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In vivo wound healing effects of S*ymphytum officinale* L. leaves extract in different topical formulations

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The present work evaluates wound healing activity of leaves extracts of *Symphytum officinale* L. (comfrey) incorporated in three pharmaceutical formulations. Wound healing activity of comfrey was determined by qualitative and quantitative histological analysis of open wound in rat model, using allantoin as positive control. Three topical formulations, carbomer gel, glycero-alcoholic solution and O/W emulsion (soft lotion) were compared. The histological analysis of the healing process shows significant differences in treatment, particularly on its intensity and rate. The results indicate that emulsion containing both extracts, commercial and prepared, induced the largest and furthest repair of damaged tissue. This could be evidenced from day 3 to 28 by increase in collagen deposition from 40% to 240% and reduction on cellular inflammatory infiltrate from 3% to 46%. However, 8% prepared extract in emulsion presented the best efficacy. This work clearly demonstrates that comfrey leaves have a wound healing activity. The O/W emulsion showed to be the vehicle most effective to induce healing activity, particularly with extracts obtained from comfrey leaves collected in Minas Gerais state in Brazil. It shows the best efficacy to control the inflammatory process and to induce collagen deposition at 8% concentration.

1. Introduction

Symphytum officinale L., comfrey, Boraginaceae, has been considered in traditional medicine for its anti-inflammatory (Predel et al. 2005), analgesic (Goldman et al. 1985; Kucera et al. 2004; Grube et al. 2007), anti-edematous (Kucera et al. 2004) and adstringent properties (Staiger 2007). For over 2000 years, it has been widely used by the population to treat a variety of ailments and to favor the growth of new tissues in wounds and bone fractures (Staiger 2007). Additionally, numerous compounds obtained from comfrey like mucilage, allantoin, alkaloids, tannins and sugars, have had their biological activities documented (Youngken 1950). The wound healing action of comfrey has been attributed to the presence of allantoin (Saito and Oliveira 1986; Martindale 2002; Cunha et al. 2003; Carvalho 2004), Anti-irritating, hydratating and anti-inflammatory properties are atributed to mucilage (Saito and Oliveira 1986; Cunha et al. 2003) and tannins, which are astringent and hemostatic (Cunha et al. 2003).

Healing is a physiological process with the objective of repairing damaged tissue. Synthetically, this mechanism has three stages – inflammatory, proliferation and remodeling – that occur gradually and dynamically (Mondolin and Bevilacqua 1985; Serhan et al. 2008). Comfrey was cited as one of the most used plants to heal wounds and to treat external skin problems, according to the studies performed on the Brazilian population (Parente and Rosa 2001; Luz 2001; Ritter et al. 2002; Champs et al. 2003; Souza and Felfili 2006). More recently, different clinical trials demonstrated the efficacy of comfrey extracts to treat sprains, strains, muscle and joint problems (Predel et al. 2005; Kucera et al. 2005; Staiger 2007), ankle distortions (Kucera et al. 2004; Koll et al. 2004), and reducing acute back pain (Giannetti et al. 2010). To treat recent abrasions in patients, *Symphytum* herb extract cream was used and it was a reduction of time to cicatrize was observed a time reduction to cicatrize, with no adverse effects (Barna et al. 2007).

The vehicle composition can affect drug release and skin permeability properties, enhancing or reducing percutaneous penetration. The selection of an appropriate vehicle is one of the most important steps to increase the efficacy of a topically applied bioactive (Kikwai et al. 2002; Nino et al. 2010).

In this way, the therapeutic efficacy of comfrey extracts in topical formulations used to treat wounds could be dependent on the composition and physicochemical properties of the vehicle.

In this context, the aim of this study was to evaluate the wound healing ability of comfrey leave extract, incorporated in three different pharmaceutical topical formulations. A commercial extract was compared with a comfrey extract prepared in our laboratory. For this purpose a wound healing rat model was used to evaluate the effects on the microscopic aspects of wound lesions.

