Materials Science & Engineering C 92 (2018) 228–235

Contents lists available at ScienceDirect

Materials Science & Engineering C

journal homepage: www.elsevier.com/locate/msec

# Vasorelaxant effect of standardized extract of *Cecropia glaziovii* Snethl encapsulated in PLGA microparticles: *In vitro* activity, formulation development and release studies

Talitha Caldas dos Santos[a], Mariana Alves Battisti[a], Kiuanne Lino Lobo[b], Thiago Caon[a], Aurea Elizabeth Linder[b], Diva Sonaglio[a], Angela Machado de Campos[a,*]

[a] Laboratório de Farmacotécnica, Departamento de Ciências Farmacêuticas, Centro de Ciências da Saúde, Universidade Federal de Santa Catarina (UFSC), Florianópolis 88040-900, Brazil

[b] Laboratório de Farmacologia, Centro de Ciências Biológicas, Universidade Federal de Santa Catarina (UFSC), Florianópolis 88040-900, Brazil

ARTICLE INFO

*Keywords:*
*Cecropia glaziovii* Snethl
Microparticles
Response surface design
Vasorelaxant activity
Release studies
Osmotic effect

ABSTRACT

In this study, the standardized dry extract of *C. glaziovii* (SDE) provided a significant vasorelaxant effect after contractions induced by phenylephrine in rat aortic rings in an endothelium-dependent manner, confirming that endothelial factors are needed to stimulate this response. A vasorelaxation close to that of acetylcholine was achieved, justifying the development of new formulations for this plant material. In this context, microparticles were selected to encapsulate SDE and the double emulsion technique was considered because of the hydrophilic nature of plant material. Two experimental designs were applied. Firstly, the effect of formulation parameters on particle size, size distribution and encapsulation efficiency (EE) was evaluated. As low EE was achieved, the effect of the osmotic pressure of the external phase was evaluated in a second experimental design. The presence of the osmotic agent (NaCl) impacted positively on the EE and slower *in vitro* release profile was obtained, which is desired in controlled release systems. The formation of denser and less porous particle surface, which was identified by SEM analysis, contributes to explain these findings. Microstructures showed to be a promising delivery system for the *C. glaziovii* SDE considering that a sustained release was achieved.

## 1. Introduction

In folk medicine, the leaves of *Cecropia glaziovii* Snethl are used in the treatment of various diseases, including the control of blood pressure [1]. Some literature studies have reported a hypotensive effect for plant preparations [2, 3], which has been attributed to a synergism between *C*-glycosylflavonoids and proanthocyanidins as these compounds showed to be inactive when tested separately [4]. Preliminary phytochemical studies have reported the presence of catechins, procyanidins and flavonoids in standardized aqueous extracts of *C. glaziovii* [5]. Costa et al. [6], in turn, isolated the main *C*-glycosyl flavonoids in aqueous extracts from the *C. glaziovii* leaves, which were chlorogenic acid, isoorientin, orientin, isovitexin and isoquercitrin.

In preliminary pharmacological studies performed by our research team, aqueous and hydroethanolic crude extracts were prepared by decoction and maceration using 5% (w/v) of *C. glaziovii* dried leaves. The hydroethanolic extract obtained by maceration (20% v/v ethanol) demonstrated a more significant vasodilator activity [7]. The effect of temperature and the presence of preservative on the stability of chemical markers [chlorogenic acid (CGA) and caffeic acid (CFA)] during the extraction process were also evaluated. High concentrations of CGA and CFA were achieved depending on extraction conditions [8]. In this context, *C. glaziovii* crude hydroethanolic extracts were prioritized due to greater vasorelaxant effect and a standardized dry extract (SDE) with a maximum content of both markers CGA and CFA was optimized [9].

Once hydrolysis phenomena were identified in previous stability studies for chemical markers from this plant [8], microparticles were selected in order to overcome this problem. Microparticles have been characterized by providing a higher drug release control compared to nanoparticles [10], which would contribute to extending the pharmacological activity. Moreover, the denser polymer layer in microparticles reduces the water access into the particle [11], contributing to reduce degradative hydrolysis reactions of encapsulated material. In past two decades, poly lactic-*co*-glycolic acid (PLGA) has been among the most attractive polymeric candidates used to fabricate devices for drug delivery particularly due to its high biocompatibility and biodegradability

* Corresponding author at: Laboratório de Farmacotécnica, Departamento de Ciências Farmacêuticas, Universidade Federal de Santa Catarina, Campus Universitário, Trindade, Florianópolis 88040-900, SC, Brazil.
*E-mail address:* angela.campos@ufsc.br (A.M. de Campos).

https://doi.org/10.1016/j.msec.2018.06.046
Received 24 October 2017; Received in revised form 23 May 2018; Accepted 19 June 2018
Available online 21 June 2018
0928-4931/ 

[12] and thus it was selected as the polymer to prepare the microparticles. Particle size, size distribution, encapsulation efficiency and drug release rate from the PLGA microparticles also indicate that this material is promising for drug delivery systems [13]. In the literature, few studies encapsulating herbal extracts in microstructures may be found, which may be associated with matrix complexity.

Based on this fact, a detailed formulation study using a response surface methodology was performed in order to understand as process and formulation variables impact on particle size, size distribution, encapsulation efficiency and drug release properties. Firstly, the vasorelaxant activity of the standardized extract was performed, followed by formulation preparation and physicochemical characterization.

## 2. Materials and methods

### 2.1. Materials

Biodegradable polymer, poly (lactic-*co*-glycolic acid) Resomers® PLGA503 (LA:GA 50:50, i.v.: 0.32–0.44 dL $g^{-1}$) and PLGA750S (LA:GA 75:25, i.v.: 0.8–1.2 dL $g^{-1}$) were purchased from Boehringer Ingelheim (Ingelheim am Rhein, Germany) and stored in a freezer prior to use. An aqueous preparation of the freeze-dried extract of *Cecropia glaziovii* was considered for the assays. Poly(vinyl alcohol) (PVA, Mowiol 40–88, Mw ~ 205,000 g $mol^{-1}$) was purchased from Sigma Aldrich (St. Louis, USA) and used as the dispersant in the external aqueous phase. All chemicals used in the assays were of analytical grade and were used as received without any further purification step.

### 2.2. Preparation of standardized extract

Dried leaves of *C. glaziovii* Snethl (*Urticaceae*), were kindly provided by the Pluridisciplinary Center of Chemical, Biological and Agronomic Studies (CPQBA) of the University of Campinas, SP, Brazil. A specimen voucher was deposited at the CPQBA herbarium (number identification: 78). The optimized extract was obtained by maceration using 18% (w/v) drug material content, 27% (v/v) hydroethanolic solution as the extractor liquid and 3 days of maceration [6]. This liquid extract was concentrated under vacuum at 40 °C (MA-120, Marconi, Brazil) and freeze-dried for 48 h (LD1500, Terroni, Brazil), resulting in the standardized dry extract (SDE).

