

## Encapsulation of clove oil in nanostructured lipid carriers from natural waxes: Preparation, characterization and *in vitro* evaluation of the cholinesterase enzymes



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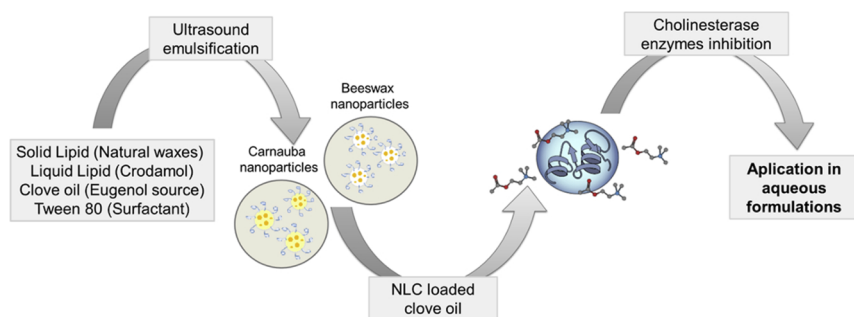
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### GRAPHICAL ABSTRACT



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### ABSTRACT

Eugenol is a phenolic compound largely found in the clove essential oil that possesses promising biological activity. However, its low water solubility is a major concern and encapsulation is an alternative to improve water affinity. The objective of this work was to produce nanostructured lipid carriers (NLC) by hot homogenization/ultrasound emulsification and to evaluate the effect of free and encapsulated clove oil on the *in vitro* cholinesterase enzymes modulation using *Drosophila melanogaster* (DM) tissue. The NLC composed of a natural wax (carnauba or beeswax) and crodamol showed an average diameter between 121 and 367 nm with good dispersion and colloidal stability. The spherical shape and solid character together with the semi-crystalline environment confirm the formation of NLC. DSC analysis indicated polymorphic transition events of the waxes. *In vitro* tests using DM demonstrated that

**Abbreviations:** NLC, nanostructured lipid carriers; DM, drosophila melanogaster; AChE, acetylcholinesterase; Ach, acetylcholine; BChE, butyrylcholinesterase; CW, carnauba wax; BW, beeswax; CO, clove oil; BSCh, butyrylthiocholine iodide; DTNB, dithiobis (2-nitrobenzoic acid); TFK, potassium phosphate buffer; Dp, average particle size; PDI, polydispersity Index; DLS, dynamic light scattering; TEM, transmission electron microscopy; FTIR, fourier transform infrared; DSC, differential scanning calorimetry; EE, encapsulation efficiency; T<sub>m</sub>, melting temperature; ΔH, melting and cooling enthalpies; ΔT<sub>1/2</sub>, width at half height; T<sub>onset</sub>, onset temperatures

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free clove oil showed a good inhibition of the butyryl and acetylcholinesterase enzymes above a concentration of 10 mM, with IC<sub>50</sub> values of 4.3 and 3.5 mM, respectively. The dispersions of the NLC loaded with clove oil showed a decrease in the IC<sub>50</sub> enzymes values, indicating the preservation of the clove essential oil and suggesting an increased in the solubility. Results indicate that NLC dispersions have good potential to be used for foods and cosmetic aqueous formulations possessing biological activity.

## 1. Introduction

Phenolic compounds are found naturally in many fruits, vegetables, and nuts, as secondary metabolites. Chemically, these compounds have one or more hydroxyl group attached to a benzene ring and play an important role in the human diet by exerting various biological effects such as free radical scavenging, metal chelation, modulation of enzymatic activity and altering signal transduction pathways [1–3]. Eugenol is the major component of clove oil, approximately 90–95%, responsible for the specific clove aroma and known as a good agent for human health due to the biological activities and also for the specific clove oil aroma [3–12].

The antioxidant potential of eugenol was extensively studied in the literature [6,10,13–17], and some studies associating this antioxidant property with acetylcholinesterase inhibition were also reported [3,7]. The acetylcholinesterase (AChE - EC 3.1.1.7) is an enzyme responsible for the termination of cholinergic signaling by hydrolyzing acetylcholine (ACh) and the inhibition of AChE is effective in the treatment and management of Alzheimer's disease, multiple system atrophy, and other conditions [3,7,18]. Acetylcholine hydrolysis can also be catalyzed by butyrylcholinesterase (BChE - EC 3.1.1.8, also known as serum cholinesterase or pseudo-cholinesterase), a related and less-specific enzyme [18], however, to the best of the authors knowledge there is no report in literature about eugenol as a potential modulator of BChE activity.

