Pharmacogn. Mag.
A multifaceted peer reviewed journal in the field of Pharmacognosy and Natural Products
www.phcog.com | www.phcog.net

ORIGINAL ARTICLE

Technological Evaluation of Emulsions Containing The Volatile Oil from Leaves of *Plectranthus Amboinicus* Lour

Pablo Queiroz Lopes[1], Fabíola Bernardo Carneiro[1], Ana Letícia Braz de Sousa[2], Sócrates Golziodo Santos[2], Elquio Eleamen Oliveira[3], Luiz Alberto Lira Soares[1,4]

[1]Programa de Pós-Graduação em Ciências Farmacêuticas, Universidade Federal de Pernambuco - UFPE, Rua Prof. Artur de Sá s/n, Recife, Pernambuco, [2]Departamento de Ciências Farmacêuticas, Universidade Federal da Paraíba - UFPB, Cidade Universitária, João Pessoa, Paraíba, [3]Centro de Ciências Biológicas e Sociais Aplicadas, Universidade Estadual da Paraíba - UEPB, Rua Horácio Trajano de Oliveira, s/n, João Pessoa, Paraíba, [4]Departamento de Ciências Farmacêuticas, Universidade Federal de Pernambuco - UFPE, Rua Prof. Artur de Sá s/n, Recife, Pernambuco, Brazil

Submitted: 30-04-2016 Revised: 10-05-2016 Published: 06-01-2017

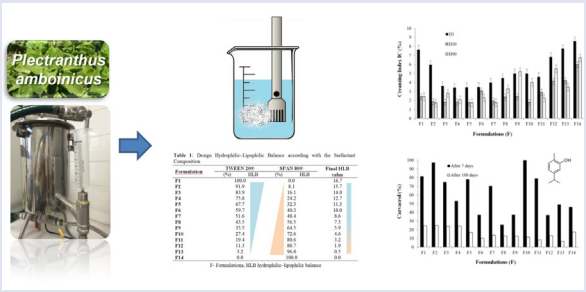
ABSTRACT

Background: *Plectranthus amboinicus* Lour is a species which is widespread throughout tropical countries where it is widely used against respiratory tract disorders such as bronchodilator, antitussive, and expectorant conditions. **Objective:** This study aims to characterize the essential oil of *P. amboinicus* (PaEO) and produce and evaluate emulsions containing PaEO. **Materials and Methods:** The essential oil was characterized by physical-chemical analyses for density, refractive index, 90% ethanol solubility, color, appearance, and identification by gas chromatography coupled to mass spectrometry detection. The emulsions were prepared following a hydrophile-lipophile balance [HLB] spreadsheet design from two nonionic surfactants (Span 80® and Tween 20®) producing HLB values ranging from 4.3 to 16.7. The products were stored at room temperature at 5°C. The emulsion stabilities were tested both in the long and short-term. **Results:** The PaEO was obtained by steam distillation and the total extraction was reached after 3 hours yielding of 0.2% (w/w). This essential oil was characterized by physicochemical analyses for density [1.5 g.ml^{-1}], refraction index [0.9167], ethanol 90% solubility [1:2], color, and appearance (yellow/clear). Nineteen components were identified in the oil, among them the sesquiterpenes: carvacrol [33.50%], p-cymene [28.20%] and γ-terpinene [14.77%]. The emulsions obtained successfully showed, for the first time, HLB values for essential oils from *Plectranthus amboinicus* [15.7]. **Conclusion:** The experimental data shows a relationship between HLB values of the surfactant mixtures contributing to the emulsified systems production containing phytopharmaceuticals. Such an approach is of great importance to the development of lipid carriers for therapeutic drugs.

Key words: *Plectranthus amboinicus,* carvacrol, essential oil, emulsion, stability

SUMMARY

- The essential oil from leaves of *Plectranthus amboinicus* was extracted by steam distillation and characterized.
- The emulsions containing essential oil were produced and the stability was performed in the short and long term.
- The critical hydrophilic-lipophilic balance (HLB) of the essential oil was 15.7 and was achieved by the combination of surfactants (Tween 80® and Span 20®).



Abbreviations used: PaEO: essential oil of P. amboinicus, HLB: hydrophilic-lipophilic balance, CI: Creaming Index, MET: micro-emultocrit technique

Correspondence:
Dr. Luiz A. L. Soares,
Laboratory of Pharmacognosy,
Department of Pharmaceutical Sciences,
Federal University of Pernambuco–UFPE,
Prof. Arthur de Sá, s/n, Cidade Universitária,
Recife-PE, Brazil,
E-mail: lals.ufpe@gmail.com
DOI: 10.4103/0973-1296.197646

Access this article online
Website: www.phcog.com
Quick Response Code:

INTRODUCTION

The species *Plectranthus amboinicus* Lour, also known as *Plectranthus aromaticus*, *Coleus aromaticus* and *Coleus amboinicus,* is a herbaceous plant. It is perennial, succulent and aromatic with brittle stems and ovate succulent leaves, an acute apex, toothed edges, and a thick petiole. This species is native to eastern Asia and it is widespread throughout the tropical countries.[1]

P. amboinicus is widely used in Brazil in the treatment of diseases such as asthma, superficial mycosis, cancer, constipation, headache, cough, colds, fever, and digestive diseases.[2-6] The literature also reports that the essential oil of the *P. amboinicus* acts as an antifungal and antibacterial agent.[7,8] The essential oil of *P. amboinicus* presents a rich composition in mono and sesquiterpenes. In several studies, the carvacrol is reported to be the major component in a concentration ranging from 40 to 90%.[6,9,10,11] Furthermore, other constituents have been reported such as eugenol, methyl chavicol, β-caryophyllene, ρ-cymene, α-humulene, γ-terpinene, and 1.8-cineole.[8,12]

Owing to its technological development, the emulsified systems containing essential oil, remain a challenge due to the volatility and instabilities and solubility of essential oil.[13] In this way, the development of emulsions as carriers of essential oils demonstrates several benefits

Cite this article as: Lopes PQ, Carneiro FB, de Sousa AB, Santos SG, Oliveira EE, Soares LA. Technological evaluation of emulsions containing the volatile oil from leaves of *Plectranthus Amboinicus* Lour. Phcog Mag 2017;13:159-67.

such as the ability to hide unpleasant flavors so they can be administered in a palatable form, and also to improve the oral bioavailability.[14,15] Additionally, emulsions are widely used in cosmetic and pharmaceutical formulations due to excellent topical administration of hydrophilic and lipophilic active ingredients.[16]

This study is aimed to produce and evaluate nonionic O/W emulsions based on the essential oil from leaves of *Plectranthus amboinicus*. For this proposal the physicochemical and phytochemical characterizations of the essential oil were performed. To produce stable O/W emulsions, the HLB system was used to determine the HLB of the essential oil.

MATERIALS AND METHODS

Plant material

A voucher specimen of the plant was identified and deposited in the Herbarium Lauro Pires Xavier of Federal University of Paraíba, with the reference numbers JPB 85943 and JPB 85944.

