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FORMULATION OF ULTRASOUND-ASSITED NANOEMULSIONS CONTAINING EUGENOL[1]

USO DE SONICAÇÃO PARA A OBTENÇÃO DE NANOEMULSÃO CONTENDO EUGENOL

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ABSTRACT

Currently, the demand for aesthetic treatments has been increasing considerably. It often causes pain, so it is used topical anesthetic prior to the treatment. The eugenol, the main compound of the ethanol extract of Indian clove (syzygium aromaticum), is responsible for many of the pharmacologic effects attributed to this plant, including the anesthetic activity. The objective of this work was the production, characterization and assay of a nanoemulsion containing eugenol. In the production of the nanoemulsion, the evaluated parameters were: mechanical agitation at speeds of 10,000, 20,000 and 26,000 rpm, 7.5 and 15.0 minutes stirring time and the active concentration of 0.50; 0.75 and 1.00%. The formulations were characterized through the average droplet diameter, polydispersity index, zeta potential and pH, as well as the determination of the active was performed via liquid chromatography high performance (HPLC). Stability studies were performed on a 75-day period in three temperature conditions. Best results were obtained when we used mechanical stirring with 20,000 rpm for 7.5 minutes stirring time and the concentration of 1.00% active, producing nanoemulsions having an average diameter droplet 101.17 ± 6.80 nm index a polydispersity 0.135 ± 0.027, zeta potential of -7.19 ± 0.713 and pH 4.84 ± 0.04. This formulation was stable for up to 30 days, keeping the results as stored at 5 and 25ºc. As for the determination of recoveries, 72 to 106% of the assets were obtained. Thus, it is concluded that the methodology used is feasible for the production of nanoemulsions containing eugenol.

Keywords: skin, nanotechnology, clove, HPLC.

RESUMO

Atualmente a procura por tratamentos estéticos tem aumentado consideravelmente, mas muitas vezes causam dor e, devido a isso, são utilizados anestésicos tópicos previamente ao tratamento. O eugenol, principal composto do extrato etanólico do cravo-da-índia (Syzygium aromaticum), é o responsável por grande parte dos efeitos farmacológicos atribuídos a planta, entre elas a atividade anestésica. O objetivo deste trabalho foi a produção, caracterização e o doseamento de uma nanoemulsão contendo eugenol. Na produção da nanoemulsão, os parâmetros avaliados foram: agitação mecânica com velocidades de 10000, 20000 e 26000 rpm, tempo de agitação de 7,5 e 15,0 minutos e, concentração do ativo de 0,50; 0,75 e 1,00%. As caracterizações

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das formulações foram diâmetro médio de gotícula, índice de polidispersão, potencial zeta e pH, assim como o doseamento do ativo foram realizados via técnica de HPLC. Estudos de estabilidade foram realizados num período de 75 dias, em três condições de temperatura. Melhores resultados foram obtidos quando se utilizou agitação mecânica com 20000 rpm, tempo de agitação de 7,5 minutos e concentração de ativo de 1,00%, produzindo nanoemulsões contendo diâmetro médio de gotícula de 101,17 ± 6,80 nm, índice de polidispersão de 0,135 ± 0,027, potencial zeta de -7,19 ± 0,713 e pH de 4,84 ± 0,04. Esta formulação foi estável por até 75 dias, mantendo os resultados quando armazenada a 5 e 25 °C. Quanto ao doseamento, recuperações de 72 a 106 % do ativo foram obtidas. Assim, conclui-se que a metodologia utilizada é viável para a produção de nanoemulsões contendo Eugenol.

Palavras-chave: *pele, nanotecnologia, cravo-da-índia, HPLC.*

INTRODUCTION

The demand for aesthetic treatments with laser and other treatments has increased considerably. Among those treatments are the basic procedures for depilation, removal of tattoos, rejuvenation, skin filling and application of botulinum toxin. These interventions often cause pain and, because of this, topical anesthetics are used. The topical anesthetics prevent the transmission of nerve impulses, promoting cutaneous analgesia by acting on dermal free nerve endings. They act by blocking nerve impulse conduction by inhibiting sodium influx, the threshold for nerve excitation increases until loss of ability to generate an action potential occurs (DEVOR, 2006; FRIEDMAM et al., 2001).

Nanoemulsions are oil-in-water droplets, stabilized by surfactants, which generally have a nanometric size of up to 500 nm (ANTON et al., 2008). These carriers show efficient skin release systems, ensuring a uniform distribution of the formulation in the skin due to the small size of their droplets (SONNEVILLE-AUBRUN et al., 2004).

