

Stability Evaluation of Propolis Topical Bases for Veterinary Use

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ABSTRACT

This study aimed to evaluate and select different dermatological bases incorporated with propolis for veterinary use as well as to analyze the chemical compounds of the propolis hydroalcoholic extract by LC-MS/MS. Thus, formulations were submitted to accelerated stability tests under different temperatures and to mechanical stress, and evaluated for the appearance, color, odor, pH, viscosity, spreadability, and the mean size of the dispersed globules from the internal phase during a period of three months. The creamy gel formulation showed satisfactory results for all the evaluated items with an excellent capability to incorporate the hydroalcoholic extract of propolis associated to the maintenance of its physicochemical properties. The propolis used in this study had been shown to possess antibacterial and antifungal in vitro activity against the main microorganisms responsible for such diseases. Therefore, the propolis creamy gel described here could be a promising formulation for use in the veterinary medicine.

Key words: Propolis, accelerated stability, phytotherapeutic, caffeic acid

INTRODUCTION

The investigation of natural products with antimicrobial activity is stimulated by the high level of bacterial resistance nowadays (Rossolini and Mantengoli 2008) and the side effects caused by the prolonged use of antibiotics (Cunha 2001). In veterinary medicine, a number of diseases related to bacterial infections show high degree of antimicrobial resistance (Pedersen et al. 2007), like external otitis and dermatitis in dogs (Brothers et al. 2002). When it became chronic, the animals are subject to side effects due to long periods of antimicrobial drugs use (Dowling 1996).

Propolis is a natural substance produced by the bees. It is relatively non-toxic and could be an alternative therapy in the treatment of infections (Jasprica et al. 2007). Its antibacterial activity is due to the presence of flavonoids, aromatic acids and esters in its composition. Moreover, the propolis aids in the healing of ulcers, and has immunostimulant, hypotensive and cytostatic activities (Bankova et al. 1995).

Considering the requirement for a non-toxic transdermal drug suitable for veterinary applications (Davidson 2003) and the fact that the propolis possessed antibacterial and antifungal activity on isolates of canine otitis (Cardoso et al.

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2010), the aim of this work was to select and evaluate the dermatological formulations suitable for incorporation of an hydroalcoholic propolis extract at minimum bactericidal and fungicidal concentration, destined to the veterinary use using accelerated stability tests. The study also aimed to analyze the presence of caffeic and rosmarinic acids in the propolis extract using liquid chromatography.

MATERIALS AND METHODS

The propolis used in this study was obtained from the municipality of São Martinho da Serra (latitude: 29° 32' 16" S, longitude: 53° 51' 17" O), located in the central region of Rio Grande do Sul, Brazil. Briefly, the propolis was cold-macerated to make an extract with ethanol 70% (brute propolis 300 g/ethyl alcohol 700 mL), resulting in a 300 µg⁻¹ mL concentration. The solution was stored at room temperature during 45 days and protected from the light. After this period, the supernatant was removed using a siphon.

Caffeic acid and rosmarinic acid, in the propolis extract, were analyzed by the HP 1100 series liquid chromatograph (Agilent®), with detection by mass spectrometry API 5000 (Applied Biosystems®). The mobile phases used were water (A) and an organic solution of methanol:water (9:1 v/v) (B), both containing 0.5% of ammonium acetate. The gradient used was 0.0 – 1.0 min 50% of A, 1.0 – 6.0 min 10% of A, returning to the initial condition up to 12 min. The flow rate was 0.5 mL min⁻¹ and the injected volume was 10 µL. The compounds were separated using a column C18 (4.6 x 150mm ID) Xbridge®, maintained at 25°C. The conditions for the mass spectrometer were optimized using the standards of caffeic acid (molecular weight = 180.1 Da) and rosmarinic acid (molecular weight = 360.1 Da) by flow injection analysis to achieve the maximum sensitivity. The optimized parameters were as below: ion spray voltage = -4500 (v), collision gas = 10, curtain gas = 18, ion source gas 1 = 55, ion source gas 2 = 50 and source temperature = 650°C. The multiple reactions monitoring (MRM) in negative mode [*M* – *H*] was used for the determination of compounds in the extract and the quantification was performed using an external calibration curve. For caffeic acid and rosmarinic acid, the precursor ions and product ions monitored were *m/z* = 179.1>107/116.8, and *m/z* =

359.1>197/133, respectively. A five points calibration curve was prepared by successive dilutions of the stock solution. Correlation coefficients higher than 0.99 were obtained in all the cases. The working range determined for caffeic acid was 9 - 180 µg L⁻¹ and 10 - 200 µg L⁻¹ for rosmarinic acid. The samples were previously diluted in methanol: water (1:1 v/v).