Although some research groups have been investigating eugenol as potential AChE inhibitor [19–22] all studies involve the use of the clove oil in the free form, and the concentration needed to ensure an effective activity as AChE inhibitor may be difficult to reach in actual biological systems due to the hydrophobic character of the essential oil. In addition, eugenol is volatile and chemically unstable in the presence of air and light, and its encapsulation may be a positive alternative since the main objective is to protect the bioactive compound against the adverse environmental conditions. In this way, the incorporation of the essential oil in a lipid matrix may improve the solubility and the bioavailability in the medium. The lipid nanoparticles are generally made by biodegradable lipids that can be solid at room and body temperature and possess several advantages like high drug loading, increased drug stability, controlled drug release, and enhanced penetration of drugs into the skin or any other target [23–25]. The nanostructured lipid

carriers (NLC) loaded with essential oils can be easily incorporated in water-rich cosmetic and food formulations, expanding its range of application and improving bioactive compound shelf life as already reported by other works [26–28].

In the present work clove oil, a great source of eugenol, was incorporated in NLC made from either carnauba wax (CW) or beeswax (BW) using the hot homogenization/ultrasound emulsification technique. The waxes obtained from natural sources like carnauba and bees are composed of hydrocarbons, alcohols, free acids, esters, and other materials, which have many advantages due to the low toxicity and biocompatibility making the final product interesting for several applications [29]. The obtained aqueous NLC dispersions were evaluated in relation to particle diameter, distribution, and morphology, clove oil encapsulation efficiency, as well as by chemical and thermal analyses. In addition, AChE and BChE activities were verified *in vitro* for the free and encapsulated clove oil using a *Drosophila melanogaster* (DM) tissue as biological model. DM is widely used in biological tests due to the great similarity with the human genetic sequence, allowing the understanding of complex biological problems [30,31] and it was used in the present study as an alternative biological model to test the NLC loaded clove oil effects in the cholinergic enzymes AChE and BChE.

## 2. Experimental

### 2.1. Materials

NLC production involved the use of carnauba wax (CW) and beeswax (BW) as solid lipids kindly donated by GM Ceras Ltda/ Brazil, crodamol GTCC (saturated triglyceride from coconut oil, Alpha Química, Brazil) as liquid lipid, clove oil (CO - 92% Eugenol) from Ferquima/Brazil as bioactive compound, polysorbate 80 (Tween 80 - Sigma-Aldrich) as surfactant, and distilled water as aqueous phase. It is worth noting that Tween 80 and crodamol are considered safe food ingredients by FDA and were used in NLC formulations [32]. For AChE and BChE activities tests were used acetylthiocholine iodine (ASCh), butyrylthiocholine iodide (BSCh), 5,5'-Dithiobis(2-nitrobenzoic acid) (DTNB, 98% purchased from Sigma-Aldrich/Brazil) and methanol (P.A., NEON). Mono and dibasic potassium phosphate (NEON) were used to prepare TFK buffer and tris-(hydroxymethyl)aminomethane

**Table 1**

Composition of the organic phase of carnauba wax (CW) and beeswax (BW) NLC, average particle diameter (Dp), polydispersity index (PDI), and encapsulation efficiency (EE) of the nanoparticles at day 1, 15 and 30.

Sample	Wax (g)	Crod (g)	CO (g)	Dp (nm)/ PDI			EE (%)		
				Day 1	Day 15	Day 30	Day 1	Day 15	Day 30
CW1	3	–	–	1489/0.27	–	–	–	–	–
CW2	2	–	–	613/ 0.31	–	–	–	–	–
CW3	1	–	–	178/ 0.27	301/ 0.43	381/ 0.52	–	–	–
CW4	0.9	0.1	–	121/ 0.08	122/ 0.09	125/ 0.12	–	–	–
CW5	0.9	0.075	0.025	126/ 0.11	128/ 0.11	130/ 0.10	58 ± 0	58 ± 0	56 ± 1
CW6	0.9	0.05	0.05	150/ 0.19	146/ 0.18	152/ 0.16	60 ± 2	61 ± 3	58 ± 1
BW1	3	–	–	443/0.165	–	–	–	–	–
BW2	2	–	–	320/ 0.12	–	–	–	–	–
BW3	1	–	–	324/ 0.15	617/0.58	698/0.64	–	–	–
BW4	1.8	0.2	–	227/ 0.09	229/ 0.09	231/ 0.12	–	–	–
BW5	1.8	0.1	0.1	272/ 0.09	242/ 0.09	260/ 0.13	66 ± 1	66 ± 0	64 ± 0
BW6	1.8	0.05	0.15	342/ 0.08	349/ 0.06	357/ 0.10	64 ± 1	65 ± 0	63 ± 2
BW7	1.8	0.02	0.18	368/ 0.10	394/ 0.12	401/ 0.14	63 ± 0	63 ± 0	60 ± 2

(Tris) from Sigma-Aldrich. All other chemicals were analytical grade and purchased from local sources. *Drosophila melanogaster* (DM) wild type flies were obtained from the National Species Stock Center (Bowling Green, OH, USA) and were used as source of biological tissue.

