Available online at www.sciencedirect.com
ScienceDirect
International Journal of Pharmaceutics 353 (2008) 149–159
INTERNATIONAL JOURNAL OF PHARMACEUTICS
www.elsevier.com/locate/ijpharm

Optimization of *Pothomorphe umbellata* (L.) Miquel topical formulations using experimental design

Peky Noriega [a,*], Cristina D. Ropke [a], Vladi O. Consiglieri [a], Maria E.S. Taqueda [b], Leoberto C. Tavares [c], André Wasicky [a], Elfriede M. Bacchi [a], Silvia B.M. Barros [d]

[a] *Departamento de Farmácia, Faculdade de Ciências Farmacêuticas, Universidade de São Paulo, Av. Prof. Lineu Prestes, 580, 05508-000 São Paulo, SP, Brazil*
[b] *Departamento de Engenharia Química da Escola Politécnica, Universidade de São Paulo, Av. Prof. Lineu Prestes, 580, 05508-000 São Paulo, SP, Brazil*
[c] *Departamento de Tecnologia Bioquímico-Farmacêutica, Universidade de São Paulo, Av. Prof. Lineu Prestes, 580, 05508-000 São Paulo, SP, Brazil*
[d] *Departamento de Análises Clínicas e Toxicológicas, Faculdade de Ciências Farmacêuticas, Universidade de São Paulo, Av. Prof. Lineu Prestes, 580, 05508-000 São Paulo, SP, Brazil*

Received 15 May 2007; received in revised form 13 November 2007; accepted 15 November 2007
Available online 10 January 2008

Abstract

Pothomorphe umbellata is a native plant widely employed in the Brazilian popular medicine. This plant has been shown to exert a potent antioxidant activity on the skin and to delay the onset and reduce the incidence of UVB-induced skin damage and photoaging. The aim of this work was to optimize the appearance, the centrifuge stability and the permeation of emulsions containing *P. umbellata* (0.1% 4-nerolidylchatecol). Experimental design was used to study ternary mixtures models with constraints and graphical representation by phase diagrams. The constraints reduce the possible experimental domain, and for this reason, this methodology offers the maximum information while requiring the minimum investment. The results showed that the appearance follows a linear model, and that the aqueous phase was the principal factor affecting the appearance; the centrifuge stability parameter followed a mathematic quadratic model and the interactions between factors produced the most stable emulsions; skin permeation was improved by the oil phase, following a linear model generated by data analysis. We propose as optimized *P. umbellata* formulation: 68.4% aqueous phase, 26.6% oil phase and 5.0% of self-emulsifying phase. This formulation displayed an acceptable compromise between factors and responses investigated.
© 2007 Elsevier B.V. All rights reserved.

Keywords: Experimental design; Formulation optimization; Ternary plots; *Pothomorphe umbellata*; Skin permeation; 4-Nerolidylchatecol

1. Introduction

Belonging to the Piperaceae family, some plants popularly known as "caapebas" or "pariparobas" were described by Pison in "De medicina brasiliensis", in 1638. Special attention was given to the *Pothomorphe* species, *Pothomorphe peltata* and *Pothomorphe umbellata*, used by the Brazilian natives for its medicinal properties (Peckolt, 1941; Riedel, 1941). The Brazilian Pharmacopoeia, in the first edition, includes *P. umbellata* (L.) Miq. with the official name of "pariparoba", being the dried roots used as medicinal drug (Silva, 1926). Kijjoa et al. (1980) isolated and characterized a secondary metabolite, named 4-nerolidylcatechol (4-NC) (Fig. 1), from the hexanic extracts from leaves and roots of *P. umbellata*.

The standardized ethanolic dried root extract of *P. umbellata* displayed no toxicity (Barros et al., 2005). Chromatographic characterization of crude drug and fluid extract was standardized previously (Moraes et al., 1984). Analytical methods for determination of 4-nerolidylcatechol were developed and validated by HPLC (Ropke et al., 2003b; Rezende and Barros, 2004).

The *N*-benzoylmescaline isolated from the aerial parts of *P. umbellata* showed significant antibacterial activity (Isobe et al., 2002). The plant *P. umbellata* has been shown to exert a potent antioxidant activity on the skin and to delay the onset and reduce

* Corresponding author. Tel.: +55 11 3091 3632; fax: +55 11 3813 197.
E-mail address: pekynoriega@hotmail.com (P. Noriega).

0378-5173/$ – see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijpharm.2007.11.039

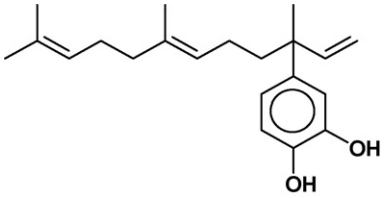

Fig. 1. Chemical structure of 4-nerolidylchatecol.

the incidence of UVB-induced skin photoaging (Ropke et al., 2003a, 2005, 2006). A recent study has shown that the *P. umbellata* root extract gel is stable for at least 3 months when stored at 5 and 25 °C and that addition of BHT does not affect the stability (Silva et al., 2005).

The ternary plots are an accurate bidimensional representation of phase behavior, in systems with three components (Osborne, 1990; Armstrong and James, 1996); this is one of the most frequently used methods to determine response surfaces and optimization regions for specific formulation characteristics (Bolton, 1991). In addition, this methodology offers the maximum return in terms of information about the interplay of multiple factors while requiring the minimum investment (Marti-Mestres et al., 1997; Martinello et al., 2006; Singh et al., 2006).

Some pioneer studies on phytotherapic formulations, such as works that studied the "optimal" concentration of various excipients to choose a formulation of propolis extract emulsions, do not include optimization techniques (Arvouet-Grand et al., 1995; Vennat et al., 1998). Another example is the development of ointment formulations, prepared by the incorporation of three *Achyrocline satureioides* spray-dried extracts combinations in a unique base (Paula et al., 1998).

Due to the importance of *P. umbellata* extract in the prevention of UV skin damage, the aim of this study was to improve *P. umbellata* formulations. The study of ternary mixtures models with constraints and graphic representation by phase diagrams was used to optimize the appearance, the centrifuge stability and the permeation of emulsions containing *P. umbellata* extract (0.1% 4-nerolidylchatecol). In this methodology, the study of the ingredient effect on some measurable characteristics of the blend (or response) is an attempt to find the formulation (or formulations) that produces the 'best' response (Cornell, 1990).

2. Materials and methods

2.1. Test materials

Methanol, ethanol, hexane Lichrosolv grade were purchased from Merck (Darmstadt, Germany). $LiCLO_4$ was from Aldrich (Milwaukee, WI, USA). Pharmaceutical grade materials acquired from Galena (Campinas, Brazil) were: cetostearylalcohol (Type A) emulsifying (cetostearyl alcohol and sodium cetostearyl sulfate mixture, commercially available as Lanette® N, Cognis Deutschland GmbH and Co.Kg), decyl oleate (Cetiol® V, Cognis Deutschland GmbH and Co.Kg); mineral oil, propylene glycol, propylparaben (Nipasol®), methylparaben (Nipagin®).