### 2.3. In vitro vascular reactivity in rat thoracic aorta

#### 2.3.1. Animals

Male Wistar rats (250–300 g) were maintained in a 12 h light/dark cycle with free access to water and standard rat chow. On the day of the assays, rats were euthanized under anesthesia with a mixture of ketamine (80 mg/kg) and xylazine (15 mg/kg) given intraperitoneally and the aorta was removed. All procedures were performed according to local institutional guidelines for the care and use of laboratory animals (authorization code: PP00706).

#### 2.3.2. In vitro measurement of isometric force generation of rat aortic rings

After the removal of fat and connective tissues, aortic rings (3–4 mm in length) were mounted in an isolated organ bath on Grass isometric transducers connected to an ADInstruments PowerLab data acquisition system [14, 15]. The aortic rings were washed with sterile physiological salt solution (130.0 mM NaCl, 4.7 mM KCl, 1.2 mM $KH_2PO_4$, 1.2 mM $MgSO_4$, 1.6 mM $CaCl_2$, 14.9 mM $NaHCO_3$, 0.03 mM EDTA and 5.5 mM glucose), maintained at 37 °C and bubbled with carbogen (5% $CO_2$,95% $O_2$). The aortic rings were gradually stretched to a basal tension of 3.5 g for 10 min and then equilibrated for 1 h with a continuous flushing every 15 min. After this period, each aortic ring was pre-contracted with phenylephrine (1 μM) and thereafter challenged by acetylcholine (1 μM) to test the tissue viability. Some assays were also performed in denuded arteries to study the interference of endothelial factors. The endothelium was mechanically removed by gently rubbing the intimal surface of the rings with a stainless steel wire. Aortic rings that relaxed at least 50% of the phenylephrine-induced contraction after addition of acetylcholine were considered as retaining functional endothelium. On the other hand, rings were considered to be denuded of functional endothelium when the acetylcholine failed to induce any relaxing response.

#### 2.3.3. Vasorelaxant effect of the standardized extract

Endothelium-denuded and endothelium-intact aortic rings were contracted by stimulation with phenylephrine (1 μM) until achieving the plateau of the contraction (approximately 15 min) and then exposed to increasing concentrations of the *C. glaziovii* SDE (0.1 to 100 μg $mL^{-1}$). In another set of experiments, concentration-effect curves for acetylcholine (1 nM to 10 μM) in endothelium-intact and -denuded vessels pre-contracted with phenylephrine (1 μM; positive control) were obtained. The maximal relaxation induced by the SDE was compared to that of acetylcholine. The contractile responses to phenylephrine were expressed as the absolute change in developed tension level (mg) and the relaxation was expressed as a percentage of the maximal contraction induced by phenylephrine. Student's *t*-test was used to compare the maximal relaxation induced by the SDE and acetylcholine and $p < 0.05$ was considered significant.

### 2.4. Microparticles preparation

The SDE-loaded microparticles were prepared by $w_1/o/w_2$ double emulsion technique, followed by solvent evaporation/extraction as previously reported [16], with minor changes. Briefly, SDE was dissolved in 0.5 mL of purified water (internal aqueous phase, $w_1$) and added to 5 mL of dichloromethane (DCM) containing the PLGA (oil phase, o). The water-in-oil ($w_1/o$) emulsion was homogenized for 2 min using a high-performance dispersing instrument (Ultra-Turrax®, T 25 basic, IKA-Werke, Staufen, Germany). This primary emulsion was further emulsified with 75 mL of 0.5% (w/v) PVA solution (external aqueous phase, $w_2$) to obtain the double emulsion ($w_1/o/w_2$).

Two approaches were considered to remove the solvent from the emulsion, the evaporation and the extraction. In the solvent evaporation approach, the formulation was kept under magnetic stirring for 3 h immediately after the preparation (final volume 75 mL). For the solvent elimination by extraction, the emulsion was subsequently diluted with 100 or 200 mL of 0.1% (w/v) PVA solution (extraction phase) and stirred for 30 min at room temperature. Moreover, the effect of osmotic pressure on extract encapsulation was evaluated considering different NaCl concentrations in the external phase. The resulting microparticles were washed three times with distilled water and finally freeze-dried (LD1500, Terroni, São Carlos, Brazil) for 48 h. The final product was stored in a desiccator at room temperature until the experimentation.

### 2.5. Experimental design

Response Surface Methodology (RSM) was used to evaluate the formation and physicochemical characteristics of the microparticles. The experimental results were analyzed by selecting the D-Optimal and IV-Optimal Desing from the Design-Expert® software, version 8.0.6 (StatEase Inc., Minneapolis, MN, USA).

In a first experimental design, four formulation parameters were statistically analyzed, including three numerical variables [amount of polymer (A) - 300, 400 and 500 mg; amount of SDE (B) - 22.2, 33.9, 55.5, 82.1 and 88.8 mg and extraction phase volume (C) - 0, 100 and 200 mL] and one categorical variable at 2 levels: type of polymer, PLGA503 and PLGA750S (D). All factor level combinations were applied in a randomized way, totaling 19 formulations. The D-Optimal Design was considered to evaluate the effect of the factors on three important particle properties: particle size, particle size distribution (span) and encapsulation efficiency (EE); assuming a model with

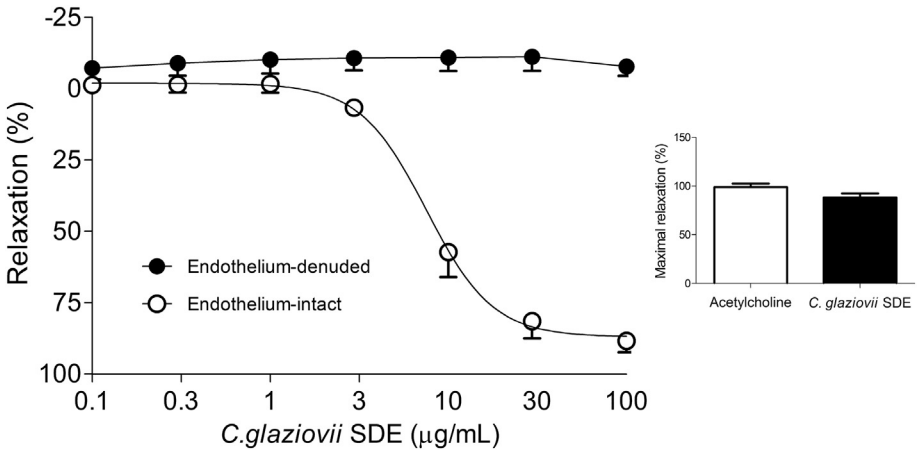


**Fig. 1.** Vasorelaxation induced by *C. glaziovii* SDE. Concentration-effect curves to SDE (0.1 to 100 μg mL$^{-1}$) were performed in endothelium-denuded (●) and –intact (○) rat aortic rings contracted with phenylephrine (1 μmol L$^{-1}$). The points represent the mean percentage ± SEM of relaxation for at least 5 different assays. Inset: Comparison of the maximal relaxation induced by *C. glaziovii* SDE (100 μmol L$^{-1}$; ■) and acetylcholine (10 μmol L$^{-1}$; □). The bars represent the mean percentage ± SEM of the maximal relaxation obtained from at least 5 different assays.

interaction among the factors (2FI) and a probability of error of $p < 0.05$.