## 2.2. Preparation of the wax nanoparticles by ultrasound emulsification

The NLC production was conducted by the hot homogenization/ultrasound emulsification technique described elsewhere with some modifications [33]. Initially, the lipid and aqueous phases were prepared separately. The aqueous phase was prepared dissolving 3%wt of Tween 80 in water (10 mL). The lipid phase was composed of different amounts of CW or BW, crodamol and clove oil as shown in Table 1. In sequence, the physical mixture of the organic components was made under heat (10 °C above the melting point of the wax) and after complete melting, the aqueous phase (previously heated to the same temperature) was added to the organic phase. Both phases were intensely homogenized for 15 min in a hot bath with orbital agitation to form a coarse emulsion. Then, the emulsion was submitted to high-energy emulsification by an Ultrasonic Dismembrator (Fischer Scientific 500, 400 W) at 50% of amplitude in pulsed regime for 1 min (30 s on 10 s off) in order to reduce the melted droplets to the nanometric range. Finally, the melted wax nanodroplets were cooled in cold water (90 mL at 4 °C) to form solid nanoparticles (final volume 100 mL).

## 2.3. Particles characterization

Intensity average particle size (Dp) and polydispersity index (Pdl) of the NLC were determined by Dynamic Light Scattering (DLS) using a Zeta Sizer Nano S (Malvern Instruments). The formulations were analyzed right after the production (Day 1) and after 15 and 30 days of storage at room temperature (Day 15 and 30). For that, the samples were properly diluted in water and measured at 25 °C in a glass cuvette. Transmission Electron Microscopy (TEM 100 kV JEM-101) was carried out to investigate the particle morphology of the CW and BW NLC. For that, each sample was diluted in distilled water up to 0.1%wt solids, and then, one drop of each diluted sample was placed on a carbon-coated 400 mesh copper grid of and dried overnight under room conditions. The chemical analysis was performed by Fourier Transform Infrared (FTIR) spectroscopy using a Shimadzu IRPrestige-21. The pure clove oil, bulk waxes, and lyophilized NLC samples were analyzed using the potassium bromide (KBr) pellet method. Spectra were taken in the range of 4000 to 400 cm<sup>-1</sup> with 32 cumulative scans. Lyophilized NLC samples and bulk waxes were analyzed by Differential Scanning Calorimetry (DSC 4000 Perkin Elmer) under inert atmosphere (N<sub>2</sub>, 20 mL.min<sup>-1</sup>), from 0 to 100 °C at a heating rate of 10 °C.min<sup>-1</sup>. The thermal history was removed under a heating rate of 20 °C.min<sup>-1</sup> and cooling rate of -20 °C.min<sup>-1</sup>. DSC heating and cooling curves were obtained from the second and first run, respectively. Calorimetric parameters including the melting temperatures (T<sub>m</sub>), melting and cooling enthalpies enthalpy (ΔH), width at half height (ΔT<sub>1/2</sub>) and onset temperatures (T<sub>onset</sub>) were analyzed using the Pyris Manager software (PerkinElmer). The crystallinity index (CI) of NLC formulations were calculated using Eq. (1):

$$CI = \frac{\Delta H_{mNLC}}{\Delta H_{mwax}} \times 100\% \quad (1)$$

where, ΔH<sub>mNLC</sub> and ΔH<sub>mwax</sub> are the melting enthalpy of the NLC sample and pure wax, respectively [29,34].

The clove oil encapsulation efficiency (EE) in the NLC matrix was determined by the analysis of the free clove oil in the aqueous phase of the NLC dispersion. The NLC dispersions containing clove oil (500 μL) were ultra-filtrated using an Eppendorf with an Amicon Ultra 0.5 coupled to a filter (Millipore®, 100 kDa) at 10,000 rpm for 10 min. An aliquot of the filtered liquid was adequately diluted in ethanol, transferred to a quartz cuvette and analyzed in the wavelength of 280 nm in a UV-vis spectrophotometer (Hitachi U-1900). The concentration of free clove oil was calculated as previously described elsewhere [35].

## 2.4. Tissue preparation

The *Drosophila Melanogaster* homogenate was prepared as described elsewhere with minor modifications [31]. Adults flies (both gender) were maintained and cultured in cornmeal medium (2% w/v sucrose, 1.6% w/v powdered milk, 0.2% salt, and 0.05% w/v methylparaben) during 12 h dark/light cycle conditions at constant temperature (22–24 °C) and humidity (45–65 %). One hundred adult flies were cold anesthetized in a domestic freezer and manually homogenized in 1 mL of Tris–HCl solution (50 mM, pH 7). In sequence, samples were centrifuged at 12,000 rpm for 10 min and the supernatant (S1) was used as DM homogenate in the *in vitro* AChE and BChE tests.

## 2.5. In vitro cholinergic activity

Acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) activity were measured by the assay described by Ellman et al. [36] considering some modifications described by Hammond and Forster [37] and Dohi et al. [7] using DM homogenate as enzymatic source. The IC<sub>50</sub> was determined plotting the graphs and line equations between the clove oil concentration versus the % to each system.