According to the preconized definition by USP XXVIII (2005) for active botanical markers, the 4-nerolidylcathecol was selected for this purpose; this substance was isolated as described previously from dried powdered roots of *P. umbellata* (Gustafson et al., 1992). *P. umbellata* roots were collected in the campus of the University of São Paulo, February 2001, the plant material was identified and a sample was deposited in the Herbarium of the Institute of Biosciences of the University of São Paulo.

2.2. Extract preparation

The roots of *P. umbellata* were washed, cut and dried in a stove with air circulation and temperature below 50 °C during 72 h, then the dried roots were first triturated in a hammer mill and after that in a ball mill (Thomas, PA, USA) to obtain a particle diameter below 840 μm. (Noriega et al., 2005). The percolation was performed with extracting solvent ethanol–water 1:1, the obtained fractions were mixed and the alcohol was evaporated under reduced pressure in a Büchi rotatory evaporator, model B-480 at 50 °C. The remaining aqueous extract material was lyophilized in an Edwards equipment, Brazil, at −40 °C and 0.9 mbar.

2.3. Formulations optimization

2.3.1. Experimental design

A Mix D-optimal Design of constrained regions for ternary mixtures was utilized in this study; the amounts of three components of emulsion: aqueous phase (x_1), oil phase (x_2) and self-emulsifying phase (x_3) were selected as the factors to systematically optimize the dependent variables: appearance, centrifuge stability and permeation (Table 1). All other formulation and processing variables were kept invariant throughout the study.

The response surface methodology of ternary system was performed with constraints for all independent variables; the phase x_2 was single component, mineral oil (emollient) and two phases (x_1 and x_3) were denoted as pseudocomponents (binary mixtures). Water and propylene glycol (moisturizing agent) composed the phase x_1. The self-emulsifying phase (x_3) consisted of (Type A) emulsifying cetostearyl alcohol and sodium cetostearyl sulfate mixture (Lanette® N, auto-emulsifying wax with anionic characteristic and HLB within 8-16, useful to produce oil/water emulsions), and decyl oleate (Cetiol® V), solubilizing agent of lipossoluble substances. The levels of the three factors were selected on the basis of the preliminary studies carried out before implementing the experimental design.

In the case of emulsions, the study of pure component (original simplex) is not interesting and the components of the mixture are restricted to intervals; the three-component constrained region, a rhomboid figure into the triangle represents a situation defined by the constrains: $0.9 \geq x_1 \geq 0.4$; $0.3 \geq x_2 \geq 0.05$ and $0.3 \geq x_3 \geq 0.05$. Table 2 displays the coordinates of the 14 design points for the constrained region, the selected design points include the vertices of the feasible region (in duplicate), axial check blends (averages of the overall center and each vertex) and overall centroid; this experimental design of ternary sys-

Table 1
Percentage composition[a] of *P. umbellata* emulsion (O/W) and constraints of the experimental design

Ingredients	Phase	Component type	Pseudocomponent proportion (%)	Minimum limit (%)	Maximum limit (%)	Factor
Propylene glycol	Aqueous	Pseudocomponent	7	40	90	A: x_1
Distilled water		Pseudocomponent	93			
Mineral oil	Oil	Component	–	5.0	30.0	B: x_2
Lanette®	Self-emulsifying	Pseudocomponent	80	5.0	30.0	C: x_3
Cetiol V®		Pseudocomponent	20			

[a] The amount of Nipagin (methyl *p*-hydroxybenzoate): 0.18% and Nipasol (propyl *p*-hydroxybenzoate): 0.02% was identical for all formulations.

Table 2
Experimental design of the ternary system with constraints for the emulsions containing dried extract of *P. umbellata,* displaying factor fraction

Formulation std	Run	Point type	Factor A (x_1); aqueous phase	Factor B (x_2); oil phase	Factor C (x_3); self-emulsifying phase
5	1	Vertex	0.900	0.050	0.050
2	2	Vertex	0.650	0.050	0.300
1	3	Vertex	0.400	0.300	0.300
4	4	CentEdge	0.525	0.175	0.300
3	5	CentEdge	0.525	0.300	0.175
11	6	Vertex	0.650	0.050	0.300
14	7	Vertex	0.400	0.300	0.030
7	8	Center	0.650	0.175	0.175
10	9	Vertex	0.900	0.050	0.050
6	10	Vertex	0.650	0.030	0.050
12	11	Vertex	0.650	0.030	0.050
8	12	CentEdge	0.775	0.175	0.050
9	13	CentEdge	0.775	0.050	0.175
13	14	Interior	0.650	0.237	0.113

tem with upper and lower constraints was based on the mixture model proposed by McLean and Anderson's extreme vertices algorithm (Cornell, 1990).

Optimization study was performed employing Design Expert software (Version 7.1, 2007, Stat-Ease Inc., Minneapolis, MN, USA). The best-fitting mathematical model was selected based on the comparisons of several statistical parameters including the determination of the Sequential Model Sum of Squares, Lack of Fit (LOF) and Predicted Residual Error Sum of Squares (PRESS) was provided by analysis of variance (ANOVA).

2.3.2. Emulsion preparation

The emulsion preparation technique was the simultaneous mixture of phases. The aqueous and oil/self-emulsifiying phases were weighted separately and heated to the same temperature, about 72 °C. The aqueous phase was gradually added to the oil/self-emulsifiying phases and stirred with high shear mixer for 5 min. Once the emulsion presented a uniform appearance, it was mixed manually. The lyophilized extract of *P. umbellata* (6.21% 4-NC) previously dispersed in propylene glycol was incorporated in a hydrophilic emulsion (final concentration: 0.1% 4-NC), when the temperature had diminished to 45 °C. Sequentially, the preservatives dissolved in propylene glycol were added, and the manual mix maintained until cooling was completed. *P. umbellata* extract is easily miscible in semi-polar solutions, displaying a good miscibility in emulsions (Ropke et al., 2002).

2.4. Response parameters

The response parameters studied were: appearance, pH, centrifuge stability and in vitro permeation using modified *Franz*'s cell. The desirable and ideal properties of a topical emulsion are: (1) the preparation must be creamy or semi fluid, spreading easily. (2) It should maintain the homogeneity, after submitted to gravitational forces and (3) the last and principal factor, the maximum skin permeation of 4-NC must be reached.

