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Hydroalcoholic extracts of *Vellozia squamata*: study of its nanoemulsions for pharmaceutical or cosmetic applications

Frederico J. O. Quintão, Renata S. N. Tavares, Sidney A. Vieira-Filho, Gustavo H. B. Souza, Orlando D. H. Santos*

Departamento de Farmácia, Universidade Federal de Ouro Preto, Brazil.

Abstract: Some species of plants are notable for the wide range of biologically active constituents in their tissues. Chemical and pharmacological studies of *Vellozia squamata* Pohl, Velloziaceae, popularly known in Brasil as "canela-de-ema" are scarce, but showed the presence of di-and triterpenoid that may be of scientific interest. In the present study the hydroalcoholic extracts from leafs and stems of *V. squamata* were submitted to phytochemical prospection to identify the principal groups of constituents, and the antioxidant activity was determined by DPPH method. The hydroethanolic extracts presented higher antioxidant activity. Thus, nanoemulsion formulations were prepared using the method of phase inversion. Accelerated stability tests, such as heat stress and centrifugation were made, and physical and chemical properties of the nanoemulsions were established. Stable formulations were obtained from both extracts from leafs and stems. By the results was possible to establish the potential application of hydroalcoholic extracts from *V. squamata* in development of products with antioxidant properties and demonstrate a promising pharmaceutical product.

Article

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Introduction

The high concentration of reactive species of oxygen can lead to damage in nucleic acids, proteins and other cellular structures leading to a huge variety of diseases such as atherosclerosis and immune dysfunction (Su-Ying et al., 2007). The presence of antioxidant compounds can minimize the undesirable effects of reactive species of oxygen. Antioxidants have being defined as substances that undergo oxidation prior to another, the first of which would be important to stay in the state of natural oxidation, and this occurs by different mechanisms (Souza et al., 2007). Nowadays it is of great interest to search for new antioxidant compounds mainly from natural origin. Generally, polyphenols and flavonoids have being associated to antioxidant properties observed in plant extracts. Nanoformulations containing plant extracts represent an alternative for its use topically.

Velloziaceae is a small family of approximately 250 species of fibrous shrubby plants, which grow mainly in edaphically dry localities of South and Central America, Africa and Madagascar (Garcia & Diniz, 2003; Jacobi & Del Sarto, 2007). Individuals are typical elements of tropical rock hilltops, and bear resistance properties such as quiescence, drought enduring leafs (sclerophylly), and adventitious roots with multi-layered velamen which allow them to quickly absorb any available water from rain or mist, and are also adapted to fire. The family has many representatives in the 'campo rupestre' vegetation (high-altitude rocky grasslands) of Brazil (Jacobi & Del Sarto, 2007).

The flavonoids 3,5,7,3′,4′-pentahydroxy-6-prenylflavonol and 3,5,7,3′,4′-pentahydroxy-8-methyl-6-prenylflavonol were isolated from the ethyl acetate extract of sheaths of *Vellozia kolbekii Alves* (Velloziaceae) (Silva et al., 2012).

Vellozia squamata Pohl, Velloziaceae, is popularly known in Brazil as "canela-de-ema", and is used in traditional medicine as an anti-inflammatory. The popular use of "canela-de-ema" is mainly through the infusion from its leafs (Almeida et al., 1998; Soares & Garcia, 2007).

The development of nanoformulations involving emulsified systems is an alternative for the use of drug via better administration routes. Nanoemulsions are basically constituted by a small size structure (20 to 500 nm) and its transparence is dependent on the size particle [translucent <~200 nm > milky] (Friberg et al., 1988; Forgiarini et al., 2000). The use of nanoemulsions is interesting because of their properties that are closely associated with the droplet size. These bring advantages when nanoemulsions are used as vehicle of pharmaceutical products or

constituents of cosmetics, mainly those for skin cares. Nanoemulsions systems are basically produced by two principal processes: 1) the spontaneous emulsification method based on temperature of inversion phase (TIF) or by reverse-phase composition (RPC) (Tadros et al., 2004); and 2) by using a mixer of high shearing power (Fernandez et al., 2004). This process allows better control of the droplet size, as well as become possible the choice of a greater number of components (Oliveira et al., 2011a).

The objectives of this work were the preparation and characterization of the hydroalcoholic extracts of the stem and leafs of *V. squamata*, and further development of nanoemulsions to be used in pharmaceutical or cosmetic formulations.