Extraction and characterization of the essential oil from *p. amboinicus*

The fresh leaves of *P. amboinicus* were extracted by steam distillation using the Linax extractor (model D2 Mini, São Paulo, Brazil). The leaves were transferred to the reservoir, which features a continuous water cycle coming directly from the water system. This water was not in contact with the sample, the water vapor was passed through the reservoir, which contains the leaves, and drags down the volatile constituents. The samples were stored in glass vials under refrigeration to prevent possible losses of volatile constituents. Different extraction times [1 to 6 h] were used to optimize the estimate of the best yield.

Physical properties of the *P. amboinicus* essential oil (PaEO)

Relative density

The relative density was determined by calculating the ratio between the mass and the volume of the sample at 20°C (Eq. 1), used in the previously calibrated 5ml pycnometer with distilled water at 20°C. The analysis was performed in triplicate.

$$d_{20}^{20} = \frac{m}{v} \text{ (Eq.1)}$$

Where: d = density ($g.ml^{-1}$); m= mass and v = volume

Refractive index

The refractive index was determined by using a Reichert refractometer [AR-200, California, USA]. The oil samples were put directly over the prism of the refractometer at 25ºC. The analysis was performed in triplicate.

Solubility

A solution of ethanol in water 90% (v/v) was used to determine the essential oil solubility. To maintain the constant volume of essential oil (0.5 ml), an alcoholic solution was added in equal quantities to the essential oil until the complete solubilization of the essential oil was achieved.[17] The analysis was performed in triplicate.

Gas chromatography - mass spectrometry analyses

The identification of the PaEO components was performed by gas chromatography coupled to mass spectrometry detection in Ultra GC-MS (Shimadzu, Kyoto, Japan). The autosampler was an AOC-20 series injector (Shimadzu), the gas chromatograph was a GC-2010 Plus (Shimadzu), the mass spectrometer was a GCMS-QP2010 Ultra (Shimadzu) and ion trap detector (MS, model 4000) (Shimadzu), with electron ionization-mass spectrometry (EI-MS 70 eV). The analysis was developed in a Rtx®-5MS (Restek, Bellefonte, PA, USA) capillary column (Shimadzu), 30 m long, with a 0.25 mm thickness and 0.25 mm i.d. with Helium at 1 ml/min as carrier gas. Temperature program: injector at 250ºC with a column-oven from 60 to 240 ºC at 3 ºC /min. The retention index (RI) was calculated for all the volatile constituents using an n-alkane homologous series, ranging from C8 to C40, using a linear temperature programmed equation.[18] The individual components were identified by comparing the mass spectra and GC retention data with those of authentic compounds previously analyzed and stored in the database from the National Institute of Standards and Technology (NIST). The interpretation of RI values was assisted by the FFNSC (Flavor and Fragrance Natural and Synthetic Compounds) Library.[13]

Development and evaluation of emulsion systems containing PaEO

Hydrophilic-lipophilic balance spreadsheet design

The emulsions were prepared following the spreadsheet design shown in Table 1. This spreadsheet includes two surfactants: one of a lipophilic nature [Span 80®, HLB=4.3] and the other of a hydrophilic nature [Tween 20®, HLB=16.7]. The final HLB value of each system varied according to the relative proportion of each surfactant.[19]

Preparation of the emulsions

The emulsions were obtained by the phase inversion temperature method.[19] The preparation of W/O emulsions by the PIT method is a widely used technique in obtaining emulsified systems, in particular, emulsions containing the essential oil.[20-25] According to studies presented by Esquena, Sankar,[26] the PIT method requires the system whose phase inversion temperature is above the freezing point of water, and below the temperature where degradation of substances occurs. This can be achieved by choosing the surfactant with the nonionic appropriate HLB value for the system. The oil in water emulsion composition was 5% (w/w) of PaEO, 2% (w/w) of surfactants, and 93% (w/w) of water. Initially, the continuous phase was prepared by dispersing the required Tween 20® in distilled water. The dispersed phase was obtained by adding Span 80® into the essential oil. Both phases were heated separately to 70°C and one phase dispersed within the other. The studies report the

Table 1: Design hydrophilic-lipophilic balance according with the surfactant composition (%).

Formulation	TWEEN 20®		SPAN 80®		Final HLB value
	(%)	HLB	(%)	HLB	
F1	100.0	16.7	0.0	0.0	16.7
F2	91.9	15.4	8.1	0.3	15.7
F3	83.9	14.0	16.1	0.7	14.7
F4	75.8	12.7	24.2	1.0	13.7
F5	67.7	11.3	32.3	1.4	12.7
F6	59.7	10.0	40.3	1.7	11.7
F7	51.6	8.6	48.4	2.1	10.7
F8	43.5	7.3	56.5	2.4	9.7
F9	35.5	5.9	64.5	2.8	8.7
F10	27.4	4.6	72.6	3.1	7.7
F11	19.4	3.2	80.6	3.5	6.7
F12	11.3	1.9	88.7	3.8	5.7
F13	3.2	0.5	96.8	4.2	4.7
F14	0.0	0.0	100.0	4.3	4.3

F-Formulations; HLB- hydrophilic-lipophilic balance

use of up to 70°C to obtain emulsions containing essential oil.[11,19,21,22,27-30] The final emulsions were obtained after homogenization using an Ultra-Turrax [IKA, model T-18, Frankfurt, Germany] at 15,500 rpm for 10 min. Two batches of 14 emulsions with varying HLB values [Table 1] were obtained and stored at 25 ± 2°C and 5 ± 1°C in tube assay to evaluate the IC. Furthermore, 14 flasks of 50ml each, were stored at room temperature to complete the pH and conductivity tests.

Characterization of the emulsions

Creaming index

The creaming rate was determined experimentally by the measurement of the creaming index (CI) from Eq.2. The CI values were obtained from the ratio between the total height of cream layer (CC) and the total height of emulsion layer (CT). CC and CT were measured directly into a storage glass flask with the help of a graduate scale.

$$\%CI = \frac{CC}{CT} \cdot 100 \ (Eq.2)$$

pH

The measurements of pH were obtained from the probe inserted directly into the emulsion container at room temperature. The values were obtained using a pre-calibrated pH meter (model PG1800, São Paulo, Brazil). The analysis was performed in triplicate.

Conductivity

The conductivity was measured by directly inserting the probe into an emulsion container at room temperature, using a portable conductivity meter [MITE, model CD30] previously calibrated with a standard solution of 0.1 N KCl. The analysis was performed in triplicate.