In a second experimental design, two numerical factors were considered: stirring speed of the primary emulsion (13,500, 17,500 and 21,500 rpm; E) and concentration of NaCl in the external aqueous phase (1, 2 and 3%, w/v; F). A total of 16 formulations were obtained and the effects were evaluated. The IV-Optimal Design was used to analyze the effect of the factors on the particle size, morphology, EE and loading capacity, assuming a quadratic model and a probability of error of $p < 0.05$.

### 2.6. Physicochemical characterization

#### 2.6.1. Determination of particle size, size distribution and shape

Particle size and particle size distribution were determined by laser diffraction using a Mastersizer 2000 size analyzer (Malvern Instruments, Malvern, UK). The morphology of microparticles was observed by using a scanning electron microscope (SEM, JEOL JSM-6390LV, Peabody, US), where dried microparticles were coated with gold on a sputter coater.

#### 2.6.2. Entrapment efficiency and loading capacity

In a tube glass, exactly 50 mg of microparticles was dissolved in 1 mL of DCM, followed by the addition of 4 mL of ethanol 27% (v/v). After a vigorous agitation (vortex) for 5 min, the sample was centrifuged (2500 rpm, 5 min) and the hydroethanolic solution (supernatant) was quantified. The concentration of phenolic compounds in the supernatant was spectrophotometrically measured at 287 nm (UV-1800; Shimadzu, Kyoto, Japan). A calibration curve was prepared using known SDE concentrations, considering a linear range from 20 to 300 μg mL$^{-1}$ ($y = 0.0029x + 0.0037$; $R^2 = 0.9996$). The results were expressed as the amount of phenolic compounds per unit volume of supernatant. The encapsulation efficiency percentage was calculated using the following equation: EE (%) = (A/B) × 100; where "A" is the amount of phenolic compounds from the microparticles and "B" is the initial amount used to prepare the formulation. The loading capacity for each formulation was expressed as mg phenolic compounds per 100 mg of microparticles.

#### 2.6.3. In vitro release studies

*In vitro* release profiles were performed with the optimized formulations and free SDE (15 mg). The study was performed using a USP II dissolution apparatus (Model 299, Nova Ética, São Paulo, Brazil). The freeze-dried microparticles (500 mg) were suspended in 300 mL phosphate buffer (0.05 M, pH 6.8) and then incubated at 37 °C at 70 rpm. The amounts of free SDE and microparticles added to dissolution medium were defined by considering solubility data, maintaining a sink condition. At pre-determined time intervals, an aliquot of 5 mL was withdrawn for analysis. The medium was replaced by fresh dissolution medium at each interval.

The samples were filtered and the absorbance was measured at 280 nm in a spectrophotometer. Calibration curves with different known concentrations of SDE were prepared. A linear range from 15 to 100 μg mL$^{-1}$ was considered ($y = 0.0073x - 0.0304$; $R^2 = 0.9995$). The results were expressed as the percentage of the released phenolic compounds from triplicate assays.

## 3. Results and discussion

### 3.1. Vasorelaxant activity

Cardiovascular effects have been often reported for some *Cecropia* species such as *Cecropia glaziovii*, *Cecropia hololeuca* and *Cecropia pachystachya* [4]. Although a vasodilator activity for the *C. glaziovii* using endothelium-intact aortic rings has already been reported by our research team [7], endothelium-denuded aortic rings were not considered in this first study. This analysis is relevant to confirm if the relaxation effects depend on the endothelial factors. Unlike the endothelium-intact aortic rings, the treatment with different concentrations of SDE did not affect the vasorelaxation in endothelium-denuded aortic rings (Fig. 1), confirming the need for endothelial factors to observe this phenomenon. Several other agents are very well-known by providing relaxation effects through the release of endothelium-derived relaxing factors. The acetylcholine neurotransmitter, for example, provides the relaxation of the aorta in an endothelium-dependent manner [17] by stimulating the release of endothelial nitric oxide. Assays performed with the *C. lyratiloba* have also demonstrated endothelium-dependent relaxation effects, which would be mediated by nitric oxide [1].

No mechanistic investigations for the *C. glaziovii* were performed in our study. Interestingly, the efficacy of the SDE of *C. glaziovii* as a vasodilator was higher than that of *C. lyratiloba* preparations. While the extract of *C. lyratiloba* induced a maximal relaxation of contracted aortic rings at 500 μg mL$^{-1}$ (approximately 60%) [1], the SDE of *C. glaziovii* achieved a maximal activity at 100 μg mL$^{-1}$ (approximately 93%). This relaxation value of SDE of *C. glaziovii* is statistically similar to that of 1 μM acetylcholine, a compound used as positive control in this assay. Taken together, these findings justify the developing of new formulations containing this standardized extract.