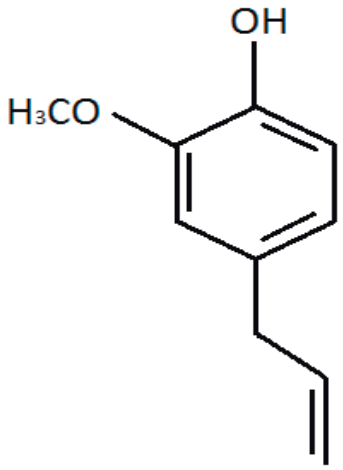
Thus, carrier systems may be an option to improve the penetration of various substances in the skin (STECOVA et al., 2007). Since epidermal lipids are the first constituent of the topical penetration barrier, lipid-based carriers can adhere to the surface of the skin and allow interaction between the carrier constituents and those of the outer layers, in the stratum corneum, favoring permeation (SCHAFER-KORTING et al., 2007).

Eugenol (4-allyl-2-methoxyphenol) - figure 1, the main derivative of Indian clove, has anti-inflammatory, healing and analgesic effect and it is also effective in fighting and reducing the bacteria that are present in the mouth. Its medicinal effects comprise the treatment of nausea, flatulence, indigestion and diarrhea. With antibacterial properties it is also used as an anesthetic and antiseptic for the relief of tooth pain (NASCIMENTO et al., 2000).

The development of novel drug delivery systems such as, nanoemulsions and nanoparticles of eugenol, would contribute to the enhancement of its therapeutic efficacy (PRAMOD et al., 2012; PRAMOD et al., 2015).

This work aimed to produce a nanoemulsion containing eugenol, as well as the co-validation (SARAN et al., 2013) of a method for the quantification of the active by liquid chromatography (HPLC) in the formulations produced, considering the anesthetic potential of the active, with the possibility of its application in future formulations for topical use.

Figure 1 - Chemical structure of eugenol (4-allyl-2-methoxyphenol).



Source: Adapted from Affonso et al. (2012).

MATERIAL AND METHODS

NANOEMULSION CONTAINING EUGENOL

The Eugenol (4-allyl-2-methoxyphenol) and sorbitan monooleate (Span 80®) were purchased from Sigma-Aldrich. Polyoxyethylene sorbitan monooleate (Tween 80®) was acquired from Labsynth.

The methodology used to produce Eugenol-containing nanoemulsions was adapted from Shahavi et al. (2015). To obtain the formulations, the reagents were weighed in Falcon tubes (polypropylene, 50 mL capacity) in analytical balance, in the order: Tween® 80, Span® 80, Eugenol and water (for the final volume of 10 mL). Afterwards, these components were submitted to mechanical agitation (Polytron PT 3100D), according to the data content in table 1. Initially, a pilot study was carried out and 24 samples were prepared. The mechanical stirring speed (10,000, 20,000 and 26,000 rpm), the agitation time (7.5 and 15 minutes) and the concentration of the active (0.50, 0.75 and 1.00%), can be seen in table 1. In all formulations 5% Tween 80® and 5% Span 80® were used, the volume (10 mL) was checked with purified water in Milli-Q® system. After preparation, the formulations remained in the refrigerator for 24 h and then they were characterized.

Table 1 - Optimization for the nanoemulsion preparation containing Eugenol.

10,000 rpm		20,000 rpm		26,000 rpm	
7.5 min	0.50% active	7.5 min	0.50% active	7.5 min	0.50% active
	0.75% active		0.75% active		0.75% active
	1.00% active		1.00% active		1.00% active
15.0 min	0.50% active	15.0 min	0.50% active	15.0 min	0.50% active
	0.75% active		0.75% active		0.75% active
	1.00% active		1.00% active		1.00% active

PHYSICAL-CHEMICAL CHARACTERIZATION OF NANOEMULSIONS

The nanoemulsions containing eugenol were analyzed through the distribution of the average particle diameter, polydispersity index, zeta potential by electrophoretic mobility, using the ZetaSizer Nano-ZS® equipment (Malvern, UK), pH using Digimed DM22 potentiometer® (Brazil). From these characterizations the working formulation was selected.

CHROMATOGRAPHIC DETERMINATION OF EUGENOL CONTENT

High performance liquid chromatography (HPLC-DAD) was performed with a Shimadzu Prominence Auto Sampler (SIL-20A) HPLC system (Shimadzu, Kyoto, Japan), equipped with Shimadzu LC-20AT reciprocating pumps connected to a DGU 20A5 degasser with a CBM 20A integrator, SPD-M20A diode array detector and LC solution 1.22 SP1 software. The chromatographic separations were reverse phase and were carried using C18 column (4.6 mm x 150 mm) packed with 5 µm diameter particles, Phenomenex®. The chromatographic conditions were: mobile phase with a mixture of methanol: water (55:45 v/v), flow rate of 1.0 mL / min, wavelength 215 nm and the injection volume of 30 µl. Methanol (grade HPL) was purchased from Carlo Erba and ultra-pure water (Milli-Q®). All the samples and mobile phase were filtered by a 0.45 µm membrane filter (Millipore®) and then degassed by ultrasonic bath prior use.