CG-FID analysis

The quantification studies were performed on a Shimadzu Gas Chromatograph (model GC-2010 Plus, Kyoto, Japan) equipped with a capillary column DB-1 dimethylpolysiloxane (30mx 0.25 mm, 0.25 micron), and a flame ionization detector was used to analyze the samples.[31] The carrier gas was N_2 with a flow of 1.3 ml/min., split 1:100, injector temperature 260°C, detector temperature 280°C, initial column temperature equal to 60°C heated at a rate of 10°C. min^{-1} up to 92°C for 3 minutes, followed by a heating rate of 10°C/min up to 120°C and a rate of 20°C/min up to 280°C. The injection volume was 1.0 µl. The calibration curve from a commercial sample of carvacrol [Sigma-Aldrich, Chicago, IL, USA] was obtained by recording the peak areas against the known injected amount contained in the same injected volume (1 ml). The analysis was performed in triplicate.

Stability studies

Long-term stability

The macroscopic aspect, CI, pH, and conductivity were evaluated on storage days 1, 3, 10, 15, 30, 60, 90, 120, 150, and 180. The samples were stored at room temperature (25 ± 2°C) and at a low temperature (5 ± 1°C). These parameters enabled the evaluation of the emulsion system stability and therefore the prediction of the chemical stability of the components of the emulsion.

Short-term stability

The short-term stability was evaluated by the micro-emultocrit technique (MET).[32] The formulations were stored in closed containers at room temperature and were not homogenized before testing. The heparin-free capillary tubes were filled to 75% with each formulation and placed in a micro-centrifuge (Quimis, model Q10.500, São Paulo, Brazil) at 10,500 rpm for 10 min. The evaluation was realized at room temperature on the storage days 1 (D1), 30 (D30) and 90 (D90). After the centrifugation cycle, the IC calculations were performed in accordance with (equation 2). The short-term stability realized by the micro-emultocrit technique revealed not only, that the emulsion stability was highly influenced by the gravity acceleration, but also managed to predict influences provided in the HLB values. The Stability measurements were made in triplicate for each of the three days.

Statistical analysis

The mean, standard error of the mean, test-t and graphics were calculated by using SigmaPlot v.10.0 [Systat, USA], and $p<0.05$ was considered to be statistically significant

RESULTS AND DISCUSSIONS

Extraction and characterization of the essential oil

The essential oils extraction conditions are important as they are one of the main physical and chemical parameters which are directly related to the quality of the essential oil. A rapid distillation can lead to a product containing predominantly volatile constituents with better organoleptic and chemical characteristics. However, a prolonged extraction of the product makes the process more expensive and can also increase the amount of less desirable compounds.[33,34] In our study, the PaEO extraction was performed in a period from 1 to 6 hours [Figure 1]. The data showed higher extraction efficiency after 3 hours. After that, the extractions from three hours, showed constant behavior. Under these conditions, 0.9 ml of PaEO was obtained from 2 kg of fresh leaves of *P. amboinicus*.

The higher yield was obtained after 3 hours of extraction. Thus, 0.9 ml of PaEO was extracted from a mass of 2 kg of plant fixed to 2 l of distilled water at 100°C. The yield was 0.067% ± 0.3 [n= 3] relative to the weight of fresh material according to the Equation.[3]

$$\%Yield = \frac{v.d.100}{m} (Eq.3)$$

Where: d = density ($g.ml^{-1}$); m= mass (g) and v = volume (ml)

The extraction performance is in accordance with the essential oil content report in the literature for this herbal drug.[35,36]

The essential oil characterization plays an important role in the establishment of quality specifications for the quality control for industrial production of herbal products. The full characterization (physico-chemical), including chemical profile and thermal profile, as well as the biological activity of the essential oils according to their toxicological or pharmacological properties contributes to improving safe and effective therapeutic use of the species.[37,38] *Plectranthus* is a genus with an economic potential in various sectors, and it is attracting attention due to its medicinal value. A large number of species are not toxic and so may be taken orally, whilst others can be used topically on the skin. It is a promising plant for the development of medication.[1,39] The essential oil of *P. amboinicus* (PaEO) was characterized by determination of its physicochemical properties such as relative density, refractive index, and solubility in 90% ethanol.

The essential oil, obtained from *P. amboinicus* leaves, presents itself as a clear yellow liquid with strong aromatic odor and the physicochemical properties as summarized in Table 2.

Generally, oil yield and chemical composition are taxon-dependent and are strongly influenced by several factors, including harvest date, storage,

Table 2: Physical properties of the essential oil extracted from leaves of *P. amboinicus* (PaEO).

Physicochemical Properties	PaEO
Density ($g.ml^{-1}$)	1.5 ± 0.03
Refractive index (N_D 25°)	0.9167 ± 0.04
Solubility in 90% ethanol (v/v)	01:02 ± 0.07
Yield (%)	0.2 ± 0.06

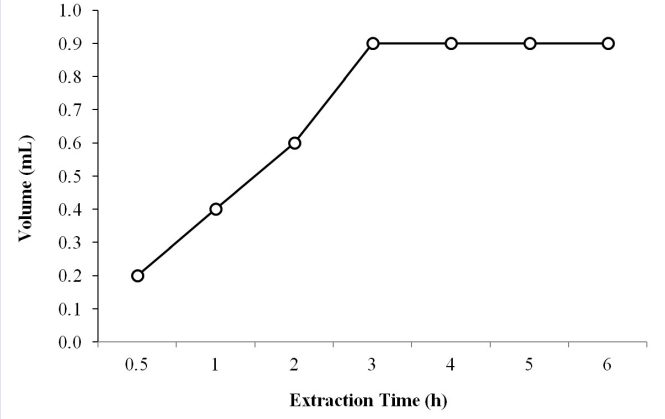


Figure 1: The extraction performance of the essential oil from the leaves of *P. amboinicus*.

environment, stage of maturity, and tested plant parts.[40] Therefore, the products marketed are usually obtained from pools of several oil charges to achieve some uniformity. Nevertheless, comparing the results obtained in this work with the literature, it could be observed that there are similarities among some quality parameters analyzed, such as: relative density, refractive index, and solubility in ethanol.[41,42]

Identification of components by GC / MS

The identification of the chemical constituents of the PaEO performed by GC-MS as well as the percentages, retention time and Kovats Index; are presented in Table 3. The chromatographic data allowed the identification of 19 components, corresponding to 99.99% of the total oil composition. The main constituents were carvacrol [33.5%], *p*-cymene [28.20%], γ-terpinene [14.77%], E-caryophyllene [4.63%], acid phthalic [4.32%], γ-bergamotene [3.16%], α-terpinene [2.52%], and β-myrcene [2.03%].

The qualitative data of the major compounds are in agreement with data previously reported for *Plectranthus* sp. oils from Brazil.[35] However, the quantitative data were different for: carvacrol [33,5%], p-cyneme [10.3%], and γ-terpinene [5.9%]. Other studies on the composition of the *P. amboinicus* oil also showed carvacrol as the main constituent, but the concentrations ranged from 28.65% to 90-98%.[6,8,43,44] The variations observed among the various reports can be attributed to the methodology used in the extraction process, seasonal variations, soil type, climate, genetic aspects, and geographical variations of plant.[8] According to Haddouchi, Chaouche[31] the knowledge about essential oil composition is fundamental in its correct application as a bioactive product. To control the types of components and the essential oil yield in the plant, it is necessary to standardize the cultivation conditions as well the harvest and postharvest procedures. Thus, the extraction of high quality essential oils is feasible.[45]

Development and evaluation of emulsions containing essential oil from P. amboinicus.