2.4.1. Physicochemical assays

The general appearance was evaluated macroscopically. (Vennat et al., 1998; Fresno et al., 1999). The qualitative parameter was transformed in a numeric value; in this case we have a non-continuous response, since the response comes from a set of ordered categories (e.g., poor, fair, good, very good, excellent) likewise, we can assign the categories ordered integers (e.g., 1, 2, 3, 4 and 5) and conduct the analysis in the same manner for quantitative response or continuous response. (Schmidt and Launsby, 1998). The arbitrary scale used for the classification of the macroscopic aspect was: non-homogeneous, 1; stiff, 2; semi-stiff, 3; creamy, 4; semi-fluid, 5. The pH values were measured in a dispersion of 10% of the emulsion diluted in water, in a potentiometer Incibras, at room temperature. The centrifuge stability was determined using 5 g of the preparation in round bottom tubes and centrifuged at 3500 rpm for 20 min (Hir, 1997). The formation of emulsion, separation of phases, precipitation or another type of instability was analyzed. The scale used was:

non-homogeneous, 1; sediment, 2; foamy surface, 3; pearly, 4; homogeneous, 5. The emulsions were evaluated after 72 h, enough time to the syneresis phenomena takes place. A panel of six trained individuals evaluated twice the appearance of each sample in randomized order.

2.4.2. In vitro skin permeation (Franz's cell)

The measurement of 4-nerolidylcatechol in the skin was used as parameter to evaluate the skin permeation of the active principle from the release module. Skin permeation studies were carried out with modified *Franz*'s cells in a thermostatic bath at 37 ± 0.5 °C (Flowscience, Brazil). In vitro studies with intact hairless mouse skin (HRS/J, male) were performed as follows: immediately after ether anesthesia, full thickness skin was obtained from 10-week-old mice; and the dorsal skin without adhering subcutaneous fat was mounted on a modified Franz diffusion cell (1.61 cm^2 surface area). Test formulations (600 μL) were applied to the epidermal surface of the skin, completely covering the exposed skin area. The receptor solution compartment contained 12 mL of sodium phosphate buffer 0.1 M, pH 7.0, to ensure sink conditions. The total experiment time to collect the material aliquots was 24 h. The 4-nerolidylcatechol quantification was carried out by HPLC method, adapted from Ropke et al. (2003b) and described in the following section.

2.4.3. High efficiency liquid chromatography—HPLC

The 4-nerolidylcatechol was identified and quantified by a validated HPLC technique by comparison with a 4-nerolidylcatechol standard previously isolated in our laboratory and identified by spectrometric analysis (Ropke et al., 2003b). The HPLC equipment consisted of a Waters Model 510 pump, a 7161 Rheodyne injector equipped with a 20 μL loop and a Hewlett_/Packard HP 1049A Electrochemical Detector. The cell contained a solid state, in situ Ag/AgCl reference electrode as a standard with the refillable electrode, and a glass carbon electrode as the standard working electrode. The detection conditions were the following: potential 0.600; polarity: oxidation, mode: amperometry. Integration of the chromatographic peaks was achieved with a SP 4600 Thermoseparation Products integrator. Chromatography was performed on a Supelcosil LC-8, 3 μm, 75 mm × 4.6 mm column (Supelco, Bellefonte, PA, USA) with a mobile phase of methanol:water (9:1) containing 20 mM $LiCLO_4$ and 2 mM KCl. The flow rate was set at 1.0 mL/min. The retention time of 4-nerolidylcathecol was 2.4 min. Each sample was filtered through 0.22 μm cellulose acetate filters (Costar®) and an aliquot of 20 μL was injected into the HPLC apparatus.

2.5. Calibration

The HPLC method was previously validated, including sensitivity, precision, accuracy, stability with a linear relationship between the concentrations, for ranges of 0.15–2.5 μg/mL of 4-NC (Ropke et al., 2003b). The stock solution of 4-NC to obtain calibration curve in ethanol–water (1:1) was prepared from 1 mg 4-NC in 1 mL 1:1 in ethanol–water solution. From this solution 8 aliquots were prepared: 0.1; 0.01; 0.005; 0.0025; 0.00125; 0.000625; 0.000313; 0.000156 mg/mL, in duplicate, as a control of the validated method.

3. Results and discussion

The aim of this work was to optimize the appearance, the centrifuge stability and the permeation of emulsion containing *P. umbellata* (0.1% 4-nerolidylchatecol). Experimental design was used to study ternary mixtures models with constraints and graphical representation by phase diagrams (response surface and contour plots). The equation resulting from each experiment was an empirical equation which approximately describes the response pattern in the simplex space (Bolton, 1991).

Table 3 summarizes the results of the response parameters studied from the 14 emulsion formulations. The statistical treatment of data (ANOVA) for each measured response was reported in Table 4, the models were expressed in terms of actual components. To choose the optimized formulation we used the

Table 3
Results of the physicochemical and permeation assays of *P. umbellata* emulsions

Formulation run	Assay			
	Macroscopic appearance	Centrifuge stability	Amount of 4-NC in the skin (24 h) (μg/cm^2)	pH
1	Semi-fluid (5)	Foamy surface (3)	1.39	6.52
2	Stiff (2)	Homogeneous (5)	2.73	6.60
3	Non-homogeneous (1)	–	–	–
4	Stiff (2)	Sediment (2)	12.11	6.62
5	Non-homogeneous (1)	–	–	–
6	Stiff (2)	Homogeneous (5)	10.83	6.67
7	Non-homogeneous (1)	–	–	–
8	Semi-stiff (3)	Homogeneous (5)	1.89	6.48
9	Semi-fluid (5)	Foamy surface (3)	3.89	6.62
10	Creamy (4)	Sediment (2)	42.15	6.45
11	Creamy (4)	Sediment (2)	35.04	6.57
12	Creamy (4)	Homogeneous (5)	12.03	6.64
13	Semi-stiff (3)	Pearly (4)	6.00	6.60
14	Semi-stiff (3)	Homogeneous (5)	11.01	6.58

Table 4
Statistical results of the physicochemical and permeation assays of *P. umbellata* emulsions

Statistical parameter	Assay		
	Appearance	Centrifuge stability	Permeation
Model significant	Linear	Quadratic	Linear
Square sum	23.17	27.32	1212.06
Freedom degree	2	5	2
Square mean	11.58	5.46	606.04
F	50.05	5.21	7.54
p	<0.0001	0.0202	0.0144
Lack of fit			
Square sum	2.55	8.394	580.12
Freedom degree	7	4	5
Square mean	0.36	1.0493	116.02
R-squared	0.9010	0.7650	0.6500
F	–	–	5.51
p	–	–	0.0953
PRESS	3.80	26.16	1118.58

desirability function; Cox graph was used to obtain the right interpretation of the influence of the components on response variables since this is most suitable for mixture problems with constraints.

Although the excipients used in the formulations were within the limits described in the literature, phase separation was observed in the run formulations numbers 3, 5 and 7 (3 and 7 are replicates of the same point inside of triangle), showing interactions with others factors, that could be due to the incorporation of *P. umbellata* extract in the formulations. This measured property of the final product was affected by the percentages or proportions of the individual ingredients that are present in the formulation (Barros et al., 1996; Cornell, 1990).