Material and Methods

Plant material

Leafs and stems of the *Vellozia squamata* Pohl, Velloziaceae, were collected in "Serra de Ouro Branco" located at Ouro Branco-MG, Brazil (20°31'2"S, 43°42'0" W). The plant material was identified by the botanist Dra. Maria C. T. B. Messias and a voucher specimen (OUPR-4145) was deposited in the *Herbarium* Prof. José Badini, Universidade Federal de Ouro Preto, MG, Brazil. The leafs and stems were separated and dried in an oven at 45 °C and under air circulation. Then, each part was fragmented in a knife mill to obtain a fine powder.

Hydroalcoholic extracts preparation

The powder of dried leafs (100 g) and stems (100 g) of *V. squamata* were respectively submitted to exhaustive extraction with ethanol-water solution (70:30 v/v) in a stainless steel percolator, at room temperature. After 48 h, the solutions were filtered and the solvent recovered in a rotatory evaporator at 40 °C, under vacuum. The solvent proportionality was adjusted in an Abbe refractometer and reused in the percolation process until complete extraction. By these processes were obtained the hydroalcoholic extract of leafs (9.1 g; 9.1%) and stems (7.3 g; 7.3%).

Pharmacognostic study

The pharmacognostic analysis of the hydroethanolic extracts were performed according to the methodology suggested by Wagner & Bladt (1996) for detection of fatty acids, anthraquinones, alkaloids, coumarins, flavonoids and terpenoids through thin layer chromatography (TLC). Specific chemical compounds were identified through methodology described by Matos (1988), through which are analyzed the presence of phenols, tannins, triterpenes, flavonoids, saponins, xantones, coumarins and alkaloids.

Initially, the extracts were fractionated by liquid/liquid partition with chloroform to separate the lipophilic compounds. The presence of secondary metabolites of the classes listed above was determined in water-alcohol fraction using the following reactions: 1) the presence of phenols and tannins was determined by reaction with ferric chloride ($FeCl_3$) at 2 % (w/v); 2) the presence of flavonoids was investigated by Shinoda reaction and was observed by comparative thin layer chromatography; 3) to analyze the presence of xanthone was used magnesium in hydrochloridric acid; 4) index of foam was used to indicate the presence of saponins; 5) the Liebermann-Bouchard reaction was used to investigate the presence of triterpens and steroids; and 6) after acid-base extraction, the presence of alkaloids was determined by general reactions of precipitation.

Antioxidant activity

The hydroethanolic extracts from leafs and stems of *V. squamata* were respectively evaluated for their antioxidant activity using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method. The assay is based on discoloration of DPPH free radical after phenols addition, assessing their ability to transfer H atoms/electrons to radicals - a likely mechanism of antioxidant protection (Molyneux, 2004; Klen & Vodopivec, 2012). Ethanol solutions of each extract were prepared at different concentrations (10.0, 25.0, 75.0, 150.0, 300.0 and 400.0 µg/mL) to a final volume of 2.5 mL and to each one was added 1.0 mL of 0.004 % (w/v) ethanol solution of DPPH•. After 30 and 60 min of reaction at room temperature (25±2 ºC) the absorbance was measured at 518 nm in a spectrophotometer (Thermo Spectronic, model Helios α). Solutions of each extract (2.5 mL) in ethanol (1.0 mL) were respectively used as blank. DPPH• 0.004 % (w/v) solution (1.0 mL) with ethanol (2.5 mL) was used as negative control. All samples were kept protected from light until reading. The procedure was adapted from methodology suggested by Rosa and co-workers (2010). The antioxidant activity assay was also realized with formulations containing the extracts and dissolved in ethanol to reach concentration curve similar to those obtained using correspondent extract. In accordance to Molineux (2004), the decrease in absorbance (ABS) of the solutions was measured and calculated using the formula:

$$\% I = (ABScontrol-ABSsample/ABScontrol) \times 100 \quad \text{(Eq. 1)}.$$

Total phenolic content

The phenolic compounds were determined using the Folin-Ciocalteu method, based on the reduction of phosphor-wolframate-phosphomolybdate complex by phenolics to a blue reaction. From the extracts solution (15.0 mg/mL), 67 µL were added to 3 mL of water and 250 µL Folin-Ciocalteu reagent. The mixture was stirred for 1 min, and 1 mL of saturated sodium carbonate (15 g/100 mL) was added. The final volume was measured to 5.0 mL and the solution was homogenized for 1 min. After 2 h, the absorbance was measured at 750 nm. The data were calculated by comparison between a standard curve (10 to 350 µg gallic acid/mL) (Figure 1) with the absorbance of each samples. The data were expressed as µg gallic acid equivalents (GAE) per gram of dry extract.