The essential oils, obtained from different plant species, have been extensively studied as bioactive products for therapeutic purposes in the pharmaceutical industry due to their antimicrobial, antiviral, anti-inflammatory, and repellent properties.[46,47] Furthermore, essential oils have showed lower toxicity and resistance in comparison to synthetic products.[48] On the other hand, the chemical complexity and variability of the herbal matrix, in addition to other deleterious properties such as susceptibility to degradation, volatility and, water insolubility, makes the technological development of pharmaceutical systems to improve the oil characteristics fundamental, in order to assure the biological properties and its effectiveness.[49]

Table 3: Percentage chemical composition of the essential oil from leaves of *P. amboinicus* by gas chromatography-mass spectrometry.

Peak	Compounds	$R_{t/min.}$	Area (%)	KI
1	α-Thujene	5.757	0.83	9.275.044
2	α-Pinene	5.969	0.38	9.350.088
3	Camphene	6.392	0.11	9.499.823
4	Vinyl amyl carbinol	7.170	0.74	9.775.221
5	β-Myrcene	7.567	2.03	9.915.752
6	α-Phellandrene	8.068	0.31	10.069.947
7	α-Terpinene	8.480	2.52	10.179.521
8	p-Cymene	8.783	28.2	10.260.106
9	Limonene	8.921	0.84	10.296.809
10	γ-Terpinene	10.04	14.77	10.594.415
11	α-Terpinolene	11.190	0.19	10.900.266
12	Linalool	11.599	0.15	11.007.907
13	L-4-terpineol	14.972	1.52	11.792.326
14	Carvacrol	20.608	33.5	13.065.455
15	E-Caryophyllene	25.710	4.63	14.233.216
16	γ-Bergamotene	26.352	3.16	14.384.452
17	α-Humulene	27.162	1.17	14.575.265
18	Caryophyllene oxide	32.558	0.62	15.878.484
19	Acid Phthalic	43.094	4.32	21.647.484

$R_{t/min.}$ - Retention Time (min.); KI - Kovats Index

These systems are defined as heterogeneous mixtures that consist of droplets of a liquid dispersed in a second continuous immiscible liquid phase forming a thermodynamically unstable system. To produce stable emulsions, surfactants are added to the system.[21,30] The selection of the surfactant used is fundamental in the production of formulations with a good stability. The first step in the selection of the correct surfactants for the emulsion system is the identification of the HLB value of the oil fraction. The concept of the hydrophile-lipophile balance [HLB] was first described by Griffin[51] and is used as a semi-empirical scale for selecting surfactants to form stable emulsions. A well established methodology to identify the HLB of essential oils is the study of long-term stability of a bath of emulsions produced with the essential oil, and ranging the HLB value of the surfactants.[19] The HLB value of the more stable emulsion is considered the HLB of the essential oil.

Long-Term stability study

The PaOE containing emulsions were evaluated for a period of 180 days to assess the stability of the systems. In spite of the various HLB values of the formulations, the process of emulsification by phase inversion was able to produce stable emulsions with a milky aspect and which remained stable on the day of preparation.

As expected, the emulsions showed different behavior to creaming during the storage time, according to the concentration of the emulsifying agent used. The cream index (CI) reflects the emulsion stability by measuring the height of the separated cream layer with

time, but also indirectly demonstrates the process of separation of droplets in the creaming.[52]

The studies by Guerra-Rosas, Morales-Castro,[53] showed that after the emulsification, the droplets migrate to the surface of the sample (coalescence) after a few hours of emulsification, forming a layer of cream. Consequently, there is a decrease of the CI over storage time in which the emulsion is preparing for phase separation.

The emulsions at room temperature showed significant creaming formation in the first 24 h (D1) [Table 4]. As the storage period increases, the emulsions showed a gradual increase in the creaming index (CI). The emulsions F5, F6, F7, F8, F9, F10, and F11 showed a phase separation process after 120 days. Therefore, phase separation of the emulsion F12 and F13 in D180 demonstrated instability of these formulations. The F14 formulation showed a high CI in D180. On the other hand, there was no phase separation of emulsions for F1, F2, F3, and F4. However, among these emulsions, the F1 had the lowest rate of creaming [Table 4].

When comparing the formulations at ambient temperature with 5°C, the temperature influences on stability were observed.[54] According to studies conducted by Hosseini, Jafari[55] and Leal, Sousa,[29] high temperatures promote a high frequency of collisions of the droplets, speeding up physical-chemical and chemical reactions, favoring the coalescence process resulting in a lower stability of the emulsions.

The formulations stored at 5°C presented a better stability when compared to the formulations stored at room temperature [Table 5].[56] There was an increase of IC with increasing storage time in the formulations. However, the emulsions F1, F2, F3, F4, and F5 showed the least CI, and the formulation F2 had the lowest CI, proving to be the most stable at the temperature 5ºC [Table 5].

Determination of pH

The measurements of pH were obtained from the probe inserted directly into the emulsion container. The evaluation of the pH values has the objective to investigate changes that affect the stability of the formulation. The pH values for emulsions stored at room temperature indicated no important influence on the surfactant composition. However, the storage time promoted a decrease in the pH for all formulations. Throughout the experimental period, the pH of all the formulations reduced, as well as the pH range between the formulations also reduced 2.5-4 (D180) [Figure 2]. The range of values may indicate the occurrence of undesirable chemical reactions or degradation of the products which could be attributed to a possible oxidation in the oil.[19,21,57] This result indicates that all formulations showed loss or degradation of the essential oil.

Conductivity

According to Masmoudi, Le Dréau,[58] modifications could be observed to the conductivity values of the emulsions before seeing the instabilities of the systems. Studies by Bernardi, Pereira[59] observed modifications to the conductivity values of the emulsions before seeing the instabilities of the systems. The studies by Abdullah, Abdulkarim[60] and Mahmood and Akhtar[61] attributed the continuous increase of conductivity values to the process of coalescence of the internal phase. This was followed by a reduction, which may be attributed to phase separation. Nevertheless, F1 and F2 presented an increase in conductivity during all the experiment, and they were the only formulations that did not have a decrease in conductivity during the period of 180 days [Figure 3]. Furthermore, F2 showed a lower value in conductivity than F1 and a better stability.

Short-term stability

The results presented in Figure 4 suggest that the method was able to detect not only the major influence of the centrifugal stress but also the individual contribution of each HLB [surfactant composition] on the system stability.