The quantification of 4-NC carried out in samples of lyophilized extract, emulsions and mice sink was done from the equation of calibration curve: area = 556181 × concentration − 3585.6 (r^2 = 0.9992).

3.1. Physicochemical assays

The physicochemical data of appearance and centrifuge stability were represented by response surface and contour plots, generated from the best regression models (Figs. 2 and 3; Eqs. (1a) and (2a)). The pH variable did not present statistical significant variations and the values were not plotted.

3.1.1. Appearance

The optimization process generated a model equation that provided a means of evaluating changes in response due changes in the independent variables levels. The statistical analysis of the data of appearance (Tables 2 and 3) suggested the polynomial equation:

$$y_1 = 5.629 \times x_1 + 0.741 \times x_2 - 5.483 \times x_3 \quad (1a)$$

that revealed a linear model, in agreement to the generic model:

$$y = b_1 \times x_1 + b_2 \times x_2 + b_3 \times x_3 \quad (1b)$$

y is the response, b is the coefficient for multiple regression and x is the component proportion

The absolutes values of the factors studied indicated that $b_1 \approx b_3 > b_2$, Eq. (1a). This equation showed that the aqueous phase and the self-emulsifying phase had practically the same influence; however the aqueous phase displayed positive effect on improving the appearance, producing semi-fluid or creamy formulations. The negative value (minus sign) for self-emulsifying phase showed that the increasing of this phase promoted worse appearance characteristics, i.e., stiff or semi-stiff formulations. On the other hand, the oil phase concentration practically did not alter the obtained values; therefore the formulations appearance was almost independent of the proportion of this individual ingredient in the formulation (Fig. 2).

3.1.2. Centrifuge stability

The analysis of the emulsions centrifuge stability results (Table 2), suggested a quadratic model (Table 3), according to the equation:

$$y_2 = 1.117 \times x_1 - 62.557 \times x_2 - 12.323 \times x_3 + 96.276 \times x_1x_2 + 32.276 \times x_1x_3 + 78.139 \times x_2x_3 \quad (2a)$$

In compliance with generic model:

$$y_2 = b_1 \times x_1 + b_2 \times x_2 + b_3 \times x_3 + b_{12} \times x_1\, x_2 + b_{13} \times x_1\, x_3 + b_{23} \times x_2x_3 \quad (2b)$$

Eqs. (2a) and (2b) contains the same three elements for the linear Eqs. (1a) and (1b), plus others three elements representing the interactions between the factors, summarizing six terms for the quadratic equation. The absolute values indicate that $b_2 > b_3 > b_1$. The evaluation of the isolated parameters demonstrated that the oil phase was the principal factor that negatively influences the stability; probably due to the fact that the increasing of the inner phase destabilizes the proportions balance of the o/w emulsion, and the system had a tendency to phases inversion. Concomitantly, the increasing of the self-emulsifying phase impaired the stability in lesser degree; this fact could be explained by the excess of surfactant in the interface oil–ater, since it acts until an optimum value (critic micellar concentration). The aqueous phase had the minor influence of all, because it is the continuous phase and an increase on its percentage in the formulation did not alter the emulsion type (o/w). Observing the interactions $b_{23} > b_{13} > b_{12}$, we could deduce that there were strong positive interactions, principally between the oil and the self-emulsifying phase (b_{23}), which suggests that the excesses of the internal phase of the emulsion is stabilized as droplets by the increase of self-emulsifying phase. The interaction of self-emulsifying with aqueous phase (b_{13}) also improved this response parameter, probably due to a suitable hydrophilic/lipophilic balance between the phases. The last interaction (b_{12}) confirmed that it was necessary an adequate proportion of these ingredients to maintain the original emulsion (o/w) without phases inversion (w/o) or other type of instability. Summarizing, the mixture of these three different ingredients increased the stability with a synergistic action and the

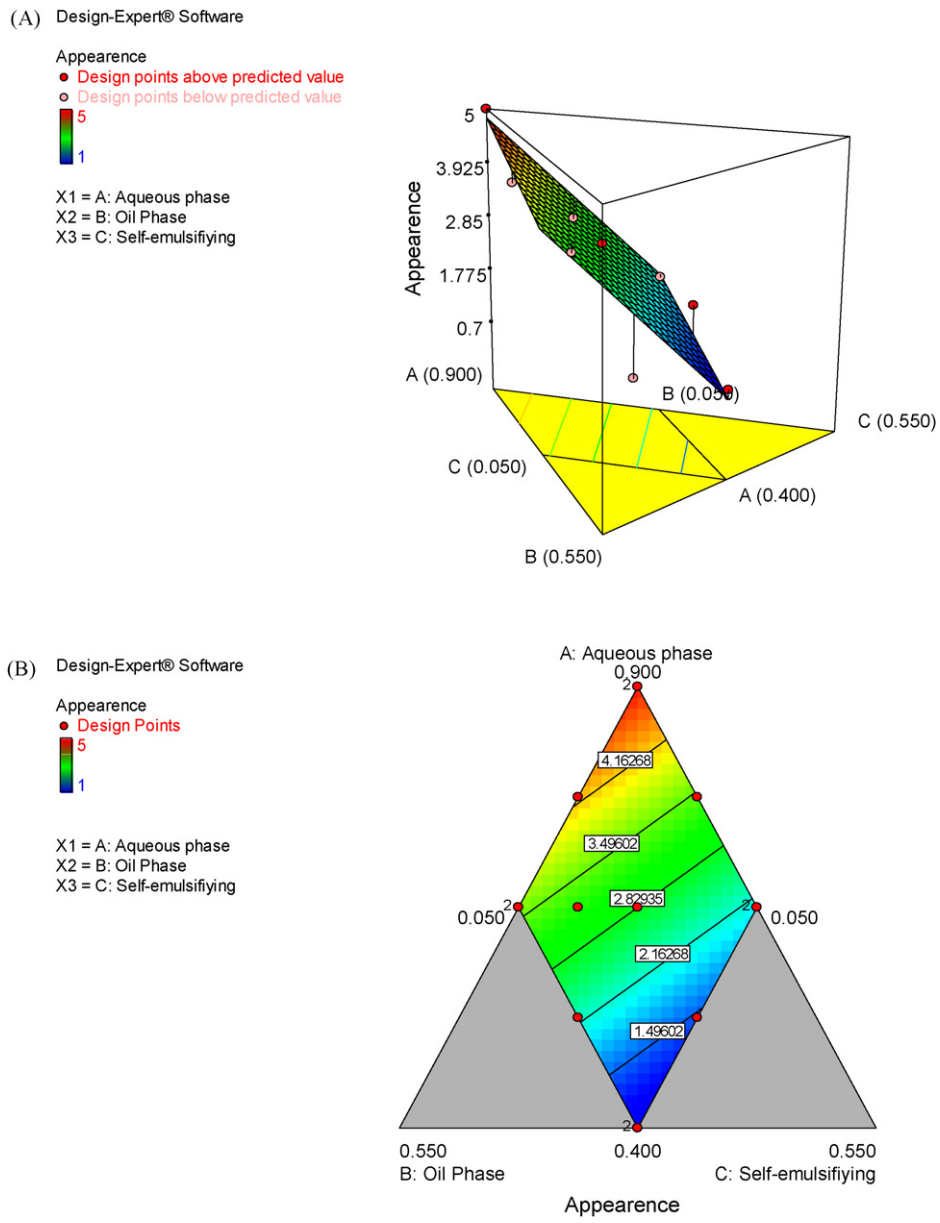

Fig. 2. Ternary response surface (A) and contour (B) plot of appearance of emulsions containing *P. umbellata*. *The scale used was: non-homogeneous, 1; stiff, 2; semi-stiff, 3; creamy, 4; semi-fluid, 5.

response surface and contour diagram corroborated these results (Fig. 3).