The determination of the active in the nanoemulsion was proceed as follows: 0.1g of the formulation was weighed into a 10mL volumetric flask, the volume was completed with methanol and taken to ultrasound, for 15 minutes. After this, 1.5 mL of solution was pipetted and placed in a 10 mL volumetric flask, the volume was measured with methanol (theoretical value 15 µg / mL). The same production process of the sample was performed for the blank nanoemulsion (without the active in the formula), to perform the specificity test. Prior to the injection of the samples into the chromatograph, they were filtered on a 0.45 µm cellulose acetate membrane.

The preparation of the standard solution was weighed the equivalent of 0.100 g of Eugenol and transferred to a 10 ml volumetric flask, the flask was measured with methanol (10.0 mg / ml). From this solution, 0.1 mL was pipetted and transferred to another 10 mL flask, and checked with

methanol (100 µg / mL). From this standard solution, the points were prepared for the calibration curve (5.0 to 25.0 µg / mL). The chromatographic condition obtained and chose for the conduction of the experiments is described in table 2.

Table 2 - Analytical parameters used for the quantification of Eugenol present in the formulation, using high performance liquid chromatography.

Parameters	
Chromatographic Column	Reverse Phase, C18 (150 x 4.6mm, 5µm)
Guard-column	C18 (4 x 3mm, 5µm)
Wavelenght	215nm
Movel phase	water:methanol (55:45)
Elution mode	Isocratic
Flow rate of movel phase	1,0 mL/min
Injection volume	30µL
Oven	30 ºC

PHYSICO-CHEMICAL STABILITY STUDY OF NANOEMULSIONS CONTAINING EUGENOL

Nanoemulsions containing 1.0% of Eugenol (in triplicate), were stored at room temperature (RT, 25 ºC), refrigerator (RE, 5 ºC) and climatic chamber (CC, 40 ºC) and evaluated at day 1, 7, 15, 30 and 75 days (mean particle diameter, polydispersity index, zeta potential, pH and active content).

STATISTICAL ANALYSIS

The statistical methodology of the data included a descriptive analysis of variables such as mean, standard deviation, coefficient of variation, correlation studies, simple linear regression, ANOVA and Tukey test, considering significance levels of 0.05. Data were generated using GraphPad Prism® and Assistat® software.

RESULTS AND DISCUSSION

FORMULATION OF NANOEMULSION

The agitation speed, agitation time and the concentration of the active in the production of the 24 formulations prepared for the pilot study were evaluated, it was also evaluated the average particle diameter, polydispersity index, Zeta potential and pH, as shown tables 3, 4 and 5.

Table 3 - Optimization of the procedure for obtaining nanoemulsion-containing Eugenol. Containing 5.0% Span 80® and 5.0% Tween 80® in high purity water. Rotation speed of the mechanical stirrer: 10,000 rpm.

	7.5 min			15.0 min		
	0.50%	**0.75%**	**1.00%**	**0.50%**	**0.75%**	**1.00%**
Size (nm)	221.0	253.8	134.3	549.6	410.5	472.5
PDI	0.596	0.513	0.233	0.651	0.937	0.802
Zeta	-10.10	-7.84	-9.39	-11.00	-9.49	-8.46
pH	5.24	5.02	5.16	5.32	5.25	5.76

Table 4 - Optimization of the procedure for obtaining nanoemulsion-containing Eugenol. Containing 5.0% Span 80® and 5.0% Tween 80® in high purity water. Rotation speed of the mechanical stirrer: 20,000 rpm.

	7.5 min			15.0 min		
	0.50%	**0.75%**	**1.00%**	**0.50%**	**0.75%**	**1.00%**
Size (nm)	86.2	99.8	115.8	77.6	90.7	113.3
PDI	0.231	0.219	0.131	0.271	0.154	0.155
Zeta	-8.07	-7.37	-7.95	-7.65	-7.82	-5.36
pH	4.96	5.09	5.34	5.55	5.50	5.18

Table 5 - Optimization of the procedure for obtaining nanoemulsion-containing Eugenol. Containing 5.0% Span 80® and 5.0% Tween 80® in high purity water. Rotation speed of the mechanical stirrer: 26,000 rpm.

	7.5 min			15.0 min		
	0.50%	**0.75%**	**1.00%**	**0.50%**	**0.75%**	**1.00%**
Size (nm)	103.1	128.3	137.8	114.1	87.9	187.00
PDI	0.152	0.147	0.252	0.331	0.200	0.289
Zeta	-8.65	-6.95	-9.26	-7.80	-7.53	-14.70
pH	5.19	5.23	5.45	5.54	5.28	5.37

In terms of particle size, the values obtained in this optimization were similar (except for 15 minutes at 10,000 rpm). The PDI with shaking at 10,000 rpm (Table 2) was poor (poor> 0.3) for both procedures. This excluded the possibility of using stirring at 10,000 rpm. Comparing stirring at 20,000 rpm (Table 3) and 26,000 rpm (Table 4) the increase in stirring speed as well as a longer stirring time (7.5 to 15.0 minutes) did not provide improvements in the characteristics of the nano-emulsion, compared to the parameters evaluated. Thus, in this work, it was chosen to produce nano-emulsions containing eugenol with a stirring speed of 20,000 rpm, at a time of 7.5 minutes and in the highest active concentration, 1.0%. Although obtaining a relatively larger droplet than in the other tests, it was decided to use this optimization, in view of the topical application, since a droplet size of up to 400 nm is acceptable.