*Free clove oil*: Initially, solutions with appropriate clove oil concentrations were prepared by sequential dilution of the essential oil in methanol. In sequence, TFK (25 μL, 100 mM) and S1 (12.5 μL) were placed in a 96-well microplate and dissolved with 12.5 μL of each previously prepared clove oil methanolic solution. At this point, the time zero (abs t<sub>0</sub>) reading was performed in a microplate reader (Thermo-Plate Reader) at 405 nm. After a pre-incubation at 37 ± 1 °C for 15 min, DTNB (62.5 μL, 2 mM) and substrate (12.5 μL, ASCh or BSCh 8 mM) were added into the wells and then incubated at room temperature for 15 min. The absorbance of the final mixtures was measured at the end of the incubation time (abs t<sub>final</sub>). A control mixture was prepared by using 12.5 μL of pure methanol instead of the methanolic oil sample. The inhibition percentage was determined by Eq. (2).

$$I(\%) = 100 - \frac{Abs_{sample}}{Abs_{control}} \times 100 \quad (2)$$

where, Abs<sub>sample</sub> is the absorbance of the sample containing clove oil and Abs<sub>control</sub> is the absorbance of the control without the essential oil.

*Clove oil-loaded NLC*: Initially, appropriated NLC concentrations were prepared by sequential dilutions of the NLC aqueous dispersion in water. In sequence, 12.5 μL of TFK (100 mM), 12.5 μL of methanol, and 12.5 μL of DM homogenate were placed in a 96-well microplate and mixed with 12.5 μL of each previously prepared NLC dispersion. All the other procedures including incubation time and temperature, absorbance reading, the addition of DTNB and substrates, and percentage of inhibition (I%) were the same as those described for “Free clove oil as inhibitor”. Otherwise, the control mixture was prepared using NLC dispersion with no clove oil (blank sample) but with the same solids content.

## 2.6. Statistical analyses

Biological results are presented as mean ± standard deviation. One-way ANOVA was used to investigate the significant difference in the *in vitro* experiments following by Tukey *post hoc* test with 5% significance level. Analyses were conducted on GraphPad Prism 5 software.

## 3. Results and discussion

### 3.1. Particles characterization

The NLC were produced using BW or CW and crodamol as matrix material, and different formulations were tested with varying amounts of wax as solid lipid, crodamol as liquid lipid, and clove oil as bioactive compound. The formulations and Dp, Pdl and EE results are presented

in Table 1. Transmission Electron Microscopy images of selected samples are showed in Fig.1.

The use of carnauba wax as matrix material systematically resulted in particles with higher Dp and PDI when compared to beeswax. However, in all cases, Dp values between 120 and 1489 nm with narrow distributions were found, which may be interesting depending on the desired application. The carnauba wax formulations containing a single lipid in organic phase (CW1, CW2, and CW3) showed a pronounced increase of the Dp with increasing wax amount used, as compared to the BW formulations, which may be associated to the CW higher chain length, and consequently, higher viscosity [38]. The use of 10 wt% (related to the organic phase, CW4 and BW4) of crodamol as liquid lipid favored a noteworthy reduction in Dp as well as in the PDI for formulations with either CW or BW. A similar effect was observed in other studies [33,34] which related the reduction of the Dp to the decrease in the organic phase viscosity, decreasing the resistance of the melted droplets disruption during emulsification/sonication.

After the incorporation of the clove oil in the formulations, a slight increase in Dp was visualized for all preparations, probably due to the reduction of the crodamol percentage in the organic phase, but it was still ensured a monomodal and homogeneous dispersion with PDI below 0.2.

The presence of the liquid lipid played an important role in the particle stability of the formulations. The absence of crodamol in the formulation resulted in a great increase in Dp and PDI after storage, while the formulations containing 10 wt% of crodamol (related to the organic phase) showed just a slight increase in Dp and distributions for both waxes, still showing a good colloidal stability. Results indicate that the SLN formulations containing only wax were more susceptible to aggregation with an increase on the Dp and PDI. On the other hand, the binary lipid NLC formulations showed a slight change in Dp and PDI after storage, indicating more stable structure, consequently, a lower disposition to aggregation.

TEM images confirmed the nanometric range of the lipid particles with diameters compatible with those measured by DLS. The spherical shape (almost oval for the BW NLC), smooth surface, and solid character indicate that no core-shell morphology was formed, but rather a

solid particle with the crodamol and the bioactive compound distributed uniformly in the lipid sphere. This solid/no core morphology is usually expected for the NLC formulations composed of the mixture of solid and liquid lipids. Although the use of a liquid lipid is essential for drug accommodation it just ensures distances between the long fatty acids chains from waxes forming imperfections in the crystal, allowing room to active compound loading but not room enough to form a core even at high liquid lipid amounts [32,39].