The treatment of ear injuries mainly caused by fungi such as *Malassezia pachydermaris* led to test the incorporation of the propolis extract in non-oily formulations aiming the auricle use. The formulations were chosen considering their adhesive characteristics necessary for topical application. Therefore, three formulations described in the National Formulary of the Brazilian Pharmacopoeia (BRASIL 2005) were selected, the cold cream (cream type W/O), lanolin and vaseline ointment, and creamy gel, which had good adhesion, oiliness and occlusion in order to stay for a longer period of time at the application site. The composition and bases preparation was done according to the National Formulary (BRASIL 2005). Briefly, the cold cream contained sodium borate (1%), methylparaben (0.25%), white wax (12%), liquid vaseline (30%), glycerin monostearate (2.5%), solid vaseline (30%), anhydrous lanolin (10%), butylhydroxyanisole (0.01%), butylhydroxytoluene (0.05%), propylparaben (0.15%) and distillate water q.s. 100%. The lanolin and vaseline ointment had 30% anhydrous lanolin, 0.02% butylhydroxytoluene and solid vaseline q.s. 100%. The creamy gel was composed by polyacrylamide and C13-14 isoparaffins and lauryl alcohol ethoxylate 70E (4%), preservative solution of parabens (3.3%), preservative solution of imidazolidinyl urea 50% (0.6%) and distillate water q.s.100%. The propolis extract was incorporated into the bases at the concentration of 30 mg mL⁻¹ immediately after the production of each formulation and transferred 60 g in each plastic container with double wall. This propolis concentration was chosen because it was above the minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MCF) of this extract as previously published (Cardoso et al. 2010).

The samples were subjected to accelerated stability studies during three months at room temperature (RT: 20 - 25°C); low temperature (LT: 3 - 8°C) and heating (HE: 40 ± 2°C) (ANVISA 2004) and then were evaluated for the appearance, color, odor, pH, mean size of the dispersed

globules from the internal phase, viscosity and spreadability. The formulations were also submitted to freeze-thaw cycle of 21 days and heating combined with centrifugation. For each condition, three samples were tested.

Appearance, color and odor were sensorially evaluated after 24 h, 30th and 90th days after the production of each formulation. The sample under low temperature (LT) was defined as the reference. The levels of alteration in the physical appearance were defined as follows: normal, without any change (I), slightly separated or slightly precipitated (II) and separated or precipitated (III). Likewise, changes in the color and odor were classified according these levels: normal color and odor, without any change (I), slightly modified (II), modified (III), intensely modified (IV) (ANVISA 2004). The pH was measured directly in the samples after 24 h, 30th and 90th days after the production of each formulation.

The mean size of the dispersed globules from the internal phase was determined only for the cold cream, because the other formulations were not biphasic. Prior to the analysis, the sample was diluted at 1:20 ratio with liquid vaseline (Martin 1993). After rapid mixing, a drop of this preparation was observed under an optical microscope with ocular of 12.5x and objective of 45x of magnification containing a calibrated scale (μm) for two-dimensional measurement.

The spreadability was determined as described by Knorst (Knorst 1991). Briefly, a circular glass plate (diameter = 20 cm; thickness = 0.2 cm) containing a central hole of 1.2 cm of diameter was placed on a support glass plate (20 x 20 cm). Under these plates, a sheet of graph paper was placed. After filling the hole with the sample, the circular plate was removed and a glass plate with known weight was placed on the sample. After one minute, the surface covered by the sample was determined by measuring the perpendicular diameters, subsequently the average diameter was calculated. This procedure was repeated, adding new plates and registering the surface covered and the plate weight added. The spreadability (S_i) at 25 °C was calculated using the equation $S_i = d^2 \cdot \pi / 4$, with S_i = spreadability of the sample (mm^2) for the weight i and d = average diameter (mm). Measurements were performed in triplicate at the 7th and 90th days.

The apparent viscosity was measured in a Brookfield viscometer rheometer using the speed of 0.3 rpm and the spindle number 4 for all the formulations according to ASTM D2196-05 (ANSI 2005). Prior to the measurements, the formulations were kept at $23 \pm 2^\circ\text{C}$, which was the same condition used to perform the tests. After setting the speed factor, the viscosity was measured three times in each sample maintained under the different conditions.