### 3.2. Preliminary formulation screening and experimental design

In the first experimental design, technological factors such as the ability to form particles and the presence of traces of free polymer in the solution were the criteria used to select the experimental domain of each independent variable. The amount of polymer in formulation ranged from 300 to 500 mg. Irregular and non-homogenous

**Table 1**
Particle size, span and encapsulation efficiency of *C. glaziovii* SDE-loaded PLGA microparticles (Design 1).

| Run | Factor | | | | Particle size (μm) | Span | EE (%) |
|---|---|---|---|---|---|---|---|
| | A | B | C | D | | | |
| 1 | 500 | 22.2 | 200 | PLGA503 | 106 | 0.67 | 4 |
| 2 | 500 | 22.2 | 200 | PLGA750S | 188 | 0.73 | 13 |
| 3 | 500 | 22.2 | 0 | PLGA750S | 197 | 0.94 | 10 |
| 4 | 300 | 88.8 | 200 | PLGA503 | 91 | 0.69 | 6 |
| 5 | 500 | 22.2 | 200 | PLGA750S | 220 | 0.79 | 13 |
| 6 | 400 | 82.1 | 100 | PLGA503 | 118 | 0.73 | 3 |
| 7 | 500 | 88.8 | 0 | PLGA750S | 366 | 1.02 | 12 |
| 8 | 300 | 88.8 | 200 | PLGA750S | 142 | 0.88 | 2 |
| 9 | 500 | 88.8 | 200 | PLGA503 | 132 | 0.99 | 8 |
| 10 | 300 | 88.8 | 0 | PLGA750S | 94 | 0.89 | 2 |
| 11 | 500 | 88.8 | 200 | PLGA503 | 190 | 0.73 | 7 |
| 12 | 500 | 88.8 | 0 | PLGA750S | 185 | 0.80 | 4 |
| 13 | 500 | 88.8 | 0 | PLGA503 | 130 | 0.83 | 5 |
| 14 | 300 | 22.2 | 0 | PLGA503 | 94 | 0.67 | 5 |
| 15 | 400 | 33.9 | 100 | PLGA503 | 124 | 0.80 | 5 |
| 16 | 300 | 22.2 | 200 | PLGA750S | 148 | 0.64 | 6 |
| 17 | 300 | 88.8 | 200 | PLGA750S | 126 | 0.98 | 2 |
| 18 | 500 | 22.2 | 0 | PLGA750S | 201 | 0.69 | 12 |
| 19 | 300 | 55.5 | 100 | PLGA750S | 146 | 0.85 | 3 |

nanoparticles were obtained for polymer amounts lower than 300 mg. For polymer amounts higher than 500 mg, highly viscous systems presenting suspended polymer filaments were found after the homogenization by Ultra-Turrax®. The SDE range was defined according to the dry residue (2.45%; w/w) of the liquid standardized extract ($d = 0.9504\ g\ mL^{-1}$) in order to maximize the loading of chemical markers in the microparticles. Based on this information and maintaining a constant volume of 500 μL as the inner aqueous phase ($w_1$), SDE amounts ranged from the 22.2 to 88.8 mg, which correspond 0.5 and 2 mg of the dry residue, respectively. After the formation of the double emulsion, 100 and 200 mL were considered as extraction phase volume aiming to provide a faster solidification of the microparticles. This event would not be observed in a system where the extraction phase was not added.

In this first experimental design, the effect of polymer, amount of extract, extraction phase volume and type of polymer on particle size, size distribution (span) and encapsulation efficiency was investigated (Table 1). As a first step, aleatory samples were analyzed by SEM to confirm the microparticle formation. The presence of porous on particle surface was detected (Fig. 2). During the solidification of the microparticles, water channels connecting the internal and external aqueous phases are usually formed, particularly due to the osmotic effect that causes water influx into the microparticles [13, 18, 19].

The particle size was significantly affected by both polymer amount

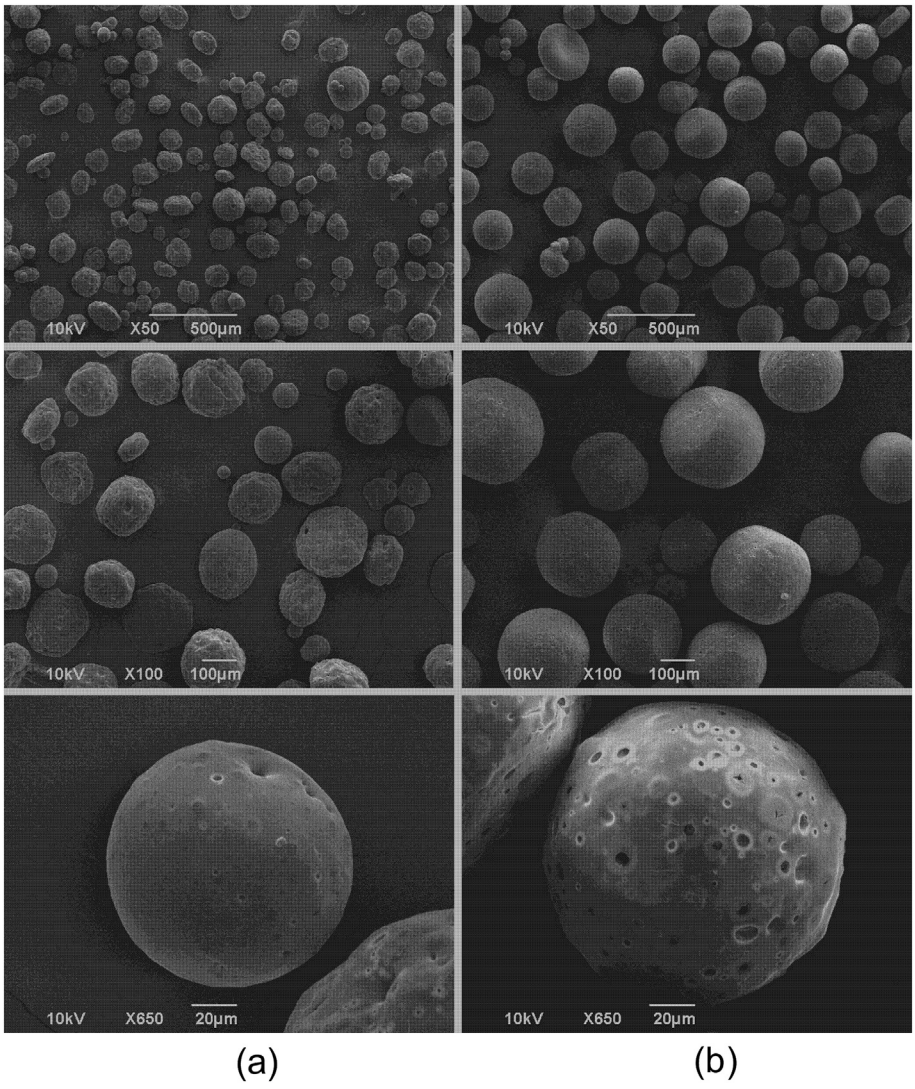


**Fig. 2.** SEM photographs of SDE-loaded PLGA microparticles prepared with PLGA503 (a) and PLGA750S (b).

**Table 2**
Analysis of variance ($p$ values) for the responses in Design 1.

| | Particle size (linear model) | | Span (linear model) | | EE (linear model) | |
|---|---|---|---|---|---|---|
| Polynomial term* | Coefficient | $p$ value | Coefficient | $p$ value | Coefficient | $p$ value |
| M- intercept | 139.54 | 0.0004 | 0.79 | 0.0719 | 5.98 | 0.0052 |
| A- amount of polymer | 29.57 | 0.0002 | 0.023 | 0.3907 | 2.60 | 0.0017 |
| B- amount of SDE | −3.26 | 0.5885 | 0.070 | 0.0145 | −1.18 | 0.0951 |
| C- extraction phase volume | 5.38 | 0.3813 | −0.011 | 0.6866 | 0.40 | 0.5707 |
| D- type of polymer | 24.19 | 0.0008 | 0.047 | 0.0709 | 1.11 | 0.1014 |
| Lack of fit | 0.6480 | | 0.9624 | | 0.4636 | |

* The polynomial model in terms of the coded factors was defined as in Arend et al. [8]; where Y is the measured response associated with each independent variable level combination, M is the mean value, and A, B, C and D are the main variables (or factors). The polynomial model and the effects of the factors were considered significant when $p < 0.05$. Lack of Fit was considered appropriate when $p > 0.05$.