2. Investigations and results

2.1. Characterization of Symphytum officinale L. extracts

The physic-chemical parameters of the two extracts of *S. officinale* L. were compared and both extracts were translucent,

Table 1: Physico-chemical characterization of *Symphytum officinale* L. extracts, CE and PE

Analysis	Specifications*	Commercial extract	Prepared extract
Propyleneglycol concentration	35.0–50.0%	40	40
pH	4.00–8.50	6.20	6.90
Density (25 °C)	0.989–1.05	1.030	1.028

*Specifications provided by the manufacturer of the fluid glycolic comfrey extract

presenting characteristic smell and dark brown color. They are also soluble in water, alcohol and propyleneglycol. The results of propyleneglycol concentration, pH and density analysis are shown in Table 1. The HPLC chromatograms highlighted the same profile for both comfrey extracts. However allantoin is peaking better in the extract prepared in the our laboratories than in the commercial extract (Fig. 1).

2.2. In vivo evaluation of the wound healing process

2.2.1. Histological analysis

The presence of comfrey extract in formulations induced a more effective wound healing process compared to the wounds not treated, treated with excipients or treated with allantoin 5%. The rate and intensity of the wound healing process, as well as the structural organization of the tissue observed during the treatment with comfrey were more favorable to induce a normal pattern of the skin remodeling. In this context, among the formulations studied, the O/W emulsion containing comfrey extract 3%, CE3%.E group induced a better and faster repair of damaged tissue (Fig. 2). This can be identified by the presence of a less intense inflammatory process and more collagen deposition on the 3rd day (Fig. 2 C and L) compared to the control group (C) (Fig. 2 A and J) and the group treated with allantoin (AE) (Fig. 2 B and K). Moreover, on the 14th day the group treated with comfrey emulsion (CE3%.E) presented a better resolution of the inflammatory process, as well as better tissue organization with a greater replacement of collagen fibers (Fig. 2 F and O). On the 28th day it was observed that the treatment with comfrey emulsion (CE3%.E) (Fig. 2 I and R) induced to a more organized tissue, very close to a healthy skin. Based on these findings, the O/W emulsion was selected as the pharmaceutical formulation to be used in order to compare the commercial and prepared extracts of comfrey and the results are shown by the morphometric analysis.

In accordance with qualitative analysis, the quantitative results of morphometric analysis showed that the commercial comfrey extract at 3% (CE3%) had a positive effect on the wound healing process induced by the three formulations studied, reducing the amount of inflammatory cells present at the injury site and promoting the collagen deposition.

This could be evidenced from day 3 to 28 by reduction of cellular inflammatory infiltrate from 3% to 46% and increasing collagen deposition from 40% to 240% (Table 2). Moreover, the O/W emulsion containing comfrey (CE3%.E group) induced the best wound healing process (Fig. 3 A1 and B1) compared to gel (CE.G group) and solution (CE.S group), probably due to the significant reduction of inflammatory cells number on the 21st day (Fig. 3 A1).

Figure 3 (A2 and B2) shows the inflammatory process and collagen deposition induced in the wounds treated with the O/W emulsion containing commercial extracts at 3% (CE3%.E group) and 8% (CE8%.E group) compared to the untreated group (C group) and with the positive control group (allantoin at 5%, AE group). The higher concentration of commercial comfrey extract (CE8%) in emulsion influenced positively the wound healing process, reducing significantly the amount of inflammatory cells on the 3rd, 14th and 21st days (Fig. 3 A2) and increasing significantly the deposition of collagen on 7th and 21st days (Fig. 3 B2). On the other hand, the results of morphometric analysis of the wound healing process induced by PE at 3% (PE3%.E group) and at 8% (PE8%.E group) (Fig. 3

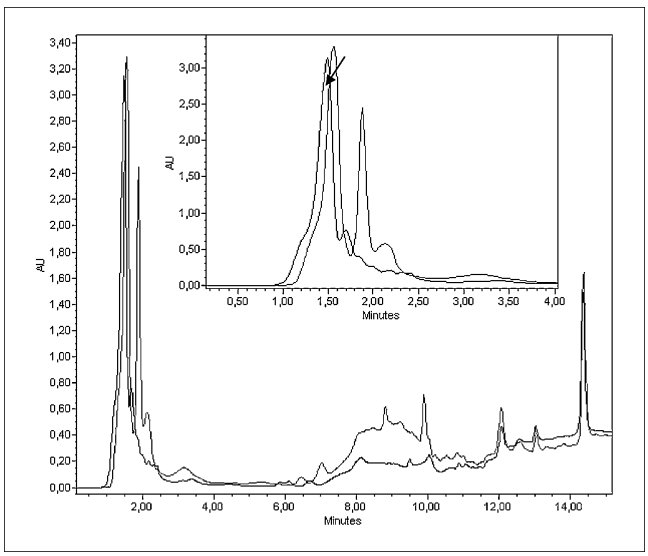

Fig. 1: Comfrey extracts chromatograms obtained at 210 nm. Prepared extract (PE): gray line; commercial extract (CE): black line. The arrow indicates the allantoin peaks at the same retention time as internal standard