3.2. In vitro skin permeation (Franz's cell)

Many factors may influence the extent of skin permeation of a chemical, one such factor is the vehicle in which it is applied to the skin; the quantification of the amount of 4-NC in the in vitro permeation studies considered parameters like *Franz*'s cell area, weight of applied emulsion, volume of receptor solution, amount of homogenized skin and concentration of 4-NC in each formulation.

The results of the statistical analysis of the skin permeation of 4-NC emulsions suggested two models, the different values of Predicted Residual Error Sum of Squares—PRESS (a measure of how the model fits each point in the design) were compared for linear and quadratic models and the last model was selected (Tables 2 and 3), according to the equation:

$$y_3 = 1.788 \times x_1 + 103.144 \times x_2 - 8.043 \times x_3 \quad (3)$$

This equation and the triangular-dimensional surface and contours diagram (Fig. 4) showed that higher proportions of oil phase promoted the penetration of 4-NC in skin, followed in a smaller proportion by the aqueous phase, while the self-emulsifying phase decreased the penetration.

This phenomenon could be explained by differences in the partition coefficient in the skin, according with physicochemical characteristics of the active chemical. The results support the fact that the permeation could be affected by the oil nature

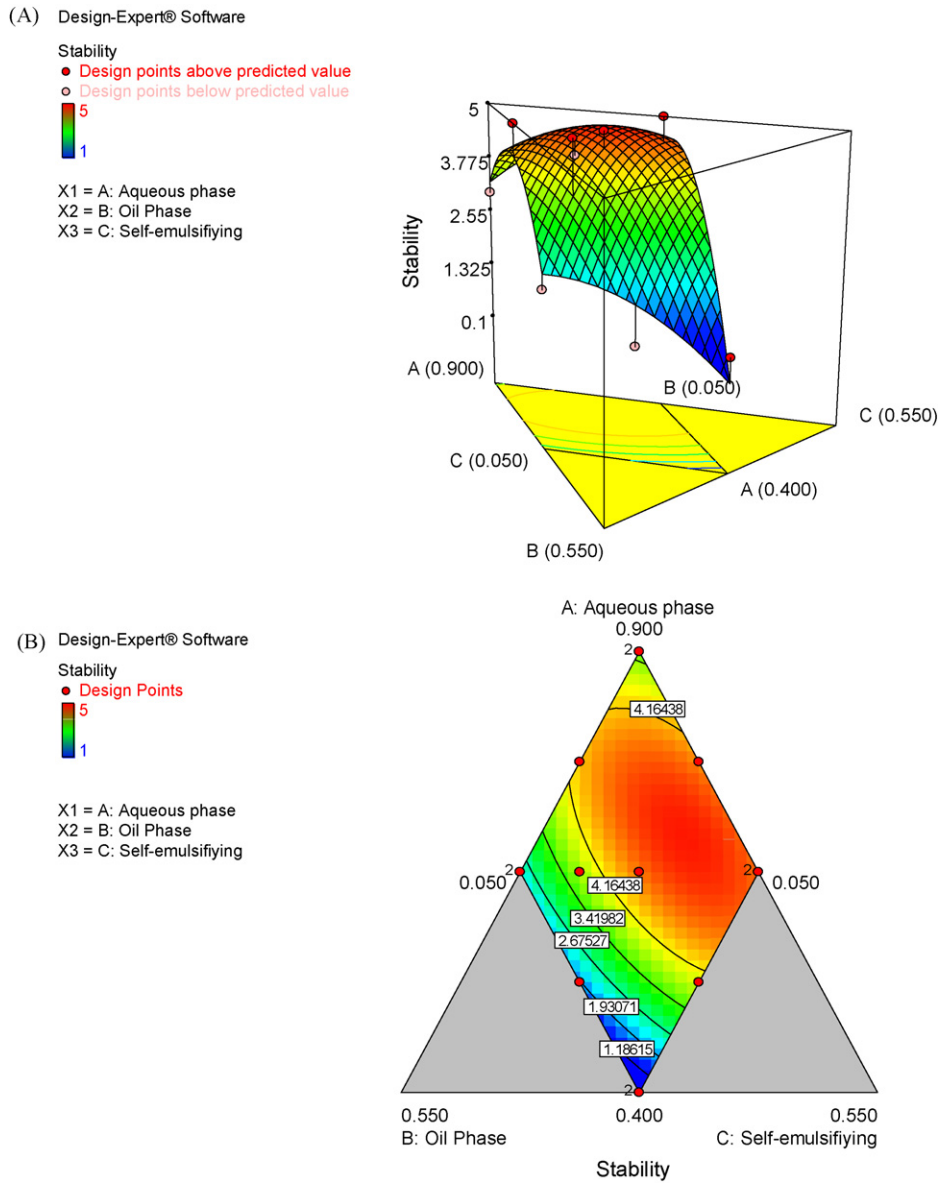

Fig. 3. Ternary response surface (A) and contour (B) plot of centrifuge stability of emulsions containing *P. umbellata*. *The scale used was: non-homogeneous, 1; sediment, 2; foamy, 3; pearly, 4; homogeneous, 5.

of 4-NC, calculated partition coefficient: 4 (Freitas, 1999). Considering the influence of the vehicle, other fact that could have improved the 4-NC permeation was the strong emollient action, besides of some occlusive effect of mineral oil. Certain amount of water in the formulation was necessary to permeate the different skin layers, but on the other hand, the auto-emulsifying phase probably stabilized the 4-NC in the emulsion so that it was poorly released from the cession module.

The major skin penetration corresponded to formulations 10 and 11 (replicates of the same point), for this response parameter this was the best formulation (0.65:0.30:0.05) that displayed higher amounts of 4-NC in the skin in comparison with a standard formulation investigated in a previous permeation study (Ropke et al., 2002).