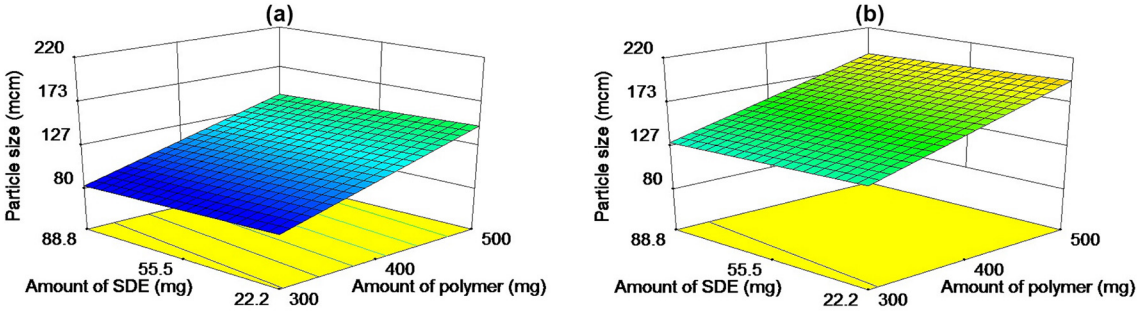


**Fig. 3.** Influence of polymer type and SDE amount on the particle size (extraction phase volume = 100 mL). PLGA 503 (a), PLGA 750 S (b).

**Table 3**
Particle size, span, encapsulation efficiency and loading capacity of *C. glaziovii* SDE-loaded PLGA microparticles (Design 2).

| Run | Factor E rate (rpm) | Factor F NaCl (%) | Size (μm) | Span | EE (%) | Loading capacity (%) |
|---|---|---|---|---|---|---|
| 1 | 21,500 | 3.00 | 126 | 0.67 | 35 | 3.56 |
| 2 | 21,500 | 3.00 | 103 | 0.73 | 30 | 3.36 |
| 3 | 21,500 | 0.00 | 182 | 0.94 | 3 | 0.35 |
| 4 | 17,500 | 2.00 | 73 | 0.69 | 20 | 1.99 |
| 5 | 13,500 | 3.00 | 114 | 0.79 | 40 | 3.96 |
| 6 | 13,500 | 0.00 | 128 | 0.73 | 2 | 0.21 |
| 7 | 13,500 | 3.00 | 106 | 1.01 | 24 | 2.38 |
| 8 | 17,500 | 2.00 | 96 | 0.88 | 21 | 2.06 |
| 9 | 21,500 | 0.00 | 140 | 0.99 | 3 | 0.30 |
| 10 | 13,500 | 3.00 | 110 | 0.89 | 25 | 2.48 |
| 11 | 13,500 | 0.00 | 160 | 0.73 | 2 | 0.23 |
| 12 | 17,500 | 3.00 | 100 | 0.80 | 42 | 4.24 |
| 13 | 17,500 | 0.00 | 173 | 0.83 | 6 | 0.63 |
| 14 | 17,500 | 1.00 | 125 | 0.67 | 11 | 1.09 |
| 15 | 21,500 | 1.00 | 111 | 0.80 | 16 | 1.66 |
| 16 | 13,500 | 1.00 | 129 | 0.64 | 9 | 0.87 |

and type ($p < 0.05$; Table 2; Fig. 3). This result seems to be associated with the viscosity of the primary emulsion, which varies depending on the viscosity and concentration of the polymer [20, 21]. During the preparation of microparticles by w/o/w double emulsion technique, the emulsification of highly viscous solutions is more difficult and leads to the formation of larger size microparticles [22, 23]. In fact, the presence of copolymer PLGA750S in microparticles, which is characterized by a higher inherent viscosity, resulted in a larger particle size.

The span value, an indicator of the particle size distribution, is established from the relation of the values for the particle diameters corresponding to 10, 50 and 90% of the cumulative distribution for each sample [24]. This response was only affected by the amount of SDE (factor B). The span value was around 0.81, indicating a narrow size distribution.

The amount of SDE was the only factor that had a significant impact on the particle size distribution (Table 2).

The EE and loading capacity of the microparticles are dependent on the material type and parameters used during the preparation of the microparticles [21]. An EE range from 2 to 13% was obtained for the tested samples. The factor A (amount of polymer) demonstrated a significant effect on this response, which could be explained by two main reasons: (*i*) polymer precipitates faster on the surface of the dispersed phase and inhibits drug diffusion at higher concentrations [25]; (*ii*) the viscosity of the organic solution increases with the concentration of the polymer, retarding the drug diffusion within the polymer droplets [26–28]. Although no statistical difference in EE values was detected when the type of polymer was changed ($p > 0.05$; Table 2), PLGA750S provided a slight increase in EE when compared to PLGA503. Microparticles characteristics such as EE are not easy to control when the double emulsion method is used [19, 32].

The solvent removal step from the emulsion, for example, is very critical because it takes much time and may cause the reduction in EE and an initial burst release of encapsulated material. During the preparation, microparticles are exposed to considerable amounts of water to remove the solvent. While the solvent is removed from the emulsion, previously encapsulated molecules can diffuse out and also can accumulate on the surface of microparticles, which would explain the high initial burst release [19]. When the solubility of a compound in the external phase is higher than that in the organic phase, the drug will easily diffuse into the external phase [26, 28]. For the *C. glaziovii* preparation, which is rich in hydrophilic compounds, this phenomenon could be observed considering that the external phase is water, explaining the low EE values.

Once low EE values were obtained (Table 1; Design 1), a new experimental design was proposed (Table 3). In this step, the effect of the osmotic pressure of the external aqueous phase on the EE of SDE was investigated. Parameters such as SDE amount (55.5 mg), internal phase volume (0.5 mL), polymer type and amount (PLGA 503/500 mg), volume of dichloromethane in the organic phase (5 mL), external phase volume (75 mL) and extraction phase volume (100 mL) were fixed according to the Design 1 results. The objective of this new experimental design was to reduce the diffusion of SDE to the external phase.