The micro-emultocrit technique (MET) was first described by Macedo, Fernandes,[32] as an appropriate tool to evaluate and predict influences provided by small variations in the HLB values on emulsions. Thus, besides the simplicity, the ability to use a low amount of sample and with a short time of execution made this method an excellent tool to evaluate and/or optimize formulation parameters and their respective HLB values. The results of the short term stability study are shown in Figure 4. Regarding the creaming index data, the lowest CI rates were observed at time D1 for the formulations F3, F4, F5 and F6, where CI were 3.62%, 3.43%, 3.47%, and 3.51%, respectively. After 30 days (D30), lower CI were observed for F1, F2, F3, F4, and F5 (2.42%, 1.88%; 1.81%; 1.83% and 1.78%, respectively). And, on the last day of analysis (D90), the CI for F1, F2, F3 and F4 were 2.43%, 1.75%; 2.9%, and 1.73% respectively.

By comparing the values obtained by the micro-emultocrit technique it became apparent that the results for formulations containing PaEO

Table 4: Analysis of creaming index (%) for the long-term stability study at room temperature.

Formulations	HBL	D1	D3	D10	D15	D30	D60	D90	D120	D150	D180
F1	16.7	2.44	3.66	6.17	6.17	6.25	6.41	6.67	6.85	5.71	5.80
F2	15.7	2.41	5.06	6.41	6.41	6.58	6.85	7.14	8.82	7.69	8.20
F3	14.7	5.00	5.00	7.69	7.69	7.89	8.11	7.14	7.25	7.58	6.25
F4	13.7	5.13	6.41	6.41	5.13	6.58	8.00	6.85	6.94	7.25	7.46
F5	12.7	5.13	6.41	6.58	6.67	6.76	8.45	5.97	PS	PS	PS
F6	11.7	4.88	6.10	7.41	7.50	7.59	9.09	5.41	PS	PS	PS
F7	10.7	3.90	5.19	6.67	5.33	6.76	8.22	5.71	PS	PS	PS
F8	9.7	5.00	6.25	7.50	7.69	7.79	9.21	6.85	PS	PS	PS
F9	8.7	5.13	5.13	6.41	7.79	7.89	6.67	6.94	PS	PS	PS
F10	7.7	3.75	5.00	6.49	6.76	8.82	10.17	8.93	PS	PS	PS
F11	6.7	3.75	5.00	7.59	7.69	7.79	9.21	8.11	PS	PS	PS
F12	5.7	3.75	5.00	8.86	7.69	7.79	6.67	9.46	9.86	9.87	PS
F13	4.7	1.23	2.47	6.25	6.33	6.41	9.33	10.96	9.86	8.82	PS
F14	4.3	2.50	3.80	6.41	7.79	7.89	8.22	11.43	11.94	7.81	13.11

F - formulation; D - day; HBL - hydrophilic-lipophilic balance; PS - phase separation.

Table 5: Analysis of creaming index (%) for the long-term stability study at 5°C degree.

Formulations	HBL	D1	D3	D10	D15	D30	D60	D90	D120	D150	D180
F1	16.7	2.44	3.66	6.17	6.17	6.25	6.41	6.67	6.85	5.71	5.80
F2	15.7	2.41	5.06	6.41	6.41	6.58	6.85	7.14	8.82	7.69	8.20
F3	14.7	5.00	5.00	7.69	7.69	7.89	8.11	7.14	7.25	7.58	6.25
F4	13.7	5.13	6.41	6.41	5.13	6.58	8.00	6.85	6.94	7.25	7.46
F5	12.7	5.13	6.41	6.58	6.67	6.76	8.45	5.97	PS	PS	PS
F6	11.7	4.88	6.10	7.41	7.50	7.59	9.09	5.41	PS	PS	PS
F7	10.7	3.90	5.19	6.67	5.33	6.76	8.22	5.71	PS	PS	PS
F8	9.7	5.00	6.25	7.50	7.69	7.79	9.21	6.85	PS	PS	PS
F9	8.7	5.13	5.13	6.41	7.79	7.89	6.67	6.94	PS	PS	PS
F10	7.7	3.75	5.00	6.49	6.76	8.82	10.17	8.93	PS	PS	PS
F11	6.7	3.75	5.00	7.59	7.69	7.79	9.21	8.11	PS	PS	PS
F12	5.7	3.75	5.00	8.86	7.69	7.79	6.67	9.46	9.86	9.87	PS
F13	4.7	1.23	2.47	6.25	6.33	6.41	9.33	10.96	9.86	8.82	PS
F14	4.3	2.50	3.80	6.41	7.79	7.89	8.22	11.43	11.94	7.81	13.11

F - formulation; D - day; HBL – hydrophilic-lipophilic balance.

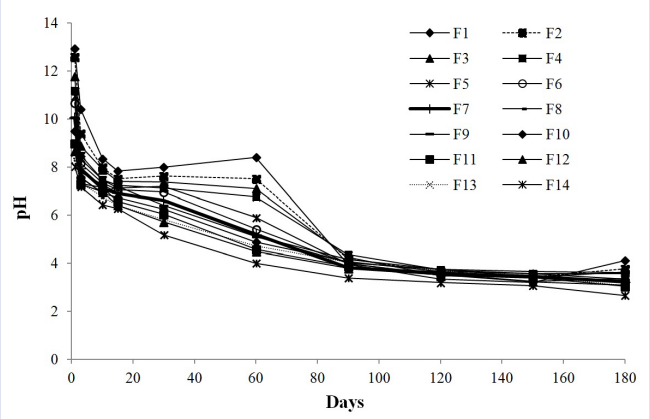


Figure 2: pH curves versus days for formulations (F) stored at room temperature.

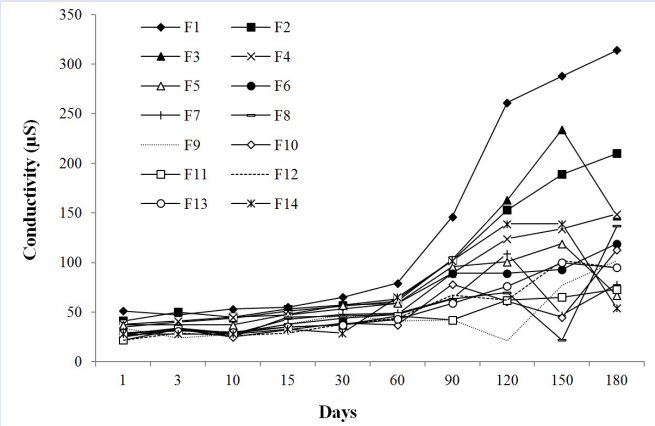


Figure 3: Conductivity curves versus days in accordance with the formulations (F) stored at room temperature.

between the days (D1, D30 and D90) showed a statistically significant difference between the formulations, when $p < 0,05$.

According to Franzol and Rezende,[62] to obtain emulsions there is a need to introduce energy in the emulsified systems, presenting very agitated droplets in the first days. This is because the emulsified system has free energy in the first days of storage which can lead to altered results. Thus, the study was conducted using the micro-emultocrit technique on three different days, where it was observed that the day D1 showed instability in relation to D30 and D90, possibly because free energy still existed in the system. The formulations on D30 and D90 had greater stability, because free energy had already dissipated promoting a kinetic stabilization. It was noted that F2 is the most stable formulation due to lower IC, based on the static data.