In the freeze-thaw cycle the samples were stored seven days in each of the following conditions: heating at 40°C, refrigerator at 3-8°C and heating at 40°C. At the end of this cycle of 21 days the samples were centrifuged (3000 rpm, 45 min) and evaluated according to these criteria: no phase separation (1), mild phase separation (2), remarkable phase separation (3), 50% of separation (4), over 50% of separation (5) (Bhargava 1987). In the heating chamber and centrifugation test combined, the samples maintained at 40°C and after 24 and 168 h aliquots were centrifuged (1 h, 3000 rpm) and evaluated according to the following criteria: no phase separation (1), mild phase separation (2), remarkable phase separation (3), 50% of separation (4), over 50% of separation (5) (ANVISA 2004).

RESULTS AND DISCUSSION

During the evaluation of the organoleptic characteristics, the appearance of the three formulations remained unchanged at room temperature (RT) and low temperature (LT); however, only the creamy gel showed good stability even in the heating condition (HE), as well as, in the additional tests of heating chamber and centrifugation combined and freeze-thaw cycle. The lanolin and vaseline ointment showed color changes and the presence of a supernatant. The cold cream also showed slight color changes and separation of the propolis extract on the surface. All the three formulations showed no changes in odor under the conditions studied. The average pH within and between the conditions (RT, LT, HE) of each formulation (Table 1) did not show significant change ($P > 0.01$). The pH from the sample with lanolin and vaseline was not measured due to the high content of oily components of this formulation.

Table 1 – Changes in pH of the formulations stored at room temperature (RT), low temperature (LT) and heating (HE).

Formulation	Time (Days)	Condition ^a		
		RT	LT	HE
Cold cream	1	7.87	7.87	7.87
	30	7.97 ± 0.18	7.54 ± 0.17	6.74 ± 0.06
	90	7.89 ± 0.1	8.03 ± 0.06	7.62 ± 0.05
Creamy gel	1	4.00	4.00	4.00
	30	4.00 ± 0.01	4.04 ± 0.01	4.17 ± 0.12
	90	3.79 ± 0.07	3.58 ± 0.02	3.63 ± 0.07

(a) 3 replicas of each sample in each condition

The mean size of the dispersed globules from the internal phase of the cold cream showed no significant difference among the conditions evaluated ($P > 0.05$) (Table 2). In the condition of heating, the significant difference observed between the beginning and at the end of the 90 days did not impair the stability of the formulation.

Table 3 – Maximum spreadability obtained by the cold cream, lanolin and vaseline ointment and creamy gel.

Sample	Day	Spreadability – Si (mm ²) ^a			
		Initial	RT	LT	HE
Cold cream	7 th		2783.9 (± 287.8)	2556.9 (± 308.2)	2443.8 (± 415.3)
	90 th	2826	2152.6 (± 184.8)	2266.7 (± 277.5)	1486.6 (± 102.5)
Lanolin and vaseline ointment	7 th		2944.1 (± 976.5)	2955.8 (± 224.8)	3003.6 (± 200.1)
	90 th	4013.1	2031.3 (± 186.7)	2337.5 (± 184.5)	1836.5 (± 170.7)
Creamy gel	7 th		2586.4 (± 302.2)	2043.4 (± 140.0)	2151.2 (± 126.1)
	90 th	1923.5	2046.9 (± 246.7)	2205.9 (± 109.4)	2116.9 (± 311.2)

(a) Average of the three samples by condition.

The apparent viscosity data gave an additional parameter to evaluate the physical changes in the formulations submitted to the stability tests. The cold cream showed a significant increasing in the viscosity, above the capability of the equipment, in all the conditions tested. The lanolin and vaseline ointment at the day 30th showed visible increase of the viscosity in all the conditions (Table 4). The creamy gel showed a slight decrease of its apparent viscosity in all the conditions. These data agreed with the results from the maximum spreadability of these formulations (Table 3). After the freeze-thaw cycle, only the lanolin and vaseline ointment showed a slight separation of the lanolin due to its low melting point and was classified as an alteration of level II. The other formulations were classified as level I because they did not have any kind of separation.

The results showed a decrease in the size of the droplets of the internal phase. This increased the homogeneity of the cream and decreased the tendency of coalescence of this phase.

Table 2 – Microscopic evaluation of the droplets average diameter of the internal phase - cold cream.

Time	Mean diameter ± SD (µm) ^a		
	RT	LT	HE
Initial	6.13	6.13	6.13
30 days	6.51 ± 0.39	6.20 ± 0.12	6.23 ± 0.14
90 days	5.26 ± 0.33	5.54 ± 0.20	4.75 ± 2.56

(a) Average of three samples, except for the initial period

The spreadability measured at the 7th and 90th days of the stability test showed significant reduction in the cold cream, as well as in the ointment with lanolin and vaseline (Table 3). The creamy gel was the formulation that showed the lowest variation in the spreadability at the end of the 90 days.