Accordingly with data reported in the literature, the measurements of permeation did not exceeded 24 h, because it is unrealistically long relative to the normal patterns of use of ointment and creams (Chiang et al., 1989) The probability value for goodness of fit test (Table 4) higher than 0.05 was probably due to differences in values of permeation observed for the formulations numbers 2 and 6; this could be explained by differences in the skin thickness or even of the integrity of the stratum corneum (Shah et al., 1992).

4. Optimization

In our study we worked with constraints and pseudocomponents for the aqueous phase and self-emulsifying phase, so that the whole area of the phase diagram was not investigated. This type of study results in a more complicated design (at the beginning) and statistical treatment of data (at the end), but offers the maximum return of information

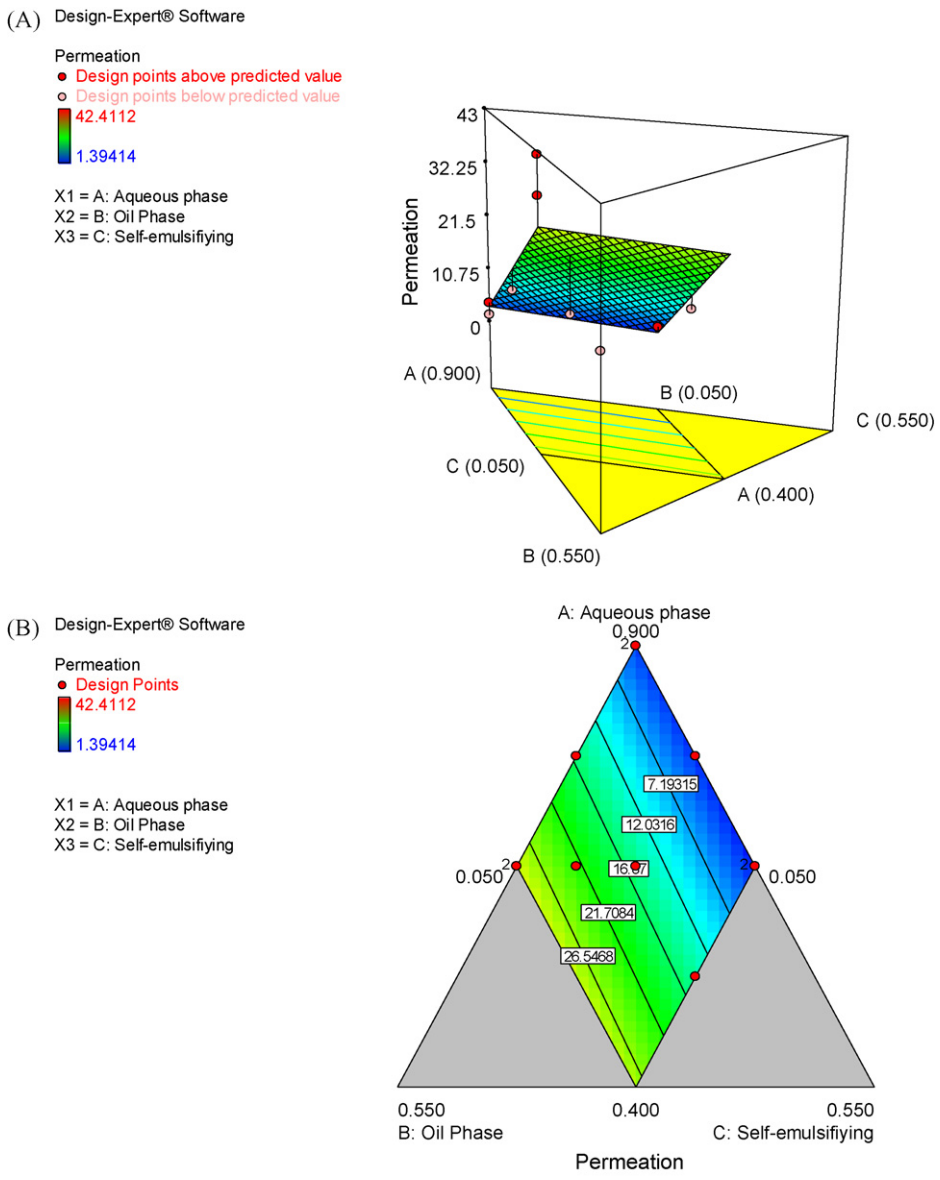

Fig. 4. Ternary response surface (A) and contour (B) plot of skin permeation of 4-NC of emulsions containing *P. umbellata*.

while reduces the number of practical experiments and time of execution.

One important application of Statistical experimental design, also called design of experiments (DOE), refers to the preparation and modification of mixtures. It involves the use of 'mixture designs' for changing mixture composition and exploring how such changes will affect the properties of the mixture (Eriksson et al., 1998). Our optimization was made mainly on basis of the permeation variable, this parameter was critical in our case because we could have discarded formulations that displayed optimal physical properties but exhibited a poor permeation.

In pharmaceutics there are many competing restrictions and the unconstrained problem is almost nonexistent. It is often necessary to trade off properties; thus, in this work the primary objective was not to optimize absolutrly, but to compromise effectively and thereby produce the best formulation under a given set of restrictions (Bohidar and Peace, 1988). Desirability is the main criteria to attend to optimization, the scale used was ranked between 0.00 (minimal) and 1.00 (maximal) (Akhnazarova and Kafarov, 1982), and the 0.60 value was considered satisfactory in the optimization of nanoparticles by Prakobvaitayakit and Nimmannit (2003).

Table 5
Criteria used to select solutions to generate optimal conditions to select the *P. umbellata* emulsion

Name	Goal	Lower limit	Upper limit	Importance
Constraints				
Aqueous phase	In range	0.4	0.9	3
Oil phase	In range	0.05	0.3	3
Self-emulsifying	In range	0.05	0.3	3
Appearance	Target = 4	1	5	1
Centrifuge stability	Maximize	1	5	2
Permeation	Maximize	1.39414	42.4112	5

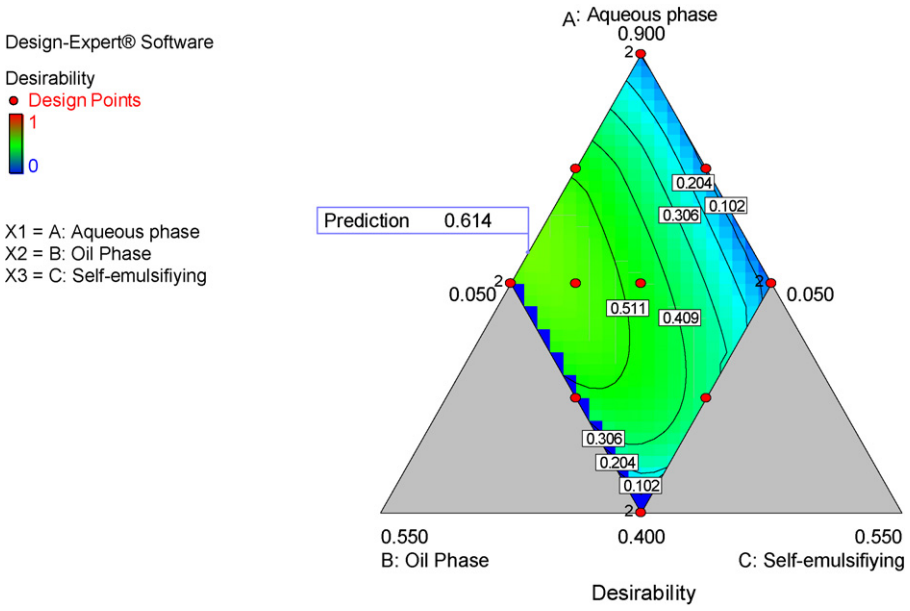

Fig. 5. Desirability plot.