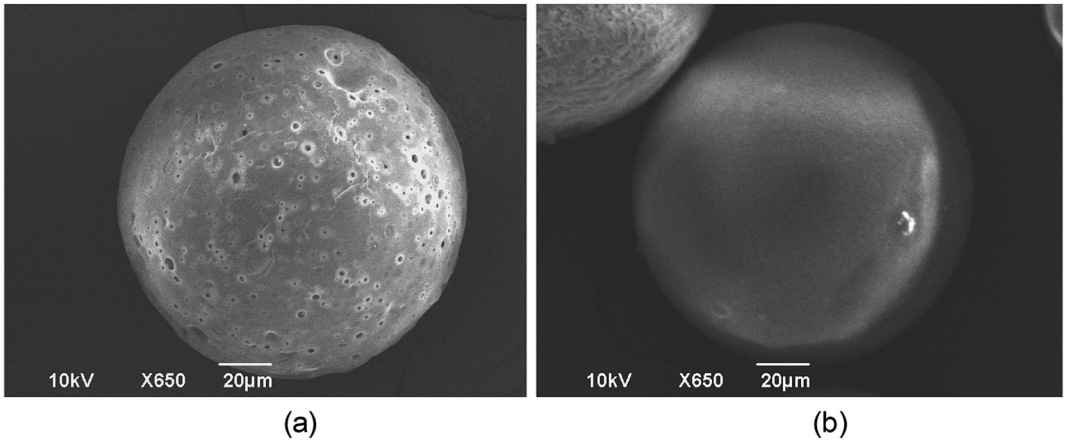


**Fig. 4.** SEM pictures of surfaces of microparticles prepared under different conditions: without NaCl in $w_2$ (a); 3% NaCl in $w_2$ (b).

**Table 4**
Analysis of variance (*p* values) for the responses in Design 2.

| | Particle size (quadratic model) | | Span (2FI model) | | EE (model) | |
|---|---|---|---|---|---|---|
| Polynomial term* | Coefficient | *p* value | Coefficient | *p* value | Coefficient | *p* value |
| M- intercept | 100.74 | 0.0104 | 0.5584 | 0.0129 | 3.84 | < 0.0001 |
| E- stirring speed | 2.37 | 0.6768 | 0.5584 | 0.5956 | 0.25 | 0.1157 |
| F- concentration of NaCl | −24.68 | 0.0010 | 0.5584 | 0.6190 | 1.93 | < 0.0001 |
| EF interaction | −2.34 | 0.7062 | 0.5584 | 0.0016 | – | – |
| $E^2$ | 2.48 | 0.8232 | – | – | – | – |
| $F^2$ | 30.53 | 0.0312 | – | – | – | – |
| Lack of fit | 0.4538 | | 0.5584 | | 0.4474 | |

* The polynomial model in terms of the coded factors was defined as in Arend et al. [8]; where Y is the measured response associated with each independent variable level combination, M is the mean value, E and F are the main variables (or factors), EF is the binary interaction between the factors, and E2 and F2 are the quadratic factors. The polynomial model and the effects of the factors were considered significant when $p < 0.05$. Lack of Fit was considered appropriate when $p > 0.05$.

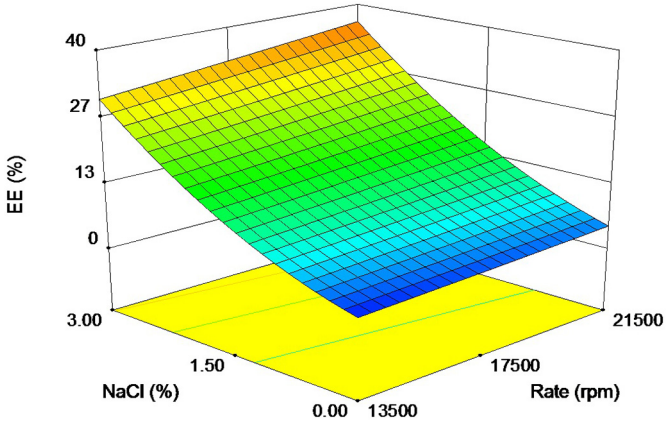


**Fig. 5.** Effect of the primary emulsion stirring speed and concentration of NaCl on the encapsulation efficiency.

**Table 5**
Values for the EE, loading capacity and size of the microparticles prepared with different concentration of NaCl for the release study.

| Sample | NaCl (%) | EE (%) | Loading capacity (%) | Method | Size (μm) |
|---|---|---|---|---|---|
| 1 | 0 | 20.03 | 2.00 | Extraction | 203.84 |
| 2 | 3 | 60.85 | 6.09 | Extraction | 176.75 |
| 3 | 3 | 49.80 | 4.97 | Evaporation | 194.32 |
| 4 | 5 | 49.98 | 5.54 | Extraction | 171.10 |

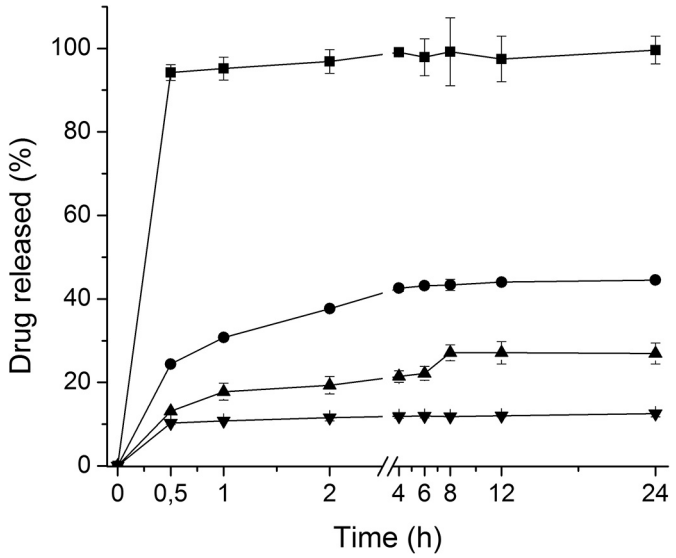


**Fig. 6.** Release profile of *C. glaziovii* SDE from microparticles prepared with different concentrations of NaCl in the external aqueous phase: Formulation 1 (■); Formulation 2 (●); Formulation 3 (▲) and Formulation 4 (▼). The assays were performed in 0.05 M phosphate buffer pH 6.8 at 37 °C. Each point represents the mean ± SEM of the percentage of phenolic compounds content released (n = 3).

