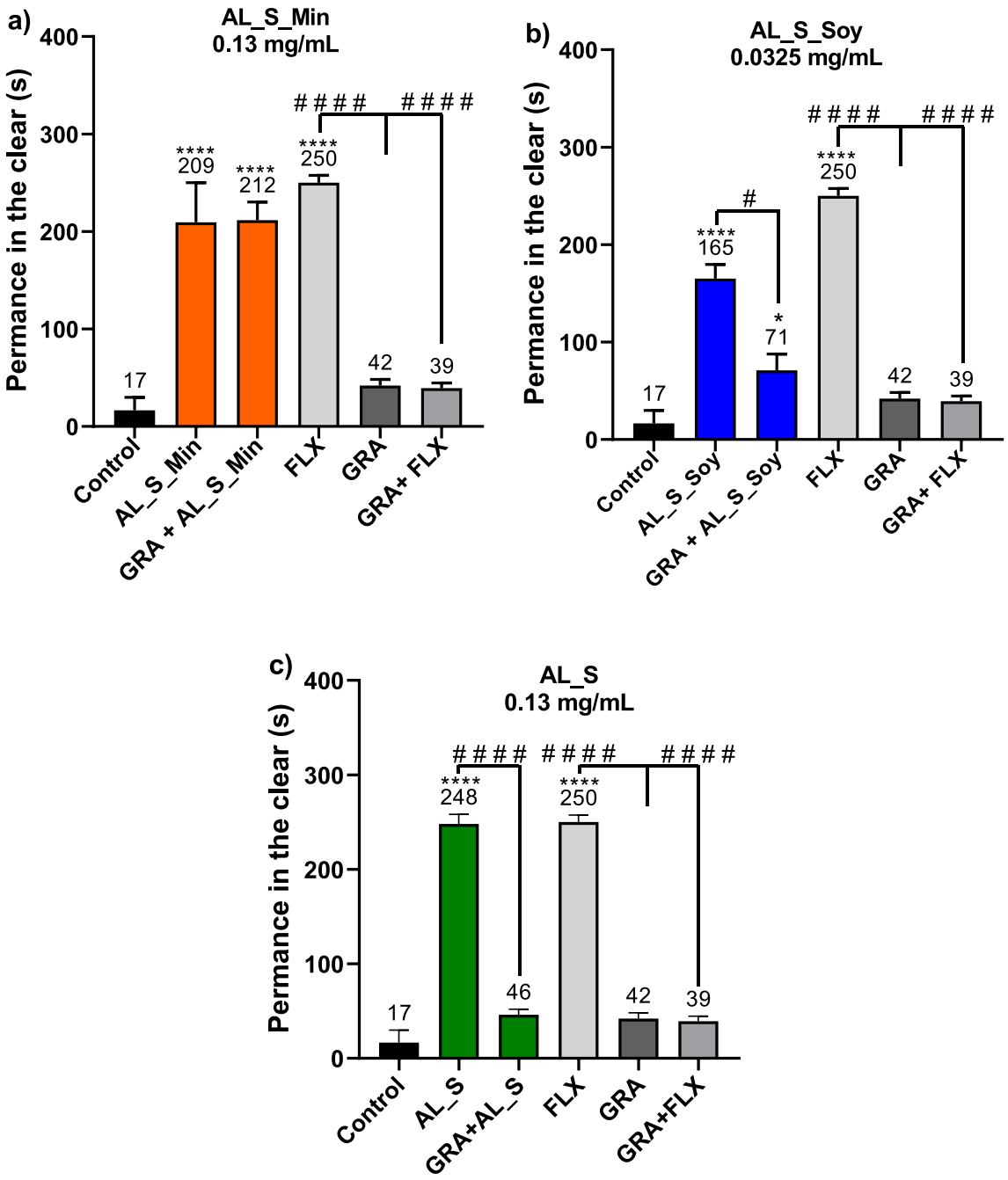


Fig. 7 Assessment of serotoninergic (5-HT) neuromodulation of zebrafish in samples **a** AL_S_Min, **b** AL_S_Soy and **c** AL_S (300 s). Data show the mean ± standard error of the mean for 6 animals per group; ANOVA followed by Tukey's test was used for statistical analysis for light/dark. (*$p < 0.05$ *vs.* Naive or vehicle, ****$p < 0.0001$ *vs.* Naive or vehicle; #$p < 0.05$ *vs.* FLX + Dzp or FLX + sample, ####$p < 0.0001$ *vs.* FLX + Dzp or FLX + sample)

(Fig. 7 b)) and AL_S (Fig. 7c)) samples, since the combination of these samples with Granisetron could reverse the anxiolytic effect of NEs. Therefore, the samples, AL_S_Soy and AL_S, have a mechanism of action with the receptors of the serotonergic system.