Preparation and analysis of nanoemulsions

The nanoemulsions of each extract were prepared by the temperature of inversion phase (TIF) method, where the oil phase with the surfactants was heated up to 80±2 °C. The water phase was heated in similar temperature conditions and was included into the oil phase, keeping the agitation speed at 80 g until complete cooling (30 min at 25 ºC) using a Fisaton agitator model 713R (Oliveira et al., 2011b). Then, each nanoemulsion was submitted to centrifugation at 2012 g for 30 minutes. After centrifugation, the nanoemulsions were macro and microscopically analyzed to determine the most stable one, in accordance with Oliveira and co-workers (2011b).

Composition of the nanoemulsions:

Components	Porcentage (w/w)
Hydroalcoholic extract	1.0
Babaçu oil	10.0
Sorbitan monoestearate	4.0
PEG-40 hydrogenated castor oil	6.0
Distilled water	79.0

Determination of nanoparticle size

The particle size distribution was determined on N5 Submicron Particle Size Analyzer (Beckman Coulter). To estimate the particle size at room temperature, 20.0 µL of the nanoemulsion were diluted in 4980.0 µL of ultrapure water (Milli-Q Millipore). The incidence angle of the laser in sample was 90°. The median and standard deviation analysis was realized in triplicate (Oliveira et al., 2011a).

Determination of pH

The measurement of pH was done through pHmeter Lutron® model PH-221. The pH value was determined in triplicate (Santos et al., 2005).

Accelerated stability assays

After 24 h of their manipulation, the stable nanoemulsions were subjected to preliminary assays to determine its stability through centrifugation and thermal stress processes (Ferrari & Rocha Filho, 2011).

Centrifugation process

An amount of nanoemulsion (3.0 mL) was inserted into the centrifugal tubes and subjected to 1398 *g*, during 1 h, using the Excelsa Baby Fanem® II Centrifuge. Then, the material was analyzed macroscopically and submitted to a new centrifugation process for creaming and separation of phases. The process was realized in triplicate. To further analysis were considered the pre-stable formulations that did not show phase separation or creaming after the assay.

Thermal stress

On water bath (WB Thermomix Braun 18 BU. Biotech International, Germany), the emulsions were gradually heated from 40 to 80 °C and the temperature rise of 5 by 5 °C, keeping each temperature for 30 min. The emulsions were macroscopically analyzed to observe the occurrence of creaming and separation of phases after each temperature increase

Rheological analyses

The rheological behavior of each formulation was performed on Brookfield Rheometer model RVDV-III cone and plate, connected to a Rheocalc Software version V 3.0, using the spindle CP 40 and with sample (0.50 g) at 25 °C. Measurements were made using a rotational speed of 250 rpm, with a variation in the range of 50 to 50 rpm, to obtain an upward curve. The downward curve was obtained decreasing the rotations from 250 to 50 rpm (Santos et al., 2011). The rheological behavior of the formulations was evaluated using the power law (Guarantini et al., 2006):

$$\tau = \kappa \cdot \gamma^n \qquad \text{(Eq. 2)}$$

Where τ: Shear stress; κ: consistency index; γ: shear rate and n: flow rate.

Results and Discussions

Pharmacognostical screening

By the chromatoplates obtained using specific reagents was possible to detect the main chemical classes of constituents present in the hydrophilic

extracts of the leafs and stems. In hydrophilic extracts of the stems were detected: anthraquinones, coumarins and flavonoids while fatty acids, alkaloids and terpenoids were not detected. All results for detection of the main chemical classes in the hydrophilic extracts of the leafs were negative. After that, confirmation of the chemicals constituents was made by the change of color, precipitation or formation of stable foam demonstrated the predominance of certain classes of organic compounds in the hydroalcoholic extracts from leafs and stems of *V. squamata*. In hydrophilic extracts of leafs were detected with strong presence steroids, flavonoids, flavanones, flavanonoids, saponins and xanthones. Catechins were detected with a in low concentrations. Aurones, catechin tannins, pirogalic tannins and triterpenoids were not detected. In hydrophilic extracts of the stems Aurones, catechin, catechin tannins, pirogalic tannins were detected. By the results was possible to consider triterpenoids as been the main constituents of this extract.