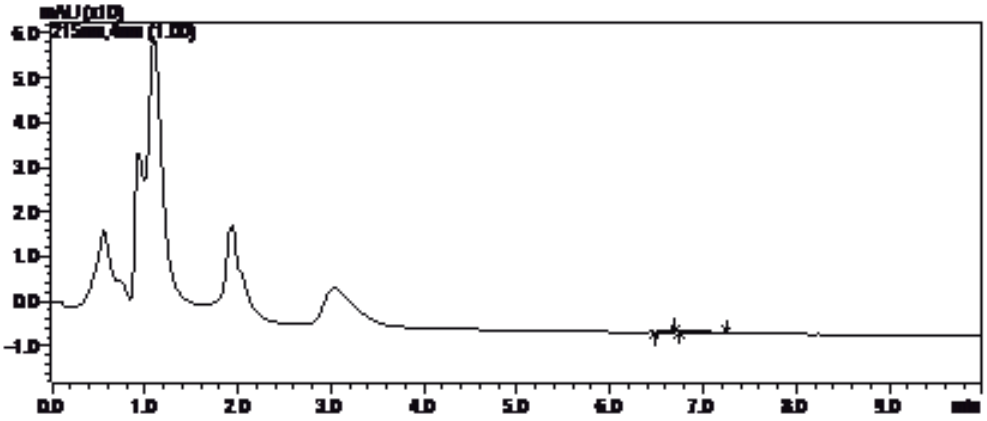
DETERMINATION OF ACTIVE BY HPLC

After the optimization of the parameters for the production of nanoemulsions containing eugenol, the determination of the active was evaluated.

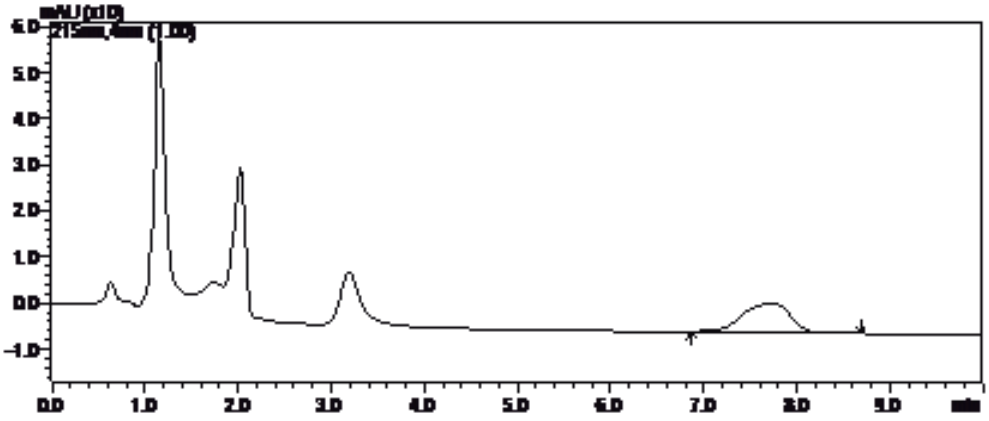
The specificity of the method was assessed by checking the peak purity. None of the formulation excipients elute at the same Eugenol retention time (RT: 6.8 min) and at the wavelength of 215 nm, as shown in figure 2.

Figure 2 - Specificity: (a) Chromatogram of nanostructured sample without active; (b) chromatogram of the standard substance Eugenol (10 µg/mL), (c) nanoemulsion chromatogram with eugenol (1.0% eugenol).

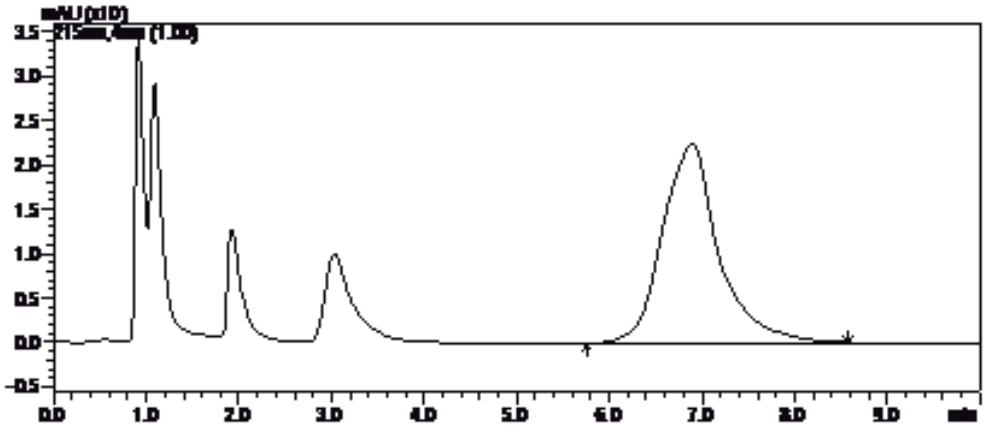
(a)