Most of these anxiolytic compounds have substituents such as dimethylamine, methoxy, nitro, hydroxyl, methyl and halogens. The chalcone DB4OCH$_3$ has two methoxy groups (Fig. 1a) which can be related to its anxiolytic effect (Higgs et al. 2019; Tan et al. 2021; Ferreira et al. 2020). The bioavailability is influenced by changes in particle sizes, which in turn explain the variation in mechanism of action due to differences in the composition of NEs. The sizes of AL_S_Soy ranged from 128.47

to 181.8 nm, while for AL_S, they ranged from 375.90 to 410.0 nm. AL_S_Min exhibited a wide range of variation, spanning from 185.27 to 3495.0 nm. As mentioned in the literature, in order for a drug to cross the Central Nervous System barriers, it must have particle sizes of less than 500 nm and is best absorbed when it is smaller than 200 nm (Karami et al. 2019; Costa et al. 2019; Nasr 2016).

Campelo et al. (2023) performed a study using polysaccharide emulsions to encapsulate volatile oil, where it was possible to reduce the fish's anxious behavior without interfering with its mobility and its anxiolytic activity is linked to the GABAergic pathway. (Adams and Valley 1995). In other research involving synthetic chalcones and derivatives, they have also been observed to cause an anxiolytic effect in zebrafish via the serotoninergic (5-HT) system (Ferreira et al. 2020; Mendes et al. 2023; Silva et al. 2021).

Conclusion

The design of the nanoemulsions altering the polysaccharide matrix and their adjuvants lead to an optimization, where alginate and soybean oil (Al_S_Soy) presented the best balance of properties considering long-term stability, low particle size and high encapsulation efficiency. In this sense, interactions between soybean oil ester groups with ether groups from T80 favored the stabilization of the hydrophilic phase with the alginate polymer matrix. Conversely, hydrophobic segments from soybean and T80 aided the compatibilization of the chalcone molecules within the droplets in the oily interface, promoting a better encapsulation of the chalcone. Anxiety tests performed using the zebrafish model identified a reduction in locomotion in fish, confirmed by light/dark test, particularly for Al_S_Soy, indicating a high anxiolytic effect with a mechanism of action with the receptors of the serotonergic system (5-HT3). The results support the potential therapeutic use of NEs produced with ALG and soybean oil with DB4OCH3 for the development of compounds with anxiolytic properties.

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Author contributions Conceptualization was done by J. F. Nascimento and F. O. M. S. Abreu. Methodology was done by J. F. Nascimento, R. M. Castelo, J. M. Guedes, and T. Holanda. Formal analysis and investigation were done by J. F. Nascimento, F. O. M. S. Abreu, and T. Holanda. Writing—original draft preparation was done by J. F. Nascimento and F. O. M. S. Abreu. Writing—review and editing was done by H. S. dos Santos and J. E. S. A. de Menezes. Funding acquisition was done by F. O. M. S. Abreu. Resources was done by F. O. M. S. Abreu, H. S. dos Santos, and J. E. S. A. de Menezes. Supervision was done by F. O. M. S. Abreu.

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Availability of data and materials The data that support the findings of this study are available from the corresponding author, [F. O. M. S. Abreu], upon reasonable request.

Declarations

Conflict of interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

Ethical approval The Project entitled "Use of Zebrafish (*Danio rerio*) as an alternative model for Investigation of the pharmacological potential of natural and synthetic products" registered with the protocol 04983945/2021, under the supervision of Jane Eire Silva Alencar de Menezes, agrees with Ethical Principles in Animal Experimentation, adopted by the Ethics Committee in Animal Experimentation of Ceará State University (CEUA—UECE).

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