All formulations showed a good capacity to incorporate different amounts of clove oil with EE around 60%, and beeswax formulations (higher amount of dispersed phase) were able to incorporate higher amounts of clove oil than the carnauba wax formulations. In addition, even after 30 days of storage, no decrease was observed in the EE showing that the bioactive compound had a good affinity for the NLC matrix.

Free clove oil, bulk CW and BW, and NLC formulations (without and with the highest amount of clove oil) were analyzed by FTIR and the spectra are shown in Fig.2.

Spectrum a shows typical eugenol peaks, including a large band related to the hydroxyl group (O–H) stretch around 3550 and 3200  $\text{cm}^{-1}$ , peaks at 2950 and 2980  $\text{cm}^{-1}$  related to axial deformation of the groups  $\text{CH}_2$  and  $\text{CH}_3$ . The peak at 1640  $\text{cm}^{-1}$  is related to weak axial deformation of the alkene group  $\text{C}=\text{C}$ , 1540  $\text{cm}^{-1}$  to the axial deformation of  $\text{C}=\text{C}$  of the benzene ring, at 1033  $\text{cm}^{-1}$  a peak related to aromatic C–H is observed and at 750  $\text{cm}^{-1}$  out-of-plane angular deformation related to C–H of the aromatic ring [40,41].

Spectra b and c show the peaks referent to carnauba wax and beeswax. The regions 2925 and 2850  $\text{cm}^{-1}$  are related to the symmetric and asymmetric vibrations of the  $\text{CH}_3$  group and the peak at 714  $\text{cm}^{-1}$  confirms the asymmetric angular deformation of the long hydrocarbon chains  $\text{CH}_2$ . Still, the peaks in the region of 1739 and 1103  $\text{cm}^{-1}$  indicate, respectively, the stretch of  $\text{C}=\text{O}$  from the o carboxyl groups and C–O stretch from the ester group [42,43]. Although in lower intensity, the same peaks were found in the NLC spectra (d, e, f, g), indicating that the matrix material maintained the original characteristics after the NLC production. On the other hand, no eugenol characteristic peak

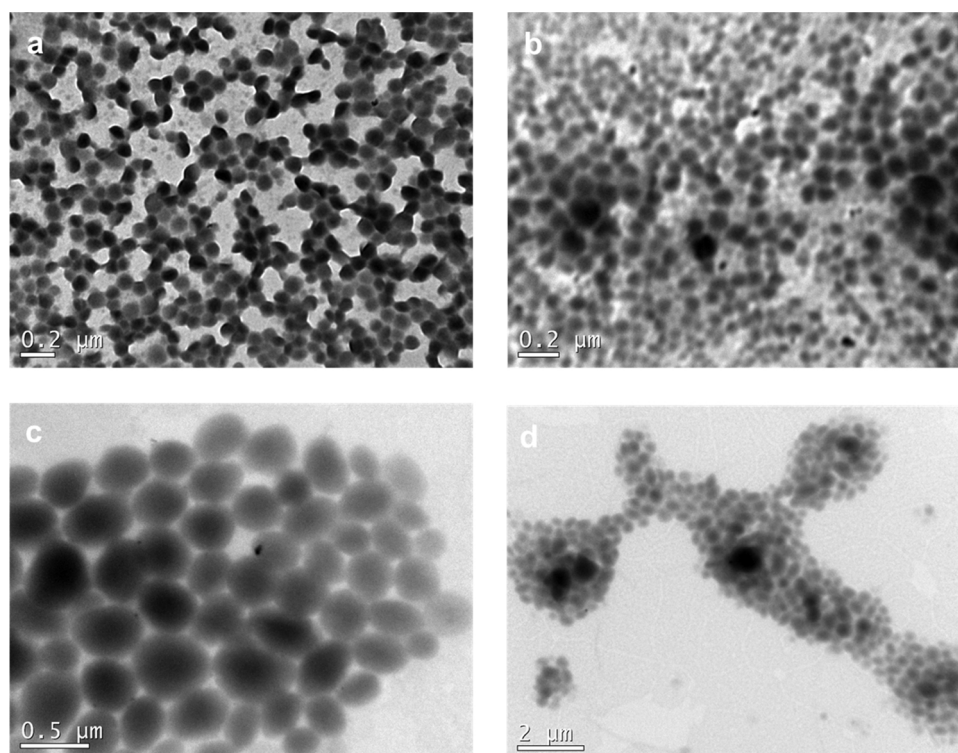


Fig. 1. TEM micrographs of the NLC formulations (a) CW4, (b) CW6, (c) BW4 and (d) BW7. The scale bar of the figures a, b, c and d corresponding, respectively, to 0.2, 0.2, 0.5 and 2  $\mu\text{m}$ .