The effect of presence and concentration of NaCl (osmotic agent) in the external phase as well as stirring speed on the properties of microparticles such as particle size, span, morphology, EE and loading capacity was evaluated. Regardless of the primary emulsion stirring speed, the particle size decreased with the increase of NaCl concentration (Table 3) in a concentration-dependent manner, which also was confirmed by SEM analyses (Fig. 4). Symmetrical pores were obtained for microparticles prepared without NaCl whereas a dense and homogeneous polymer matrix was observed for these particles after salt addition.

The addition of NaCl significantly decreases the water influx inside the microparticles due to the osmotic pressure gradients between the two aqueous phases during particle formation [18, 19, 30, 31]. Thus, the particle porosity decreased with an increase in the concentration of NaCl in the external phase. The EE and loading capacity were directly related to the increase in NaCl concentration ($p < 0.0001$; Table 4) (Fig. 5). The balancing of the osmotic pressure of the internal and external aqueous phases seems to be the key aspect of improving the EE.

### 3.3. *In vitro* release studies

Considering that the amount of released SDE from microparticles directly impacts on pharmacological activity, *in vitro* release studies were developed with the optimized formulations from the Experimental Design 2. Formulations containing NaCl 3% were characterized by higher EE. Literature reports suggest that high concentration of NaCl may increase the EE by modulating osmotic pressure [18]; however, a slower SDE release would be observed. For this reason, a formulation containing 5% of NaCl was also included in this experimental step in order to confirm this hypothesis. Samples without NaCl were named formulation 1 and used as control (Table 5). For the formulation 1, a non-controlled and rapid SDE release from the microparticles was observed. The porous surface of these microparticles (Fig. 6) leads to rapid diffusion of medium with a complete release of the SDE from the first 15 min.

The impact of solvent elimination (extraction - formulation 2 or evaporation - formulation 3) on the release profile was also considered. The formulation 3, which solvent was removed by evaporation, demonstrated a slower release profile (13 *versus* 24% at 30 min and 27 *versus* 45% at 24 h) and lower EE than formulation 2. Two different phases were identified during the release study of these preparations, a consistent behavior with many matrix-type drug delivery systems. For the latter, a rapid release phase through pores is initially observed, followed by a slow release phase through the polymer matrix [32].

The formulation 4, which was prepared under more drastic saline conditions, displayed the lowest release profile. Changes in surface morphology may explain this behavior. The presence of salt in formulation step resulted in non-porous surfaces, reducing the amount of SDE able to achieve the release medium.

Based on *in vitro* release assay results, the amount of microparticles to be considered for the formulations 3 and 4 should be 1.6 and 4.0-fold higher than those of the Formulation 2, respectively. *In vivo*, an increased release rate of the standardized extract from microparticles is expected because biological constituents contribute to greater polymer degradation. In this context, additional studies considering biorelevant release medium should be considered.

## 4. Conclusion

The SDE of *C. glaziovii* demonstrated a vasorelaxant activity similar to acetylcholine (positive control). The experimental design was a useful tool to optimize the formulation parameters. Although low EE was found for microparticles in Experimental Design 1, the inclusion of an osmotic agent improved this parameter significantly. The double emulsion solvent evaporation/extraction technique combined with an osmotic agent showed to be a promising approach to encapsulate hydrophilic compounds.

The presence of the osmotic agent impacted on particle surface structure, leading to the formation of denser and less porous polymer surface. This finding contributes to extending the release profile of *C. glaziovii* SDE from microparticles, which may result in a long-lasting relaxation effect. *In vivo* additional studies should be performed in order to confirm the real advantages of developed PLGA-microparticles in hypertension treatment (alone and combined with other synthetic drugs).

## Acknowledgements

Talitha Caldas dos Santos thanks the Instituto Nacional de Ciência e Tecnologia para Inovação Farmacêutica (INCT-IF) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for the graduate scholarship. The authors also thank Ílio Montanari Jr. for providing the plant material and Central Laboratory of Electron Microscopy (LCME) for performing the SEM analysis.

## Declaration of interest

The authors report no conflicts of interest.

## References

[1] R. Ramos Almeida, J. Montani Raimundo, R. Rodrigues Oliveira, M.A. Coelho Kaplan, C. Rocah Gattass, R.T. Sudo, G. Zapata-Sudo, Activity of *Cecropia lyratiloba* extract on contractility of cardiac and smooth muscles in Wistar rats, Clin. Exp. Pharmacol. Physiol. 33 (2006) 109–113.
[2] M.T.R. Lima-Landman, A.C.R. Borges, R.M. Cysneiros, T.C.M. De Lima, C. Souccar, A.J. Lapa, Antihypertensive effect of a standardized aqueous extract of *Cecropia glaziovii* Sneth in rats: an *in vivo* approach to the hypotensive mechanism, Phytomedicine 14 (2007) 314–320.
[3] M.F.M.L. Ninahuaman, C. Souccar, A.J. Lapa, M.T.R. Lima-Landman, ACE activity during the hypotension produced by standardized aqueous extract of *Cecropia glaziovii* Sneth: a comparative study to captopril effects in rats, Phytomedicine 14 (2007) 321–327.
[4] G.M. Costa, E.P. Schenkel, F.H. Reginatto, Chemical and pharmacological aspects of the genus *Cecropia*, Nat. Prod. Commun. 6 (2011) 913–920.
[5] M.M. Tanae, M.T.R. Lima-Landman, T.C.M. Lima, C. Souccar, A.J. Lapa, Chemical standardization of the aqueous extract of *Cecropia glaziovii* Sneth endowed with antihypertensive, bronchodilator, antiacid secretion and antidepressant-like activities, Phytomedicine 14 (2007) 309–313.
[6] G.M. Costa, C.F. Ortmann, E.P. Schenkel, F.H. Reginatto, An HPLC-DAD method to quantification of main phenolic compounds from leaves of *Cecropia* species, J. Braz. Chem. Soc. 22 (2011) 1096–1102.
[7] D.P. Arend, T.C. dos Santos, L.H. Cazarolli, M.A. Hort, D. Sonaglio, A.L.G. dos Santos, R.M. Ribeiro-do-Valle, F.R.M.B. Silva, A.M. de Campos, *In vivo* potential hypoglycemic and *in vitro* vasorelaxant effects of *Cecropia glaziovii* standardized extracts, Braz. J. Pharmacogn. 25 (2015) 473–484.
[8] D.P. Arend, T.C. dos Santos, D. Sonaglio, A.L.G. Dos Santos, F.H. Reginatto, A.M. de Campos, Experimental design as a tool to evaluate chlorogenic and caffeic acids extracted from *Cecropia glaziovii* Sneth, J. Pharm. Biomed. Anal. 54 (2011) 58–68.
[9] T.C. dos Santos, Microesferas contendo extrato padronizado de *Cecropia glaziovii* Snethl para o tratamento da hipertensão arterial, Universidade Federal de Santa Catarina, 2012, https://repositorio.ufsc.br/handle/123456789/100788.
[10] R. Singh, J.W. Lillard, Nanoparticle-based targeted drug delivery, Exp. Mol. Pathol. 86 (2009) 215–223.
[11] D. Bennet, S. Kim, Application of Nanotechnology in Drug Delivery, InTech, 2014.
[12] H.K. Makadia, S.J. Siegel, Poly lactic-co-glycolic acid (PLGA) as biodegradable controlled drug delivery carrier, Polymers (Basel). 3 (2011) 1377–1397.
[13] D. Klose, F. Siepmann, K. Elkharraz, S. Krenzlin, J. Siepmann, How porosity and size affect the drug release mechanisms from PLGA-based microparticles, Int. J. Pharm. 314 (2006) 198–206.
[14] A.E. Linder, M. Tumbri, F.F.P. Linder, R.C. Webb, R. Leite, Uridine adenosine tetraphosphate induces contraction and relaxation in rat aorta, Vasc. Pharmacol. 48 (2008) 202–207.
[15] A.E. Linder, R.P. Davis, R. Burnett, S.W. Watts, Function of the serotonin transporter in vasculature of the female rat: comparison with the male, Clin. Exp. Pharmacol. Physiol. 38 (2011) 314–322.
[16] A. Lamprecht, H. Yamamoto, H. Takeuchi, Y. Kawashima, pH-sensitive microsphere delivery increases oral bioavailability of calcitonin, J. Control. Release 98 (2004) 1–9.
[17] R.F. Furchgott, J.V. Zawadzki, The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine, Nature 288 (1980) 373–376.
[18] S. Wang, S. Guo, Disodium norcantharidate-loaded poly(ε-caprolactone) microspheres. II. Modification of morphology and release behavior, Int. J. Pharm. 353 (2008) 15–20.