(b)



(c)



A good linearity was successfully achieved in the concentration range from 5.00 µg / mL to 25.00 µg/mL. The standard curve was performed in triplicate and, with the average absolute area obtained, a plot was constructed plotting the mean area versus concentration of Eugenol (µg/mL). The standard curve and its respective equation of the line were determined through the linear regression, using the least squares method and the results were statistically evaluated through analysis of variance (ANOVA). The areas obtained in the evaluation of the curve are shown in table 6. The respective equation of the line obtained was Y = 109805X - 188841 and $R^2 = 0.9999$.

From the analysis of variance (ANOVA) from results of the standard average curve, no linearity deviation was observed and presented significant linear regression ($p < 0.05$). These results, together with the correlation coefficient (r = 0.999), show that this standard curve, in the concentration range of 5.0 to 25.0 µg/mL, can be used for the interpolation of the experimental values, aiming at the determination of this substance.

Table 6 - Values of the areas obtained from the Eugenol standard curve by HPLC (n=3).

Concentration (µg/mL)	Area	Average of area ± SD	RSD
5.0	501999 568466 514276	528247 ± 35367.46	6.7
10.0	1036118 1144876 1050677	1077224 ± 59039.14	5.5
15.0	1589783 1731890 1598409	1640027 ± 79672.23	4.9
20.0	2089983 2249744 2162350	2167359 ± 79998.20	3.7
25.0	2651076 2829005 2704818	2728300 ± 91259.10	3.3

The limit of detection (LOD) determinated was 2,18 µg/mL and the limit of quantification (LOQ) determinated was 6,54 µg/mL. Those were calculated based on the standard deviation of the responses and the slope using three independent analytical curves. LOD and LOQ were calculated as 3 and 10 σ/S, respectively (σ is the standard deviation and S is the slope of the calibration curve).

The repeatability of the method was evaluated by assaying eugenol in nanoemulsions containing 1% of the active substance in three different concentrations by a single-day (n = 3) analyst, as described below:

The equivalent of 100 mg of the nanoemulsion was analytically weighted and transferred to a 10 mL volumetric flask, accomplished the volume with methanol. Submitted to ultrasound irradia-

tion for 15 minutes. Aliquots of 0.5; 1.0; 1.5 mL were transferred to 10 mL volumetric flasks and the volumes were diluted to obtain the respective concentrations of 5.0, 10.0 and 15.0 µg/mL.

The experimental values obtained for the determination of the repeatability of the developed method are described in table 7. The average RSD for the contents in this test was 0.24%, thus reveling below as the recommended maximum limit of 5% (BRASIL, 2003).

Table 7 - Intermediate precision and repeatability of the method for determination of the active present in the samples by HPLC

	5 µg/mL	**10 µg/mL**	**15 µg/mL**
Peak Area	494286	977141	2003013
Peak Area	484838	951206	2020503
Peak Area	510431	959650	1903062
Average	496518	962665	1975526
SD	12942	13228	63362
RSD%	2.6	1.4	3.2
Average RSD%	**2.4%**		

STABILITY

The nanoemulsions produced were characterized by the average particle diameter (nm), polydispersity index, zeta potential (mV) and pH. For the blank formulations and with active, the average diameter obtained by dynamic light scattering was 101.17 ± 6.80 nm with a polydispersion index of 0.135 ± 0.027 for the nanoemulsion containing Eugenol. Diameter of 65.66 ± 4.37 nm with polydispersity index of 0.250 ± 0.004 for the nanoemulsion without Eugenol. These data were obtained 24 hours after the preparation of nanoemulsions. The results obtained are shown in table 8 for the nanoemulsion without the active and in table 9 for the nanoemulsion containing eugenol (1%).

Table 8 - Values found in the physico-chemical parameters of stability nanoemulsion without Eugenol, n = 3

		Room Temp. (25 ºC)	**Refrigerator (5 ºC)**	**Climatic Chamber (40 ºC)**
Day 01	Mean diameter ± SD	65.66 ± 4.37	65.66 ± 4.37	65.66 ± 4.37
	PDI ± SD	0.250 ± 0.004	0.250 ± 0.004	0.250 ± 0.004
	Zeta ± SD	-11.62 ± 0.77	-11.62 ± 0.77	-11.62 ± 0.77
	pH ± SD	5.73 ± 0.06	5.73 ± 0.06	5.73 ± 0.06
Day 07	Mean diameter ± SD	76.83 ± 7.09	74.91 ± 7.32	146.58 ± 4.52[(a)]
	PDI ± SD	0.359 ± 0.089	0.327 ± 0.098	0.198 ± 0.02
	Zeta ± SD	-9.42 ± 0.38	-7.56 ± 0.17	-12.93 ± 1.50
	pH ± SD	5.05 ± 0.02	4.46 ± 0.66	3.73 ± 0.25
Day 15	Mean diameter ± SD	70.02 ± 1.27	69.05 ± 6.04	- *
	PDI ± SD	0.34 ± 0.018	0.357 ± 0.024	- *
	Zeta ± SD	-7.24 ± 0.64	-6.99 ± 0.24	- *
	pH ± SD	5.01 ± 0.44	5.45 ± 0.27	- *