Quantitative analysis of carvacrol in emulsion

The quantitative analysis plays an important role in the pharmaceutical field throughout the production process from quality control to finished product to achieve a quality standard required for a drug.[63] This approach becomes more critical and challenging when complex materials such as biological matrix are used as active ingredients. Regarding the essential oils in which the chemical complexity is followed by adverse physico-chemical properties, the quantitative

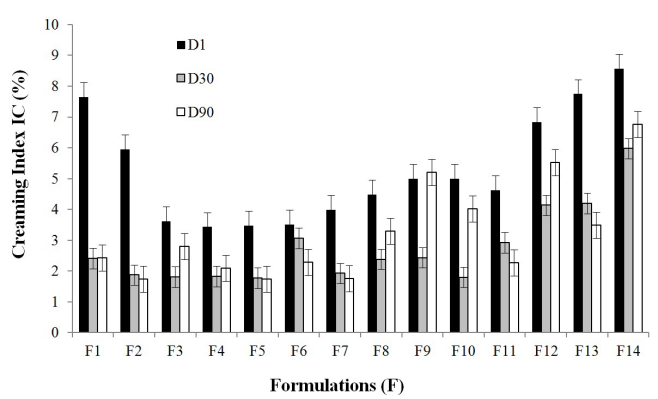


Figure 4: Analysis of creaming index [CI %] for the formulations containing essential oil *P. amboinicus* stored at room temperature. The short-term stability study performed by the micro-emultocrit technique in the days (D) 1, 30 and 90.

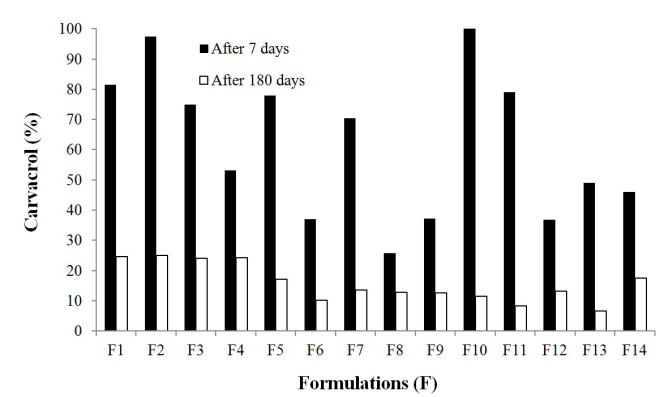


Figure 5: The quantitative analysis of carvacrol in emulsions containing essential oil *P. amboinicus*. The analysis was realized after 7 and 180 days stored at room temperature.

evaluation is used both as an indication of stability and measurement of technological success. According to Lukhoba, Simmonds,[1] and Ruzsíková, Součková,[64] the drug quantitative analysis facilitates development of medicines.

The chemical control of the emulsions was performed by the quantitative analysis of the Carvacrol, which is the major constituent of the PaEO. The contents of the chemical marker were assayed by GC, and the PaEO contents in the emulsions were evaluated, also indicating the emulsions stability. The contents of the chemical marker for each formulation were evaluated after 7 and 180 days of storage at room temperature [Figure 5]. The quantification of Carvacrol in the emulsions was carried out only at room temperature because volume loss was observed, unlike with emulsions, which are stored at 5°C. The proportions of PaEO entrapped in emulsified systems were estimated from the total volume of essential oil added to each formulation.

After 7 days, the percentages of carvacrol present in the emulsions were ranged from 25.6% to 100.00%. Maximum carvacrol content was observed in the formulations F2 [97.38%] and F10 [100.00%]. Regarding the content of carvacrol after 180 days, an important decrease was observed. The content reduction ranged from 50.3% to 89.6%, for formulations F8 and F11, respectively.

The F1, F2, F3, and F4 emulsions, which had the highest values of HLB, showed the highest carvacrol content after 180 days. Thus, this showed that the most stable formulations are those which showed higher HLB values and the highest levels of carvacrol. According to studies by Viana, Bohrer,[65] the HLB system is based on surfactants, which are amphiphilic compounds whose molecules are in the hydrophilic and lipophilic groups. The higher HLB values are assigned to more hydrophilic surfactants in which the hydrophilic groups are more suitable for the production of O/W emulsions. In fact, the essential oil properties presented a volatility which could explain the difficulty of oil entrapment and retention.

CONCLUSION

The development of emulsions containing essential oil remains a technological challenge, specifically in the improvement of their topical applicabilities. However, in the present work the feasibility of production of disperse systems was demonstrated using only the essential oil from leaves of *Plectranthus amboinicus* in the disperse phase, and its HLB value was described for the first time. Accordingly, the combination of surfactants [Span 80® and Tween 20®] leads to a required HLB value of 15.7.

To summarize, the present work demonstrated the feasibility of emulsion containing essential oil extracted from leaves of *P. amboinicus*. However, further biological studies are necessary to evaluate the activity of the PaOE containing emulsions, as well as the study of cutaneous permeation for topical use, as well as the definitive dose to be administered.

Acknowledgement

The authors thank the CNPq, FACEPE, UEPB, UFPB, and UFPE for their support. The authors are also grateful to Andrew Alastair Cumming for editing this paper.

Financial support and sponsorship

This work was supported by the CNPq [480128/2012-0, 302113/2012-6] and FACEPE [APQ-0363-. 4.03/13, APQ-0493-4.03/14].

Conflicts of interest

There are no conflicts of interest.

REFERENCES

1. Lukhoba CW, Simmonds MS, Paton AJ. *Plectranthus*: a review of ethnobotanical uses. J Ethnopharmacol 2006;103:1-24.
2. Morais SM, Dantas J da Silva ARA, Magalhães EF. Ethno-medicinal plants of Tapeba Indians from the State of Ceará- Brazil. Rev Bras Farmacogn 2005;15:169-77.
3. Oliveira RAG, Lima EO, Souza EL, Vieira WL, Freire KR, Trajano VN, *et al.* Interference of *Plectranthus amboinicus* (Lour.) Spreng essential oil on the anti-Candida activity of some clinically used antifungals.Rev Bras Farmacogn 2007;17:186-90.
4. Nogueira JCR, Diniz MFM, Lima EO. *In vitro* antimicrobial activity of plants in Acute Otitis Externa. Braz J Otorhinolaryngol 2008;74:118-24.
5. Gurgel APAD, da Silva JG, Grangeiro ARS, Oliveira DC, Lima CMP, da Silva ACP, *et al. In vivo* study of the anti-inflammatory and antitumor activities of leaves from *Plectranthus amboinicus* (Lour.) Spreng (Lamiaceae). J Ethnopharmacol 2009;125:361-3.
6. Gonçalves TB, Braga MA, de Oliveira FF, Santiago GM, Carvalho CB, e Cabral PB, *et al.* Effect of subinihibitory and inhibitory concentrations of *Plectranthus amboinicus* (Lour.) Spreng essential oil on *Klebsiella pneumoniae*. Phytomedicine 2012;19:962-8.
7. Manjamalai A, Alexander T, Grace VM. Bioactive evaluation of the essential oil of *Plectranthus amboinicus* by GC-MS analysis and its role as a drug for microbial infections and inflammation. Int J Pharm Pharm Sci 2012;4:205-11.
8. Murthy PS, Ramalakshmi K, Srinivas P. Fungitoxic activity of Indian borage (*Plectranthus amboinicus*) volatiles. Food Chem 2009;114:1014-8.
9. Bandeira JM, Barbosa FF, Barbosa LMP, Rodrigues ICS, Bacarin MA, Peters JA, *et al.* Essential oil composition of four *Plectranthus* species. Rev Bras Plantas Med 2011;13:157-64.
10. Joshi RK, Badakar V, Kholkute SD. Carvacrol rich essential oils of *Coleus aromaticus*

(Benth.) from Western Ghats region of North West Karnataka, India. Adv Environ Biol 2011;5:1307-10.