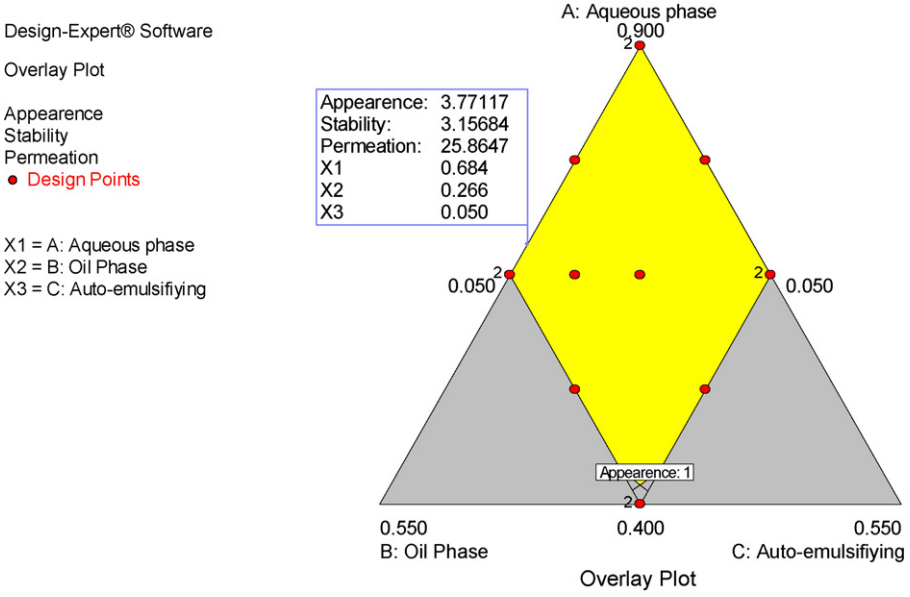

Fig. 6. Overlay plot. Region of interest of the optimized formulations (appearance, centrifuge stability and skin penetration).

Taking in account the criteria of Table 5 to obtain the solutions to generate numerical and graphical optimal conditions, the graph of desirability was plotted for the first solution found via numerical optimization (represented in Fig. 5) and the desirability value corresponded to 0.614. For this reason we chose the formulation (0.684:0.266:0.050) as the optimized formulation, sacrificing partially one characteristic for another. In our case the maximum in vitro permeation given by formulation 10 and 11 (0.65:0.30:0.05) was eclipsed in function of maximum centrifuge stability and best appearance, the overlay plot (Fig. 6) displays intersection point of the optimized formulation. The predicted data of the three responses for the optimized formulation were estimated by the polynomial equations obtained by regression analysis; the calculations results were: $y_1 = 3.77$, $y_2 = 3.16$ and $y_3 = 25.86$.

Nowadays, the application of concepts of pharmaceutical technology for obtaining phytomedicinal products is fundamental to assure the quality of phytopharmaceutical industries. This work is thus a starting point, not an ending point, for an evaluation of factors that may affect phytotherapic formulation using experimental design.

5. Conclusions

The amount of trial and error empiricism in arriving at a formulation condition was reduced, resulting in a quality final

pharmaceutical form, with creamy appearance, high centrifuge stability and high skin permeation. The ternary surface response analysis was especially useful for this work when more than one propriety of interest was present, because it allowed analyzing simultaneously three responses (appearance, centrifuge stability and permeation) while varying simultaneously the proportion of three components of the emulsion.

The graphical procedure was an important tool for understanding the region of interest. By superposition of Figs. 2–4 and the overlay plot (Fig. 6) we propose as optimized formulation: 68.4% aqueous phase, 26.6% oil phase and 5.0% of self-emulsifying phase, this formulation displayed an acceptable compromise between factors and responses investigated.

Acknowledgements

The Ph.D. studentships of Peky Noriega were supported by CONICT (Venezuela). This work was supported by CAPES and FAPESP (Brazil). We would like to thank Prof. Telma Mary Kaneko for the use of Franz's cell devices.

References

Akhnazarova, S., Kafarov, V., 1982. Experiment Optimization in Chemistry and Chemical Engineering. Mir Publisher, Moscow.

Armstrong, A., James, K., 1996. Pharmaceutical Experimental Design and Interpretation. Taylor & Francis, London, pp. 205–223.

Arvouet-Grand, A., Vennat, B., Lejeune, B., Pourrat, A., 1995. Formulation of propolis extracts emulsions. I. O/W creams based on non-ionic surfactans and various consistency agents. Drug Dev. Ind. Pharm. 21, 1907–1915.

Barros, N.B., Scarminio, I., Bruns, R., 1996. Planejamento e Otimização de Experimentos. UNICAMP, Campinas.

Barros, S., Ropke, C.D., Sawada, T.C.H., Silva, V.V., Pereira, S.M.M., Barros, M.B.S., 2005. Assessment of acute and subchronic oral toxicity of extract of *Pothomorphe umbellata* L. Miq. (Pariparoba). Braz. J. Pharm. Sci. 41, 53–61.

Bohidar, N., Peace, K., 1988. Pharmaceutical formulation development. In: Peace, K. (Ed.), Biopharmaceutical Statistic for Drug Development. Marcel Dekker, New York.

Bolton, S., 1991. Pharmaceutical Statistics. Practical and Clinical Applications. Marcel Dekker, New York, pp. 421–452.

Chiang, C.M., Flynn, G.L., Weiner, N.D., Szpunar, G.J., 1989. Bioavailability assessment of topical delivery systems: effect of inter-subject variability on relative *in vitro* deliveries of minoxidil and hydrocortisone from solution and ointment formulations. Int. J. Pharm. 50, 21–26.

Cornell, J.A., 1990. Experiments with Mixtures: Designs, Models and the Analysis of Mixture Data. Wiley, New York.

Eriksson, L., Johansson, E., Wikstrom, C., 1998. Mixture design: design generation, PLS analysis, and model usage. Chemomet. Intell. Lab. Syst. 43, 1–24.