In vitro *antioxidant activity of extracts and formulations*

The use of the stable free radical DPPH was herein considered as a quick way to estimate the *in vitro* antioxidant activity of the hydroalcoholic extracts from *V. squamata* and its respective nanoemulsions. The hydroalcoholic extract obtained from leafs and stems of *V. squamata* were evaluated for their ability to scavenge DPPH radical, observed by the absorbance decrease of the solution. The results for hydroalcoholic extract and nanoemulsions, prepared with it, revealed the antioxidant property of the *V. squamata* leaves (Figure 1 and 2). Below the median inhibition concentration (IC50) was observed that the radical inhibition properties are dependent on the concentration of the extracts (Figure 1 and 2, at left).

Djeridane et al. (2006) have demonstrated a linear correlation between the content of total phenolic compounds and their antioxidant properties. This may be related to the polyphenols which have been identified by phytochemicals tests in the leaves and stems of *V. squamata*. In accordance to Wojdyło et al. (2007) phenolic acids represent the main class of phenolic compounds that are widely distributed in the plant kingdom.

Total phenolic content

The total phenolic content of hydroalcoholic extracts from *V. squamata* were in range of 26.52 μg

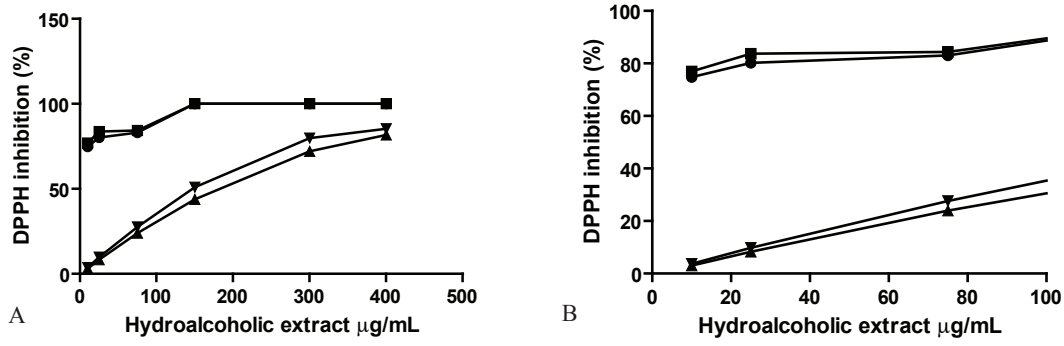

Figure 1. A. Percentage of inhibition of DPPH induced by hydroalcoholic extracts from stems and nanoemulsion prepared with it [▲ inhibition stem ● inhibition formulatiom stem after 30 min ▼ inhibition stem ■ inhibition formulatiom stem after 60 min. B. At left expansion of the inhibition observed.

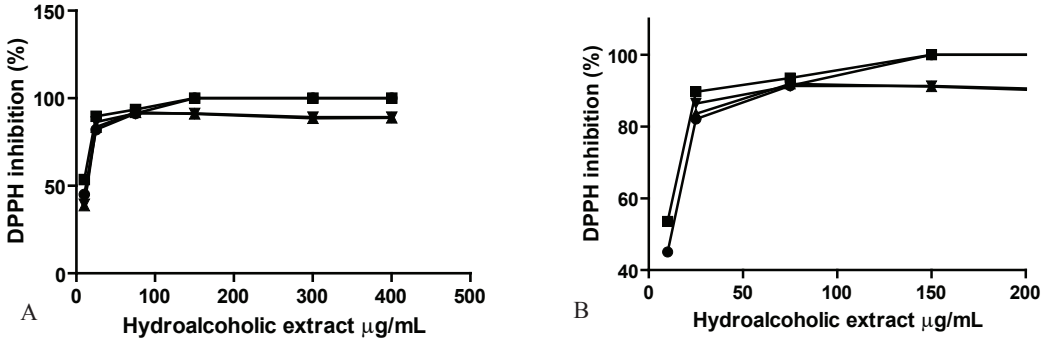

Figure 2. A, Percentage of inhibition of DPPH reagent of the hydroalcoholic extracts of the leafs and nanoemulsion prepared with it. it [▲ inhibition leaf ● inhibition formulatiom leaf after 30 min ▼ inhibition leaf ■ inhibition formulatiom leaf after 60 min. B. At left expansion of the inhibition observed.

GAE/g (leaf extract) to 48.86 µg GAE (stem extract)/g dry weight basis (Table 1). Phenolic compounds are used by plants as defense mechanisms to counteract reactive oxygen species (ROS) in order to survive and prevent molecular damage and also to hinder he action of microorganisms, insects, and herbivores (Vaya et al., 1997). Results show a positive correlation coefficient between the total phenolic content and DPPH assay of plants extracts. In this study, it seemed that, the higher total phenolic content of plants extracts resulted in higher antioxidant activity as similarly reported by Cai and co-workers (2004).