11. Pinheiro PF, Costa AV, Alves TdA, Galter IN, Pinheiro CA, Pereira AF, *et al.* Phytotoxicity and Cytotoxicity of Essential Oil from Leaves of *Plectranthus amboinicus*, Carvacrol, and Thymol in Plant Bioassays. J Agric Food Chem 2015;63:8981-90.
12. Shubha J, Bhatt P. *Plectranthus amboinicus* leaves stimulate growth of probiotic L. *plantarum*: Evidence for ethnobotanical use in diarrhea. J Ethnopharmacol 2015;166:220-7.
13. Korac R, Krajišnik D, Savic S, Pantelic I, Jovancic P, Cekic N, *et al.* A new class of emulsion systems - Fast inverted o/w emulsions: Formulation approach, physical stability and colloidal structure. Colloids Surf A Physicochem Eng Asp 2014;461:267-78.
14. Traynor MP, Burke R, Frias JM, Gaston E, Barry-Ryan C. Formation and stability of an oil in water emulsion containing lecithin, xanthan gum and sunflower oil. Int Food Res J 2013;20:2173-81.
15. Dora CL, Costa Silva LF, Mazzarino L, Siqueira JM, Fernandes D, Pacheco LK, *et al.* Oral Delivery of a High Quercetin Payload Nanosized Emulsion: *In Vitro* and *In Vivo* Activity Against B16-F10 Melanoma. J Nanosc Nanotechnol 2016;16:1275-81.
16. Otto A, du Plessis J. The Effects of Emulsifiers and Emulsion Formulation Types on Dermal and Transdermal Drug Delivery. In: Dragicevic N, Maibach HI, editors. Percutaneous Penetration Enhancers Chemical Methods in Penetration Enhancement. Springer Berlin Heidelberg 2015;223-41.
17. Monteiro OS, Souza AG, Soledade LB, Queiroz N, Souza AL, Mouchrek Filho V, *et al.* Chemical evaluation and thermal analysis of the essential oil from the fruits of the vegetable species *Pimenta dioica* Lindl. J Therm Anal Calorim 2011;106.2:595-600.
18. Van den Dool H, Kratz PD. A generalization of the retention index system including linear temperature programmed gas-liquid partition chromatography. J Chromatog A 1963;11: 463-71.
19. Ferreira MRA, Santiago RR, de Souza TP, Egito EST, Oliveira EE, Soares LAL. Development and Evaluation of Emulsions from *Carapa guianensis* (Andiroba) Oil. AAPS PharmSci 2010; 11:1383-90.
20. Friberg SE, Corkery RW, Blute IA. Phase inversion temperature (PIT) emulsification process. J Chem Eng Data 2011;56:4282-90.
21. Xavier-Junior FH, Silva KGH, Farias IEG, Morais ARV, Alencar EN, Araujo IB, *et al.* Prospective study for the development of emulsion systems containing natural oil products. J Drug Deliv Sci Tec 2012;22.4:367-72.
22. Streck L, Araújo MM, Souza I, Fernandes-Pedrosa MF, Egito EST, Oliveira AG, *et al.* Surfactant-cosurfactant interactions and process parameters involved in the formulation of stable and small droplet-sized benznidazole-loaded soybean O/W emulsions. J Mol Liq 2014;196:178-86.
23. Amaral DMF, Bhargava K. Essential oil nanoemulsions and food applications. Adv Food Technol Nutr Sci Open J 2015;1:84-7.
24. Hassan A. Effective surfactants blend concentration determination for o/w emulsion stabilization by two nonionic surfactants by simple linear regression. Indian J Pharml Sci 2015;77:461.
25. Kaviani D, Koonani M, Saghi M, Bigtan MH. Investigation of the effect of different parameters on the phase inversion temperature O/W nanoemulsions. Nanomed J 2016;3:65-8.
26. Esquena J, Sankar GSR, Solans C. Highly concentrated W/O emulsions prepared by the PIT method as templates for solid foams. Langmuir 2003;19:2983-8.
27. Pianovski AR, Vilela AFG, Silva AAS, Lima CG, Silva KK, Carvalho VFM, *et al.* Use of pequi oil (*Caryocar brasiliense*) in cosmetics emulsions: development and evaluate of physical stability. Rev Bras Cienc Farm 2008;44:249-59.
28. Frange RCC, Garcia MTJ. Desenvolvimento de emulsões óleo de oliva/água: avaliação da estabilidade física. Rev Ciênc Farm Básica Apl 2010;30:263-71.
29. Leal LB, Sousa GD, Seixas KB, Souza PHN, Santana DP. Determination of the critical hydrophile-lipophile balance of licuri oil from *Syagrus coronata*: application for topical emulsions and evaluation of its hydrating function. Braz J Pharm Sci 2013;49:167-73.
30. Fernandes CP, Mascarenhas MP, Zibetti FM, Lima BG, Oliveira RPRF, Rocha L, *et al.* HLB value, an important parameter for the development of essential oil phytopharmaceuticals. Rev Bras Farmacogn 2013;23:108-14.
31. Haddouchi F, Chaouche TM, Zaouali Y, Ksouri R, Attou A, Benmansour A, *et al.* Chemical composition and antimicrobial activity of the essential oils from four Ruta species growing in Algeria. Food Chem 2013;141:253-8.
32. Macedo JP, Fernandes LL, Formiga FR, Reis MF, Júnior TN, Soares LA, *et al.* Micro-emultocrit technique: a valuable tool for determination of critical HLB value of emulsions. AAPS Pharm Sci 2006;7:E146-E52.
33. Almeida PP, Mezzomo N, Ferreira SRS. Extraction of *Mentha spicata* L. volatile compounds: evaluation of process parameters and extract composition. Food Bioprocess Tech 2012;5: 548-59.
34. Fornari T, Ruiz-Rodriguez A, Vicente G, Vázquez E, García-Risco MR, Reglero G, *et al.* Kinetic study of the supercritical CO2 extraction of different plants from *Lamiaceae* family. J Supercrit Fluids 2012;64:1-8.
35. Galvão Rodrigues FF, Costa JGM, Rodrigues FFG, Campos AR. Study of the interference between Plectranthus species essential oils from Brazil and aminoglycosides. Evid Based Complement Alternat Med 2013; [Article ID 724161] 2013.
36. Aguiar JJS, Sousa CPB, Araruna MKA, Silva MKN, Portelo AC, Lopes JC, *et al.* Antibacterial and modifying-antibiotic activities of the essential oils of *Ocimum gratissimum L.* and *Plectranthus amboinicus* L. Eur J Integr Med 2015;7:151-6.
37. Fernández de Simón Bg, Muiño I, Cadahía E. Characterization of volatile constituents in commercial oak wood chips. J Agr Food Chem 2010;58:9587-96.
38. Azmir J, Zaidul I, Rahman M, Sharif K, Mohamed A, Sahena F. Techniques for extraction of bioactive compounds from plant materials: a review. J Food Eng 2013;117:426-36.
39. Rice LJ, Brits GJ, Potgieter CJ, Van Staden J. *Plectranthus*: A plant for the future. S Afri J Bot 2011;77:947-59.
40. Olle M, Bender I, Koppe R. The content of oils in *umbelliferous* crops and its formation. Agron Res 2010;8: 687-96.
41. El-hawary SS, El-sofany RH, Abdel-Monem AR, Ashour RS, Sleem AA. Seasonal variation in the composition of *Plectranthus amboinicus* (Lour.) Spreng essential oil and its biological activities. Am J Essent Oils Nat Prod 2013;1:11-8.
42. Noudogbessi J, Alitonou G, Djènontin T, Avlessi F, Figueredo G, Chalard P, *et al.* Chemical Compositions and Physico-chemical Properties of Three Varieties Essential oils of *Cymbopogon giganteus* Growing to the Spontaneous State in Benin. Orient J Chem 2013;29:59-67.
43. Senthilkumar A, Venkatesalu V. Chemical composition and larvicidal activity of the essential oil of *Plectranthus amboinicus* (Lour.) Spreng against Anopheles stephensi: a malarial vector mosquito. Parasitol Res 2010;107:1275-8.
44. Chen Y-S, Yu H-M, Shie J-J, Cheng T-JR, Wu C-Y, Fang J-M, *et al.* Chemical constituents of *Plectranthus amboinicus* and the synthetic analogs possessing anti-inflammatory activity. Bioorg Med Chem 2014;22:1766-72.
45. Carneiro FB, Júnior I D, Lopes PQ, Macêdo RO. Variation in the amount of ß-caryophyllene in essential oil of *Plectranthus amboinicus* (Lour.) Spreng. Lamiaceae under different conditions of cultivation. Rev Bras Farmacog 2010;20:600-6.
46. Bakkali F, Averbeck S, Averbeck D, Idaomar M. Biological effects of essential oils–a review. Food Chem Toxicol 2008;46:446-75.
47. Figueiredo AC, Barroso JG, Pedro LG, Scheffer JJ. Factors affecting secondary metabolite production in plants: volatile components and essential oils. Flav Fragr J 2008;23:213-26.
48. Otto A, Du Plessis J, Wiechers JW. Formulation effects of topical emulsions on transdermal and dermal delivery. Int J Cosmetic Sci 2009;31:1-19.
49. Flores FC, Ribeiro RF, Ourique AF, Rolim CMB, Silva CB, Pohlmann AR, *et al.* Nanostructured systems containing an essential oil: protection against volatilization. Quim Nova 2011;34:968-72.
50. Hunter TN, Pugh RJ, Franks GV, Jameson GJ. The role of particles in stabilising foams and emulsions. Adv Colloid Interface Sci 2008;137:57-81.
51. Griffin WC. Classification of surface-active agents by "HLB". J Soc Cosmet Chem 1949;1:311-26.
52. Xiang Z, Runge T. Emulsifying properties of succinylated arabinoxylan-protein gum produced from corn ethanol residuals. Food Hydrocoll 2016;52:423-30.
53. Guerra-Rosas MI, Morales-Castro J, Ochoa-Martínez LA, Salvia-Trujillo L, Martín-Belloso O. Long-term stability of food-grade nanoemulsions from high methoxyl pectin containing essential oils. Food Hydrocoll 2016;52: 438-46.
54. Varka EM, Ampatzidis C, Kostoglou M, Karapantsios T, Dutschk V. On the use of electrical conductance measurements for the stability of oil-in-water Pickering emulsions. Colloids Surf A Physicochem Eng Asp 2010;365:181-88.
55. Hosseini A, Jafari SM, Mirzaei H, Asghari A, Akhavan S. Application of image processing to assess emulsion stability and emulsification properties of Arabic gum. Carbohydr Polym 2015;126:1-8.
56. Ghosh S, Pradhan M, Patel T, Haj-shafiei S, Rousseau D. Long-term stability of crystal-stabilized water-in-oil emulsions. J Colloid Interface Sci 2015;460: 247-57.