Table 4 – Initial and final values of viscosity for the cold cream, lanolin and vaseline ointment, and creamy gel stored at different temperature conditions.

Condition	Time	Viscosity (mPa.s) ^a		
		Cold cream	Lanolin and vaseline ointment ^b	Creamy gel
	Initial	25200	56700	123480
RT	90 days	*	103380	114870
LT	90 days	*	97440	121380
HE	90 days	*	86520	113820

(a) n=3 samples for each condition.

(b) Value corresponding to day 30th.

(*) Viscosity above the reading capacity of the equipment.

In the test of heating chamber and centrifugation combined, the cold cream exhibited a slight separation of the propolis extract and the lanolin and vaseline ointment showed a slight separation

of the lanolin in the bottom of the tube after centrifugation at 3.000 rpm for 60 min and was classified as level II. The creamy gel did not show any change after the centrifugation. According to Ansel et al. (2000), the topical base of creamy gel was considered a suitable formulation, because it maintained the physical, chemical and biological properties of the active compounds and raw materials used for its production. Based on the studies of the risks and benefits associated with transdermal therapy in veterinary practice, Davidson (2005) concluded that the transdermal formulations were safe and effective, since they had less possibility to cause systemic damage to the patient. Moreover, topical treatment is not invasive, which represents a comfort to the animals and makes application more practical to the owners. According to Sartor et al. (2004), the transdermal form showed fewer gastrointestinal side effects compared to oral treatment.

Many authors such as Payne et al. (2007) and Raghukumar et al. (2010) reported a demand for new therapies with natural products. Such natural products could provide a pool of several chemical substances that may have antimicrobial activity with fewer tendencies to instigate resistance. Rossolini and Mantegoli (2008) mentioned that the antibiotic resistance was the leading public health problems. Pedersen et al. (2007) pointed out this problem in veterinary medicine, because the selection of bacteria resistant to antibiotics also occurred in the treatment of infectious diseases on domestic animal. Another drawback of the indiscriminate use of antibiotics are the side effects to which animals are subjected. For example, Dowling (1996) reported the ototoxic potential of aminoglycosides and contact hypersensitivity with topical neomycin. The propolis was considered by Bankova et al. (1995) and Kujumgiev et al. (1999) as the hold of natural active substances with a large therapeutic use. According to Sforcin (2007) *in vitro* and *in vivo* assays demonstrated that propolis activated the macrophages, increasing their microbicidal activity, enhanced the lytic activity of natural killer cells and stimulates antibodies production.

Cardoso et al. (2010) evaluated the antimicrobial potential of the propolis extract used in this work and found 21 mg mL⁻¹ and 5.3 mg mL⁻¹ as the minimum bactericidal concentration (MBC₉₀) against the isolates of *Staphylococcus* coagulase positives and minimum fungicidal concentration

(MFC₉₀) for *M. pachydermatis*, respectively. These organisms are often isolated from the dogs with otitis (Kiss et al. 1997) and cause secondary infection in atopic dermatitis (Prélaud 2005). Therefore, the creamy gel formulation containing propolis extract above the minimum bactericidal and fungicidal concentration (30 mg mL⁻¹) could be a promising treatment to canine external otitis and dermatitis in veterinary practice. However, Kujumgiev et al. (1999) found that the chemical constituents and the active substances of each propolis might vary according to the flora, seasonality, specie of bees of the region and extraction methods. Bankova (2005) reported that the chemical characterization of the propolis was the only significant way to study its biological and pharmacological activity. Results of the liquid chromatography showed that the propolis extract used in this study contained caffeic acid and not rosmarinic acid. Previous studies such as Schneidewind et al. (1979) and Kartal et al. (2003) revealed that the caffeic acid was one of the components, which were responsible for the antimicrobial activity of all the propolis worldwide. Similar results were reported by Cardoso et al. (2010).

Altogether, the obtained results showed that the creamy gel formulation had a remarkable stability to all the stress conditions studied. This formulation exhibited an excellent maintenance of its physicochemical properties such as the odor, color, viscosity, and spreadability. Moreover, it presented a great capability to incorporate the hydroalcoholic propolis extract. Such results were not achieved with the other formulations tested. The stability of this formulation associated with the *in vitro* efficacy of the propolis against microbial agents that commonly caused canine otitis (Cardoso et al. 2010) showed that *in vivo* tests should be carried out seeking its potential application in the treatment of bacterial and fungal infections in veterinary medicine.

CONCLUSION

The propolis used in this study showed antibacterial and antifungal *in vitro* activity against the main microorganisms responsible for such diseases. Therefore, the propolis creamy gel described here could be a promising formulation for use in the veterinary medicine.

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