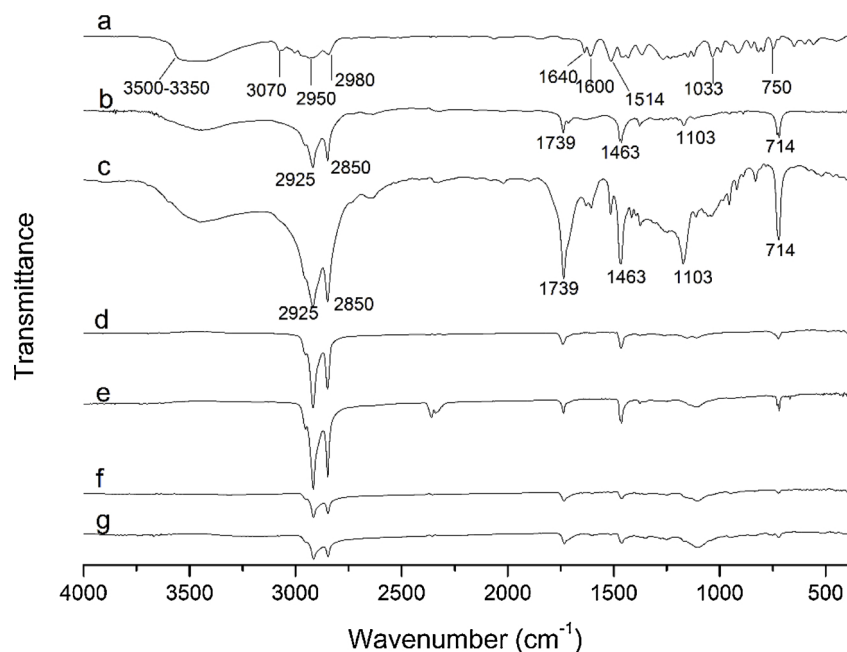


Fig. 2. FT-IR spectra of (a) pure clove oil, (b) beeswax (BW), (c) carnauba wax (CW), (d) BW4, (e) BW7, (f) CW4, and (g) CW6.

was found in spectra f and g (NLC formulations containing clove oil), indicating the overlapping of the clove oil spectra with the waxes spectra probably due to the low amount of the oil in the formulations as already reported by other authors [44,45].

Fig. 3 shows second heating and first cooling runs of bulk waxes (BW, CW), NLC formulations without (BW4/CW4) and with the highest amount of clove oil (BW7/CW6) analyzed by DSC.

The heat flow curve for bulk BW showed an endothermic melting peak with homogeneous melting and melting temperature ( $T_m$ ) 57 °C (Fig.3a). The NLC BW4 had a pre-melting (left shoulder) followed by complete melting, which is associated to the presence of a high amount of crodamol (10 wt%) in the formulation creating a less crystalline environment and, consequently, melting at a lower temperature while a more ordered structure melts at a higher temperature. The cooling curves (Fig.3b) showed that the presence of crodamol in NLC BW4 assisted the polymorphic transition of the lipid from  $\beta$ -modification to  $\alpha$ -modification [46], most dense packing and disordered aliphatic chain conformation, respectively [46–49], confirming the presence of a less ordered structure. On the other hand, formulation NLC BW7 (reduced amount of crodamol (1 wt%) and high amount of clove oil 9 wt%) showed single melting and cooling peaks similar to those found for the bulk wax, revealing the absence of polymorphic transitions. The less ordered crystal lattice is favorable for entrapping molecules and the increase in  $\Delta H$  and decrease in  $T_{onset}$ ,  $T_m$ , CI, and  $\Delta T_{1/2}$  (Table 2) for BW NLC formulations compared to the bulk wax indicates that both nanoparticulate formulations possess a portion of less ordered structures, but only BW4 with 10 wt% of crodamol presented  $\alpha \rightarrow \beta$  polymorphic transition.

Fig.3a also shows the heat flow curve for bulk CW with endothermic and homogeneous melting peak and melting temperature ( $T_m$ ) of 83 °C while the CW NLC formulations showed a pre-melting at 58 °C followed by the complete melting, consequently, reducing the  $T_{onset}$  and an increasing  $\Delta T_{1/2}$  of the NLC CW in relation to the bulk wax. The cooling curves presented in Fig.3b show the polymorphism transition from  $\beta$ -modification to  $\alpha$ -modification in bulk CW and CW NLC. The result suggests that bulk CW possesses a polymorphic nature and the presence of a shoulder in melting peaks of the CW NLC may be related to the lipid intermediates ( $\beta'$ -modifications) formed during the  $\beta \rightarrow \alpha$  polymorphic transition [38,44,49]. In addition, some authors associated these  $\beta'$ -fractions to the interaction between the lipid and the emulsifier