Day 30	Mean diameter ± SD	114.22 ± 18.03	92.21 ± 2.05	- *
	PDI ± SD	0.485 ± 0.047	0.322 ± 0.075	- *
	Zeta ± SD	-7.64 ± 1.02	-7.37 ± 1.64	- *
	pH ± SD	4.22 ± 0.06	4.22 ± 0.06	- *
Day 75	Mean diameter ± SD	71.52 ± 0.83	71.47 ± 0.29	- *
	PDI ± SD	0.198 ± 0.011	0.239 ± 0.012	- *
	Zeta ± SD	-8.23 ± 1.14	-7.07 ± 0.32	- *
	pH ± SD	3.53 ± 0.15	6.01 ± 0.06	- *

(a) Value with significant statistics indicating instability for nanoemulsion maintained in climatic camera.* Disregarded values due to the results presented since the analysis of day 7, showing that the formulation is unstable at 40ºC (climatic chamber).

Table 9 - Values found in the physico-chemical parameters of stability nanoemulsions containing Eugenol, n=3.

		Room Temperature	**Refrigerator**	**Climatic Chamber**
Day 01	Mean diameter ± SD	101.17 ± 6.8	101.17 ± 6.8	101.17 ± 6.8
	PDI ± SD	0.135 ± 0.03	0.135 ± 0.027	0.135 ± 0.03
	Zeta ± SD	-7.19 ± 0.71	-7.19 ± 0.71	-7.19 ± 0.71
	pH ± SD	4.84 ± 0.04	4.84 ± 0.04	4.84 ± 0.04
Day 07	Mean diameter ± SD	126.81 ± 7.03	102.85 ± 7.87	863.74 ± 106.29[(a)]
	PDI ± SD	0.165 ± 0.14	0.174 ± 0.039	0.356 ± 0.09[(a)]
	Zeta ± SD	-7.06 ± 0.80	-9.84 ± 0.24	-9.28 ± 0.97
	pH ± SD	4.52 ± 0.01	4.98 ± 0.33	4.71 ± 0.61
Day 15	Mean diameter ± SD	128.11 ± 3.13	102.52 ± 1.15	- *
	PDI ± SD	0.225 ± 0.12	0.143 ± 0.039	- *
	Zeta ± SD	-6.42 ± 1.89	-7.01 ± 0.36	- *
	pH ± SD	4.35 ± 1.51	4.93 ± 0.26	- *
Day 30	Mean diameter ± SD	142.53 ± 30.48	104.13 ± 7.63	- *
	PDI ± SD	0.167 ± 0.09	0.146 ± 0.033	- *
	Zeta ± SD	-9.17 ± 1.65	-7.45 ± 0.72	- *
	pH ± SD	3.86 ± 0.55	4.69 ± 0.14	- *
Day 75	Mean diameter ± SD	150 ± 30.99	92.65 ± 4.61	- *
	PDI ± SD	0.179 ± 0.08	0.152 ±0.036	- *
	Zeta ± SD	-7.32 ± 1.68	-6.75 ± 2.67	- *
	pH ± SD	4.34 ± 1.21	5.22 ± 0.16	- *