Table 1. Total phenolic content expressed as µg gallic acid equivalents (GAE) per gram of dried hydroethanolic extracts from steams and leafs of *Vellozia squamata*.

Hydroethanolic extract	ABS	µg GAE/g dry extract
Stem extract	2.6109	48.85912
Leaf extract	1.4188	26.52679

Determination of pH

The determination of pH values is of great importance in topical formulations, as they must conform to the natural pH of the skin. The formulations showed a slightly acid pH, the pH compatible with the skin that has values from 4.1 to 5.8 (Segger et al., 2008). There were changes in values after one month, but still within the expected range (Table 2).

Table 2. Determination of pH values of nanoemulsion formulations with stems and leafs hydroethanolic extracts from *Vellozia squamata*.

Nanoemulsion formulation with hydroethanolic extract	pH value of extract	
	Initial	After 30 days
Stems	5.12±0.01	5.18±0.01
Leafs	4.95±0.02	5.13±0.02

Determination of the rheological behavior of the formulations

The rheological behavior of the formulations was evaluated using the Power law given by the formula cited above. Thus after measuring the rheological behavior of the formulations, it was observed that both formulations containing leaves and stems extracts exhibit a Newtonian behavior, *i.e.*, have an ideal viscous behavior (Table 3).

It was shown that the relationship between shear stress and the shear rate is linear. This means that for a given temperature, the viscosity remained constant during its measurement, regardless of time and the shear employed (Corrêa et al., 2005).

Table 3. Parameters of rheological behavior of the nanoemulsion formulations with hydroethanolic extracts from stems and leafs of *Vellozia squamata*.

Nanoemulsion Formulation with↓	Flow index	Consistence index (cP)	Apparent viscosity (cP)
Stems extract	1.00	2.39	99.70
Leafs extract	1.00	2.39	99.70

cP: centi Poise.

Determination of droplets size

The particle size is an important parameter to evaluate nanoemulsified systems due to the fact that the particle size is the main factor responsible for the permeation enhancing effect of active components in the skin layers. In addition the particle size, it is essential to know the polydispersity index, since these factors together influence the stability of these systems. The distribution of the particle size in the nanoemulsion obtained with the hydroalcholic extracts from *V. squamata* leaves and stems were measured for seven days (Table 4). Both nanoemulsions presented small droplet size and the microstructure have not undergone significant changes over the seven days

Table 4. Distribution of particles size from nanoemulsions made with stems and leafs hydroethanolic extracts from *Vellozia squamata* in function of time after preparation.

Period of nanoemulsion Formulation	Particle size of nanoemulsion of hydroethanolic extract			
	Leaf		Stems	
	Size (nm)	P.I.	Size (nm)	P.I.
0 h	154.6±9.59	0.284±0.034	147.6±33.32	0.351±0.254
24 h	138.4±1.69	0.154±0.037	132.0±4.94	0.275±0.055
48 h	154.2±2.43	0.361±0.254	167.7±7.51	0.528±0.105
72 h	155.0±4.41	0.289±0.073	180.2±23.48	0.733±0.196
6 d	144.4±1.06	0.239±0.017	144.4±1.06	0.239±0.017
7 d	144.5±4.02	0.310±0.022	157.4±6.90	0.490±0.128

P.I.: Polydispersity index

analyzed, showing that the formulations are highly stable. Evaluation of polydispersity index shows that both nanoemulsions were homogeneous and adequate for topical application.

Conclusion

Evaluation of the antioxidant activity by DPPH showed a significant inhibition of the DPPH• by both hydroalcoholic extracts from leafs and stems. This activity is related to molecules with phenolic groups able to neutralize free radicals as flavonoids. flavanones and flavanonols which were detected by pharmacognostical screening and As can be seen ambos hydroalcoholic extracts presented in dosage test phenolic a high concentration of such compounds.

The phase inversion method was efficient in the preparation of stable nanoemulsions that are adequate for topical application of these extracts. Even in the formulation form, the antioxidant activity of the extracts remained the same as found in their solution, showing the vehicle do not influence on this action. The nanoemulsions formulations using hydroalcoholic extracts from leafs and stems have shown promise in the development of phytomedicines.

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***Correpondence**

Orlando D. H. Santos
Departamento de Farmácia, Universidade Federal de Ouro Preto
Rua Costa Sena, 171, 35400-000 Ouro Preto-MG, Brazil
orlando@ef.ufop.br
Tel: +55 31 3559 1038
Fax +55 31 3559 1628