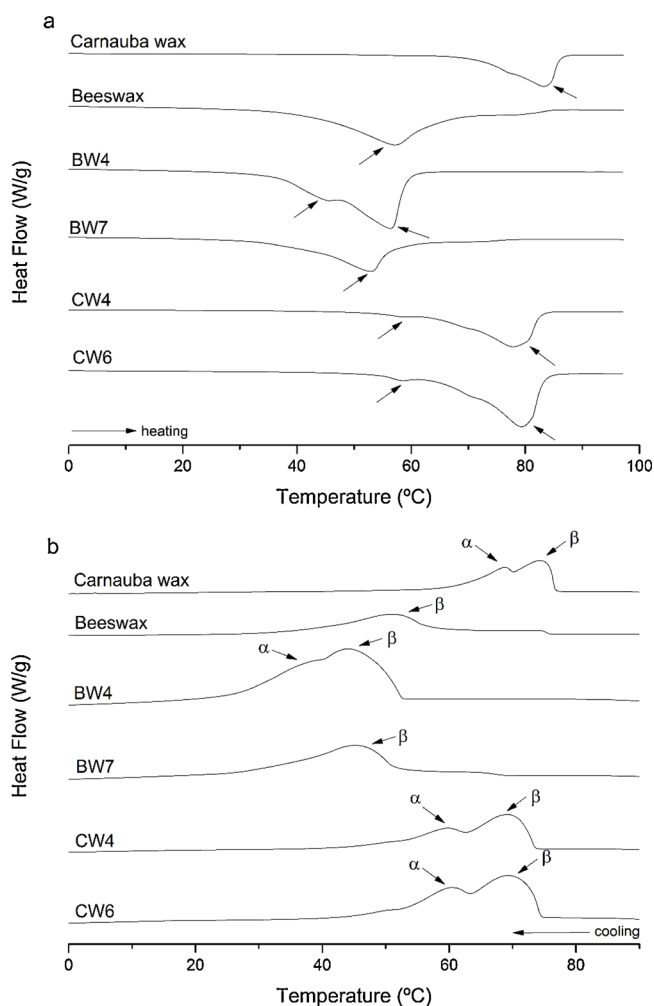


Fig. 3. DSC heating (a) and cooling (b) enthalpies of carnauba wax (CW), beeswax (BW), and formulations BW4, BW7, CW4 and CW6.

**Table 2**

DSC calorimetric parameters of waxes and NLC formulations: melting temperatures ( $T_m$ ), recrystallization temperatures, heating and cooling enthalpies ( $\Delta H$ ), width at half height ( $\Delta T_{1/2}$ ) and onset temperatures ( $T_{onset}$ ).

Melting						
Parameter	Carnauba wax	Beeswax	BW4	BW7	CW4	CW6
$T_{onset}$ (°C)	72.7	51.4	36.8	48.2	55.93	56.9
$T_m$ (°C)	83.3	57.6	56.4	52.9	77.9	79.2
$\Delta H$ (J/g)	186.0	149.3	142.1	135.5	134.1	133.3
CI (%)	100.0	100.0	95.1	90.7	72.1	71.7
$\Delta T_{1/2}$ (°C)	8.9	13.6	12.8	9.9	10.7	9.4
Recrystallization						
Parameter	Carnauba wax	Beeswax	BW4	BW7	CW4	CW6
$T_\alpha$ (°C)	68.7	–	38.3	–	59.0	60.1
$T_\beta$ (°C)	74.4	51.0	44.2	45.2	69.2	69.4
$\Delta H$ (J/g)	–197.0	–144.9	–149.5	–124.3	–135.9	–132.7

[28,44]. Other study observed an increase in the left shoulder of the DSC melting curves as the amount of carnauba wax was increased in the formulation and related this thermal behavior directly with the SLN production process and reduction of the particle size [44]. The difference in crystallization and melting temperatures, enthalpies,  $T_{onset}$ , and the sharp decrease in CI between the nanoparticulate and bulk CW samples indicates that the polymorphism of the crystals formed in the NLC are less organized than in the pure wax. The decrease in crystallinity is favorable for compounds entrapment since lattice defects of the lipid structure could offer room to accommodate the drugs, while highly crystalline lipids would lead to drug expulsion [28].

The NLC formulations containing crodamol as liquid lipid showed a semi-crystalline or less ordered structure than the bulk waxes. Similar behavior was reported by other authors [45,50] that used binary lipid combinations to entrap bioactive compounds, and the presence of a liquid lipid in the formulation decreased the crystallinity of the dispersed phase, and consequently, improved the drug encapsulation efficiency. In other words, the use of a liquid lipid increases the number of imperfections and leads to increased drug loading capacity and retention, which composes the first model of NLC called imperfect type NLC [39]. These results indicate that all BW and CW formulations containing a fraction of crodamol could be used as a potential system for clove oil delivery. Based on this, the samples containing the highest amount of clove oil were selected for the *in vitro* AChE and BChE inhibition assays.