57. Chen N, Zhao M, Sun W, Ren J, Cui C. Effect of oxidation on the emulsifying properties of soy protein isolate. Food Res Int 2013;52:26-32.
58. Masmoudi H, Le Dréau Y, Piccerelle P, Kister J. The evaluation of cosmetic and pharmaceutical emulsions aging process using classical techniques and a new method: FTIR. Int J Pharm 2005;289:117-31.
59. Bernardi DS, Pereira TA, Maciel NR, Bortoloto J, Viera GS, Oliveira GC, *et al.* Formation and stability of oil-in-water nanoemulsions containing rice bran oil: *in vitro* and *in vivo* assessments. J Nanobiotechnology 2011;9:1-9.
60. Abdullah GZ, Abdulkarim MF, Salman IM, Ameer OZ, Chitneni M, Mahdi ES. *et al.* Stability studies of nano-scaled emulsions containing ibuprofen for topical delivery. Int J Drug Dev 2011;3:74-82.
61. Mahmood T, Akhtar N. Stability of a cosmetic multiple emulsion loaded with green tea extract. Scientific World J 2013;[Article ID 153695], 2013:
62. Franzol A, Rezende MC. Emulsion stability: a case study involving anionic, cationic and nonionic emulsifiers. Polímeros 2015;25:1-9.
63. Cefali LC, Souza-Moreira TM, Corrêa MA, Salgado HRN, Isaac VLB. Development and evaluation of an emulsion containing lycopene for combating acceleration of skin aging. Braz J Pharm Sci 2015;51:579-90.
64. Ruzsíková A, Součková L, Suk P, Opatřilová R, Kejdušová M, Šrámek V, *et al.* Quantitative analysis of drug losses administered via nasogastric tube-*In vitro* study. Int J Pharm 2015;478:368-71.
65. Viana C, Bohrer D, Carvalho LM, Nascimento PC, Rosa MB. Emulsified systems for metal determination by spectrometric methods. Trends Anal Chem TrAC 2014;53:49-59.