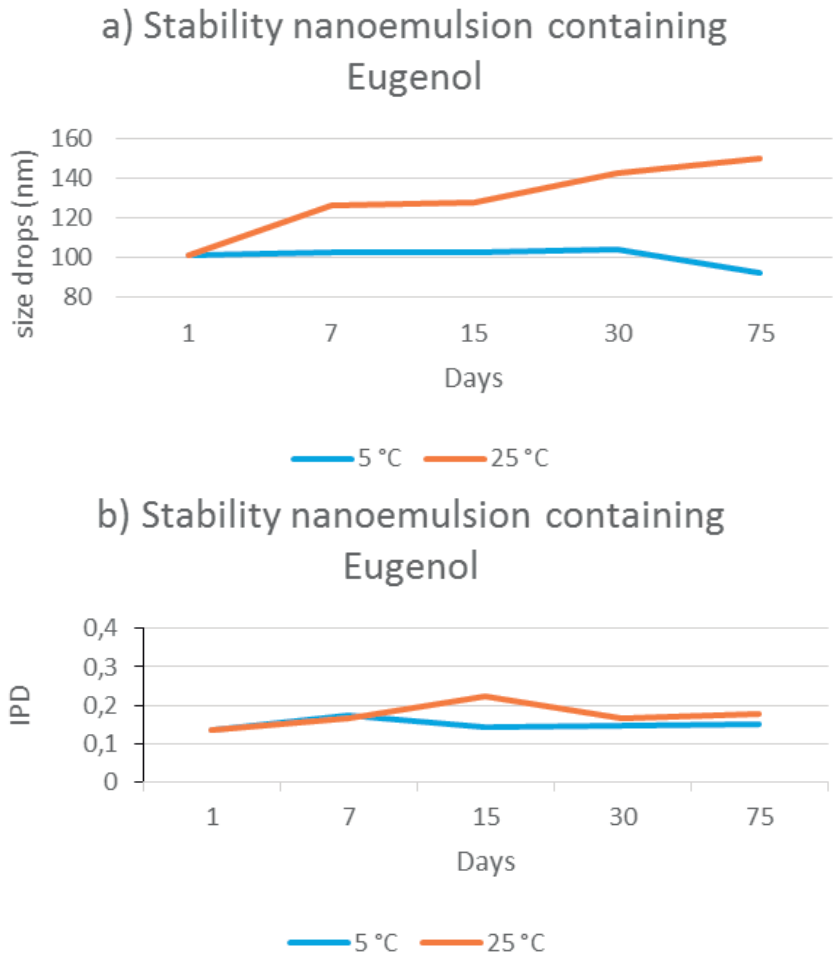
(a) Value with significant statistics indicating instability for nanoemulsion maintained in climatic camera; *Disregarded values due to results presented since the analysis of day 7, showing that the formulation is unstable at 40 °C (climatic chamber).

Two graphs showed below (Figure 3) where the size of the droplets of the nanoemulsions with the active one is highlighted, as well as the polydispersion indices in the stability evaluation period. Data on samples stored in a climatic chamber (40 ºC) were not plotted before 7 days of storage, there was a breakdown of the nanoemulsion (droplet size reached 863.74 ± 106.20 as PDI rose from 0.3, reaching 0.356 ± 0.088, indicating the instability of the system.

According to the values presented in table 10 and statistical analysis (GraphPad Prism®) there was a significant difference in the average diameter of the particles in the course of thirty days storage for the formulations at room temperature (25 ºC) and under refrigeration (5 ºC). In the sample

stored in climatic chamber, it is possible to observe significant difference as measured for the particle diameter in seven days, is due to the fact that in this condition, the degradation of the components of the formulation occurs.

Figure 3 - Storage stability of nanoemulsions at different temperatures.
(A) mean droplet size of eugenol nanoemulsions over the storage period at 5 ºC and 25 ºC;
(B) mean polydispersion index values of eugenol nanoemulsions during storage (5 ºC and 25 ºC).



For the polydispersity index, PDIs lower than 0.3 were observed over the 30 days for storage conditions RT and GE, indicating monodisperse formulations (SCHOLES et al., 1993). However, in the formulation maintained in a climatic chamber, a statistically significant difference was observed when compared to the analysis performed on day 01 of the same, exhibiting a high polydispersion index (greater than 0.3) in the seven-day analysis.

The results of the zeta potential are usually applied to predict the stability of colloidal systems, i.e. if the suspended particles exhibit a high zeta potential, they tend to repel themselves avoiding aggregation and increasing stability (LAOUINI et al., 2012). This potential depends on the degree of ionization of the emulsifying agent and is pH dependent (KLANG; BENITA, 1998).

Regarding the pH values for the Eugenol-containing nanoemulsions no statistical difference was observed during the thirty days and in the three storage conditions in the present study.

DETERMINATION OF THE ACTIVE IN NANOEMULSIONS BY HPLC

The active, Eugenol, was quantified during the stability evaluation period by HPLC under the chromatographic conditions according to the table 5. The data presented in table 10 were obtained in triplicate, containing average values and standard deviation.

Table 10 - Assay results of the active during the evaluation period of the stability of the produced nanoemulsions.

	Room Temperature	Refrigerator	Climatic Chamber
	Eugenol (µg/mL)	**Eugenol (µg/mL)**	**Eugenol (µg/mL)**
Day 1	85.80 ± 7.00	85.80 ± 7.00	85.80 ± 7.00
Recovery %	100	100	100
Day 7	77.00 ± 7.70	81.90 ± 2.90	79.20 ± 0.90
Recovery %	89.74	95.45	92.31
Day 15	90.60 ± 6.30	83.60 ± 4.60	62.60 ± 4.40
Recovery %	105.59	97.44	72.96
Day 30	83.00 ± 1.80	82.80 ± 2.10	62.60 ± 4.30
Recovery %	97.67	96.50	72.96
Day 60	85.52 ± 3.90	84.40 ± 4.00	62.00 ± 2.00
Recovery %	99.67	98.37	72.26

For the recoveries obtained from the Eugenol content present in the nanoemulsions, during the period of the stability study, it is assumed that the active did not suffer a degradation process at storage temperatures of 5 ^{o}C and 25 ^{o}C and a degradation when stored at 40 ^{o}C, from the seventh day of storage. Differences were considered significant when the results presented p-value <0.05. The IBM SPSS Version 23 software was used as a computational tool for the statistical analysis of the data. The recovery rate was close to 100% according to the recommended by the legislation that predicts recovery percentages between 90 and 100%. However, smaller values are acceptable, providing that the recovery is accurate and accurate (BRASIL, 2003).