Freitas, P.C.D., 1999. Atividade antioxidante de espécies medicinais da família Piperaceae: *Pothomorphe umbellata* (L.) Miq. e *Piper regnellii* (Miq.) C. DC. Ph.D. Thesis. São Paulo University, Brazil.

Fresno, M.J., Jimenez, M.M., Sellés, E., 1999. Galenic and dermopharmaceutical effectiveness study of an emusifield pharmaceutical form with retinoic acid. Drug Dev. Ind. Pharm. 24, 73–79.

Gustafson, K.R., Cardelina FJ.H. II., Mcahon, J.B., Pannell, L.H., Cragg, G.M., Boyd, M.R., 1992. The peltatols, novel HIV-inhibitory cathecol derivatives from *Pothomorphe peltata*. J. Org. Chem. 57, 2809–2811.

Hir, L.A., 1997. Noções de Farmácia Galênica, sixth ed. Organização Andrei Editora, São Paulo.

Isobe, T., Ohsaki, A., Nagata, K., 2002. Antibacterial constituents against *Helicobacter pylori* of brazilian medicinal plant, Pariparoba. Yakugaku Zasshi 122, 291–294.

Kijjoa, A., Giesbrecht, A.M., Akisue, M.K., Gottlieb, O.R., Gottlieb, H.E., 1980. Nerolidylcatechol from *Potomorphe umbellata*. J. Med. Plant Res. 39, 85–87.

Marti-Mestres, G., Nielloud, F., Marti, R., Maillois, H., 1997. Optimization with experimental design of nonionic, anionic, and amphoteric surfactants in a mixed system. Drug Dev. Ind. Pharm. 23, 993–998.

Martinello, T., Kaneko, T.M., Velasco, M.V.R., Taqueda, M.E.S., Consiglieri, V.O., 2006. Optimization of poorly compactable drug tablets using the mixture experimental design. Int. J. Pharm. 322, 87–95.

Moraes, M., Akisue, S., Oliveira, M.B., Akisue, F.G., 1984. Chromatographic characterization of crude drug and fluid extract of *Pothomorphe umbellata* (L.) Miq. Farm. Química 24, 1–9.

Noriega, P., Ropke, C.D., Camilo, C.M., Freitas, P.C.D., Barros, S.B.M., 2005. Evaluation of extraction conditions of 4-nerolydylchatecol from *Pothomorphe umbellata* (L.) Miq. using factorial design. Braz. J. Pharm. Sci. 41, 261–269.

Osborne, D.W., 1990. The use of phase behavior and laboratory robotics for the optimization of pharmaceutical topical formulations. In: Osborne, D.W., Amann, A.H. (Eds.), Topical Drug Delivery Formulations. Marcel Dekker, New York, pp. 143–195.

Paula, I.C., Ortega, G.G., Bassani, V.L., Petrovick, P.R., 1998. Development of ointment formulations prepared with *Achyrocline satureioides* spray-dried extracts. Drug Dev. Ind. Pharm. 24, 235–241.

Peckolt, W., 1941. Contribuição à matéria médica vegetal do Brasil: estudo farmacognóstico de *Heckeria umbellata* (L.) Kunth. Mem. Inst. Butantan 15, 59–68.

Prakobvaitayakit, M., Nimmannit, U., 2003. Optimization of polylactic-coglycolic acid nanoparticles containing itraconazole usig 2^3 factorial design. AAPS PharmaScienTech 4, 1–9.

Rezende, K.R., Barros, S.B.M., 2004. Quantification of 4-nerolydylchatecol of *Pothomorphe umbellata* (Piperaceae) in rat plasma samples by HPLC UV. Braz. J. Pharm. Sci. 40, 373–380.

Riedel, O.O., 1941. Subsídios para o estudo farmacognóstico da *Heckeria umbellata* (Linné) Kunth. Trib. Farm. 9, 269–283.

Ropke, C.D., Kaneko, T.M., Rodrigues, R.M., Silva, V.V., Barros, S., Sawada, T.C.H., Kato, J.M., Barros, S.B.M., 2002. Evaluation of percutaneous permeation of 4-nerolidylcatechol for four topical formulations. Int. J. Pharm. 249, 109–116.

Ropke, C.D., Rodrigues, R.M., Silva, V.V., Barros, S., Sawada, T.C.H., Barros, S.B.M., 2003a. *Pothomorphe umbellata* extract prevents α-tocopherol from depletion after UV-irradiation. Photochem. Photobiol. 78, 109–116.

Ropke, C.D., Ostrosky, E.A., Kaneko, T.M., Camilo, C.M., Sawada, T.C.H., Barros, S.B.M., 2003b. Validação de metodologias analíticas para determinação quantitativa de α-tocoferol e 4-nerolidilcatecol. Braz. J. Pharm. Sci. 39, 209–217.

Ropke, C.D., Sawada, T.C.H., Silva, V.V., Michalany, N.S., Barros, S.B.M., 2005. Photoprotective effect of *Pothomorphe umbellata* root extract against ultraviolet radiation induced chronic skin damage in the hairless mouse. Clin. Exp. Dermatol. 30, 272–276.

Ropke, C.D., Silva, V.V., Kera, C.Z., Miranda, D.V., Almeida, R.L., Sawada, T.C.H., Barros, S.B.M., 2006. In vitro and in vivo inhibition of skin matrix metalloproteinases by *Pothomorphe umbellata* root extract. Photochem. Photobiol. 82, 439–442.

Schmidt, S.R., Launsby, R.G., 1998. Understanding Industrial Designed Experiments, fourth ed. Air Academy Press, Colorado Springs, pp. 29–30.

Shah, V.P., Behl, C.R., Flynn, G.L., Higucchi, W.I., Schaefer, H., 1992. Principles and criteria in the development and optimization of topical therapeutic products. Int. J. Pharm. 82, 21–28.

Silva, R.A.D., 1926. Farmacopéia dos Estados Unidos do Brasil, first ed. Nacional, São Paulo, p. 649.

Silva, V.V., Ropke, C.D., Almeida, R.L., Miranda, D.V., Kera, C.Z., Rivelli, D.P., Sawada, T.C.H., Barros, S.B.M., 2005. Chemical stability and SPF

determination of *Pothomorphe umbellata* extract gel and photostability of 4-nerolidylcathecol. Int. J. Pharm. 303, 125–131.

Singh, B., Chakkal, S.K., Ahuja, N., 2006. Formulation and optimization of controlled release mucoadhesive tablets of atenolol using response surface methodology. AAPS Pharm. Sci. Technol. 7, E1–E10.

US Pharmacopeia XXVIII, 2005. US Pharmacopeial Convention, Rockville, MD, pp. 2345–2353.

Vennat, B., Grand, A.A., Pourrat, A., 1998. Skin healing preparations: compared *in vitro* diffusion of the active ingredients. Drug Dev. Ind. Pharm. 24, 253–260.