[19] M. Ye, S. Kim, K. Park, Issues in long-term protein delivery using biodegradable microparticles, J. Control. Release 146 (2010) 241–260.
[20] A. André-Abrant, J.-L. Taverdet, J. Jay, Microencapsulation par évaporation de solvant, Eur. Polym. J. 37 (2001) 955–963.
[21] F.T. Meng, G.H. Ma, W. Qiu, Z.G. Su, W/O/W double emulsion technique using ethyl acetate as organic solvent: effects of its diffusion rate on the characteristics of microparticles, J. Control. Release 91 (2003) 407–416.
[22] M. Li, O. Rouaud, D. Poncelet, Microencapsulation by solvent evaporation: state of the art for process engineering approaches, Int. J. Pharm. 363 (2008) 26–39.
[23] Y. Capan, B.H. Woo, S. Gebrekidan, S. Ahmed, P.P. DeLuca, Influence of formulation parameters on the characteristics of poly (D, L-lactide-co-glycolide) microspheres containing poly (*L*-lysine) complexed plasmid DNA, J. Control. Release 60 (1999) 279–286.
[24] G.S. Domingues, S.S. Guterres, R.C.R. Beck, A.R. Pohlmann, Micropartículas nanorrevestidas contendo um fármaco modelo hidrofóbico: preparação em etapa única e caracterização biofarmacêutica, Quim Nova 31 (2008) 1966–1972.
[25] H. Rafati, A.G.A. Coombes, J. Adler, J. Holland, S.S. Davis, Protein-loaded poly(DL-lactide-co-glycolide) microparticles for oral administration: formulation, structural and release characteristics, J. Control. Release 43 (1997) 89–102.
[26] Y. Yeo, K. Park, Control of encapsulation efficiency and initial burst in polymeric microparticle systems, Arch. Pharm. Res. 27 (2004) 1–12.
[27] R. Ghaderi, C. Sturesson, J. Carlfors, Effect of preparative parameters on the characteristics of poly (*D*,*L*-lactide-co-glycolide) microspheres made by the double emulsion method, Int. J. Pharm. 141 (1996) 205–216.
[28] R. Bodmeier, J.W. McGinity, Solvent selection in the preparation of poly(dl-lactide) microspheres prepared by the solvent evaporation method, Int. J. Pharm. 43 (1988) 179–186.
[30] J.T. He, H.B. Su, G.P. Li, X.M. Tao, W. Mo, H.Y. Song, Stabilization and encapsulation of a staphylokinase variant (K35R) into poly(lactic-co-glycolic acid) microspheres, Int. J. Pharm. 309 (2006) 101–108.
[31] K. Han, K.D. Lee, Z.G. Gao, J.S. Park, Preparation and evaluation of poly(L-lactic acid) microspheres containing rhEGF for chronic gastric ulcer healing, J. Control. Release 75 (2001) 259–269.
[32] P.B. O'Donnell, J.W. McGinity, Preparation of caprolactone microcapsules by the solvent evaporation technique, Adv. Drug Deliv. Rev. 28 (1997) 25–42.

**Talitha Caldas dos Santos**: PhD in Pharmacy at the Federal University of Santa Catarina. Her research interests focus on the standardization of herbal extracts, with emphasis on the development of drug delivery systems, polymeric micro- and nanoparticles, hydrogels and thin films.

**Mariana Alves Battisti**: PhD student in Pharmacy at the Federal University of Santa Catarina. Her research interests focus on the standardization of herbal extracts, with emphasis on the development of drug delivery systems, lipid nanocarrier for topical and buccal application.

**Kiuanne Lino Lobo**: Master in Pharmacology at the Federal University of Santa Catarina. Her expertise basically involves the in vitro/in vivo evaluation of vascular relaxing effect of different materials.

**Thiago Caon**: Professor and Researcher at Department of Pharmaceutical Sciences of Federal University of Santa Catarina. Expert in biopharmaceutical field, focusing on permeability studies in skin, buccal and intestinal mucosa.

**Aurea Elizabeth Linder**: Professor and Researcher at Pharmacology Department of Federal University of Santa Catarina with emphasis in cardiovascular pharmacology, especially the comprehension of smooth muscle contraction and relaxation mechanisms.

**Diva Sonaglio**: Professor and Researcher at Pharmaceutical Sciences Department of Federal University of Santa Catarina. Expert in statistical techniques targeted to experimental design.

**Angela Machado de Campos**: Professor and Researcher at Pharmaceutical Sciences Department of Federal University of Santa Catarina with emphasis in drug delivery systems, nano- and microstructured systems for the topical and ocular route, and standardization of herbal extracts.