CONCLUSION

For the production of nanoemulsion containing eugenol with the use of mechanical stirrer at 20,000 rpm, time of 7.5 minutes and active concentration of 1.0% was obtained for nanoemulsion without active droplet size of 65.66 ± 4, 37, PDI of 0.250 ± 0.004, zeta potential of -11.62 ± 0.77 and pH of 5.73 ± 0.06. For the eugenol-containing nanoemulsion, the droplet size was 101.20 ± 6.80 nm, PDI of 0.135 ± 0.03, zeta potential of -7.19 ± 0.71 and pH 4.84 ± 0, 04. The formulations were stable for up to 75 days when stored between 5 °C and 25 °C.

The analytical methodology proposed in this study for the quantification of eugenol in nanoemulsion by HPLC obtained very positive results for all evaluated parameters, indicating that the

technique was selective / specific, precise and linear, with results of recoveries of 72 to 106% of the active. Nonetheless, we assume that the methodology used is viable to achieve nanoemulsions containing Eugenol for biotechnological applications.

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REFERENCES

AFFONSO, R. S. et al. Aspectos químicos e biológicos do óleo essencial de Cravo da Índia. **Revista Virtual de Química**, v. 4, n. 2, p. 146-161, 2012.

ANTON, N.; BENOIT, J. P.; SAULNIER, P. Design and production of nanoparticles formulated from nano-emulsion templates. **A review Jounal Control Release**, v. 128, n. 3, p. 185-199, 2008.

BRASIL. Ministério da Saúde. Agência Nacional de Vigilância Sanitária (ANVISA). **Guia para validação de métodos analíticos e bioanalíticos**. Brasília, DF, 2003.

DEVOR, M. Sodium Channels and Mechanisms of Neuropathic Pain. **The Journal of Pain,** v. 7, n. 1, p. S3-S12, 2006.

FRIEDMAN, P. M. et al. Topical anesthesis: EMLA and beyond. **Dermatologic Surgery**, v. 27, n. 12, p. 1029-1026, 2001.

KLANG, S. H. **Design and evaluation of submicron emulsions as colloidal drug carriers for intravenous administration.** Amsterdam: Overseas Publishers Association, 1998.

LAOUINI, A. et al. Preparation, Characterization and Applications of Lipossomes: State of the Art. **Journal of Colloid Science and Biotechnology**, v. 1, n. 2, p. 147-168, 2012.

NASCIMENTO, G. G. F.; LOCATELLI, J.; FREITAS, P. C. Antibacterial activity of plant extracts and phytochemicals on antibiotic - resistant bacteria. **Brazilian Journal of Microbiology**, v. 31, n. 4, p. 247- 256, 2000.

PRAMOD, K. et al. Eugenol nanodroplet gel as novel biomaterial in nanomedicine. **Advanced Science Letters**, v. 10, n. 1, p. 1-13, 2012.

PRAMOD, K. et al. High-performance thin-layer chromatographic analysis of eugenol in developed nanoemulsion gel and nanoparticles: validation of a stability-indicating method. **Acta Chromatographica**, v. 27, n. 3, p. 571-582, 2015.

SARAN, S. et al. Validated RP-HPLC method to estimate eugenol fram commercial formulations like Caturjata Churna, Lavangadi Vati, Jatiphaladi, Sitopaladi Churno and clove oil. **Journal of pharmacy research**, v. 6, n. 1, p. 53-16, 2013.

SCHAFER-KORTING, M.; MEHNERT, W. G.; KORTING, H. C. Lipid nanoparticles for improved topical application of drugs for skin diseases. **Advanced Drug Delivery Reviews,** v. 59, n. 6, p. 427-435, 2007.

SCHOLES, P. D. et al. The preparation of sub-200nm poly(lactide-co-glycolide) microspheres for site-specific drug delivery. **Journal of Controller Release**, v. 25, n. 1, p. 145-153, 1993.

SHAHAVI, M. H. et al. Evalution of critical parameters for preparation of state clove oil nanoemulsion. **Arabian Journal of Chemistry**, 2015 (article in press).

SONNEVILLE-AUBRUN, O.; SIMONNET, J. T.; L'ALLORET, F. Nanoemulsions: a new vehicle for skincare products. **Advances in Colloid and Interface Science**, v. 108, p. 145-149, 2004.

STECOVA, J. et al. Cyproterone Acetate Loading to Lipid Nanoparticles for Topical Acne Treatment: Particle Characterisation and Skin Uptake. **Pharmaceutical Research,** v. 24, n. 5, p. 991-1002, 2007.