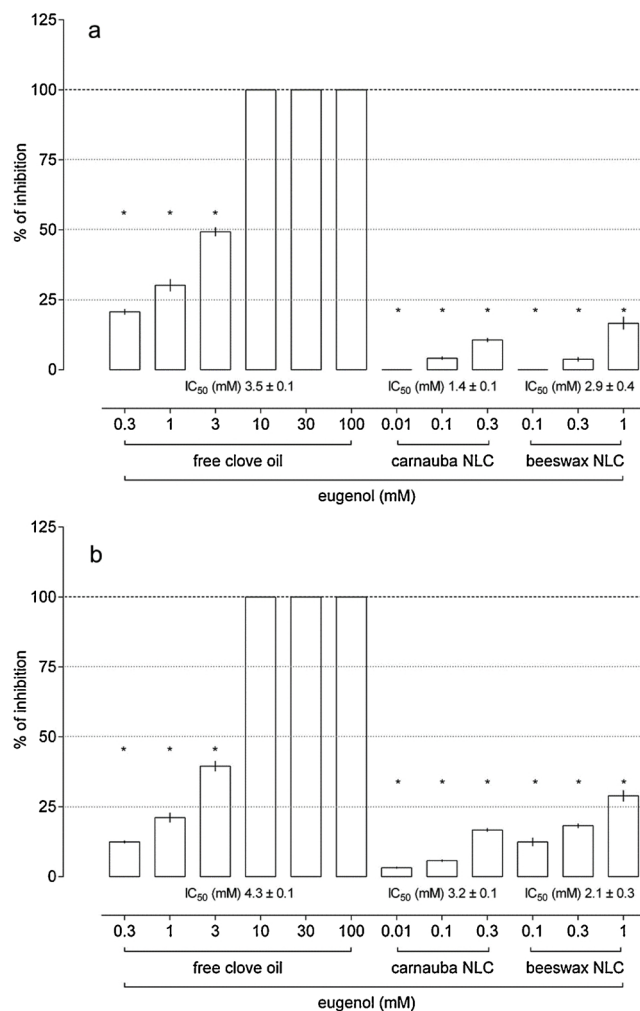
### 3.2. Acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) inhibitory activity

*Drosophila melanogaster* homogenate was employed to determine the *in vitro* inhibitory activity of AChE and BChE after the incubation with different concentrations of free and encapsulated clove oil, the activity inhibition (%) and  $IC_{50}$  (mM) are shown in Fig. 4.

*Drosophila melanogaster* is the closest invertebrate model organism to human based on the gene sequence similarity, and the high sensitivity to toxic substances favors its use as a bioindicator to evaluate the biological action of pharmacological agents [51,52]. The free clove oil dissolved in methanol showed similar efficacy in the inhibition in both cholinergic enzyme (AChE and BChE), and concentrations equal or above 10 mM were able to inhibit completely the enzymes. In this way, the  $IC_{50}$  of free clove oil for AChE inhibition (3.5 mM) was similar to the value found in a previous work [7] that utilized electric eel as the enzyme source (*Electrophorus* AChE type VI-S). Authors investigated the AChE inhibitory effect of many essential oil components, indicating that the eugenol present in the clove oil plays the same role in the oil activity. Results suggest that the free clove oil had similar activity against both AChE and BChE.

With the encapsulation of clove oil in CW and BW NLC it was possible to decrease the  $IC_{50}$  values to less than 50% when compared to

the free clove oil at the same concentrations. This result indicates that the incorporation of the essential oil in the lipid particles favored the water affinity of the oil since no solvent (such as methanol) was used. A similar result was found in other work that evaluated beta-carotene nanodispersed in water and demonstrated a decrease in the



**Fig. 4.** AChE (a) and BChE (b) inhibitory activity (%) and  $IC_{50}$  (mM) of the free clove essential oil, clove oil loaded into NLC of carnauba wax (CW6) and beeswax (BW7). Values are mean  $\pm$  SEM (n = 4 per group). Significance determined by one-way analyses of variance (ANOVA) followed by Tukey's test. \*p < 0.05 in comparison with the control (100% of inhibition).

nanoparticle average diameter increased the beta-carotene dispersibility in water and favored the AChE inhibition [31].

#### 4. Conclusion

Clove essential oil was successfully incorporated in natural nanostructured lipid carriers using beeswax or carnauba wax as matrix materials. The presence of a liquid lipid (medium chain triglycerides, crodamol) in the formulation led to the decrease in the average particles size but the sizes distribution remained monodisperse. The solid lipid nanoparticles containing crodamol in formulation presented satisfactory aqueous colloidal stability after 30 days of storage. The particles characterization showed typical nanostructured lipid carrier properties as solid morphology, semi-crystalline environment, and polymorphic transition events. The free clove oil and the solid lipid dispersion containing encapsulated clove oil showed efficient *in vitro* acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) inhibition in *Drosophila melanogaster* tissue. In addition, nanostructured lipid formulations showed high inhibition with an IC<sub>50</sub> lower than the free clove oil. The free oil was tested in the presence of a good solvent (methanol) while the nanoparticles were evaluated in water only, meaning that the oil bioactivity was potentialized in aqueous medium which is important in a commercial point of view in food and cosmetic applications.

#### Declaration of Competing Interest

None.

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