Table 2: Percentages of reduction on cellular inflammatory infiltrate and increasing in collagen deposition after the treatment with O/W emulsion containing S. officinale extracts or allantoin, compared with controls groups

Groups	Day 3	Day 7	Day 14	Day 21	Day 28
Cellular inflammatory infiltrate					
Control	665.4	700.5	609.9	529.1	335.3
E	−22,8%	4,2%	−37,8%	−33,0%	−2,3%
CE3%.E	−27,7%	−3,9%	−26,9%	−42,1%	−27,4%
CE8%.E	−43,8%	−21,5%	−37,2%	−41,8%	−36,1%
PE3%.E	−46,0%	−20,4%	−36,5%	−36,1%	−36,4%
PE8%.E	−41,0%	−24,9%	−31,3%	−45,1%	−33,2%
AE	−18,4%	−17,8%	−18,6%	−40,8%	−25,1%
Collagen deposition					
Control	39503.0	90378.0	178265.0	179639.0	194672.0
E	31,8%	18,7%	30,0%	35,5%	34,4%
CE3%.E	98,5%	40,6%	42,8%	47,0%	58,4%
CE8%.E	134,3%	140,7%	52,0%	66,0%	36,6%
PE3%.E	156,5%	129,1%	71,7%	78,6%	71,0%
PE8%.E	244,0%	125,6%	90,2%	107,4%	110,0%
AE	44,2%	30,5%	14,1%	30,1%	29,6%

Data are expressed in percentage. Cellular nucleus and the collagen present in the skin fragments were quantified in 20 randomly fields (total area covered equal to 1.5×10^6 μm^2)

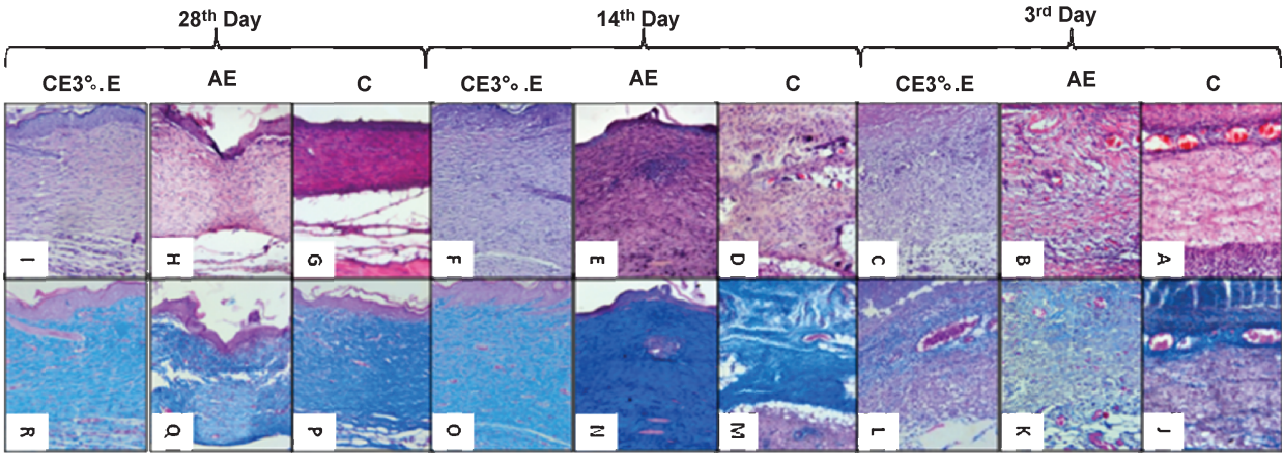

Fig. 2: Photomicrographs of rat skin showing the structural organization rate and intensity of the wound healing process. C - Control (untreated); AE – O/W emulsion containing allantoin 5%; CE3%.E - O/W emulsion containing CE 3%. Days: 3rd (A, B, C, J, K, L); 14th (D, E, F, M, N, O); 28th (G, H, I, P, Q, R). Hematoxylin-Eosin (A, B, C, D, E, F, G, H, I) and Masson Trichrome (J, K, L, M, N, O, P, Q R), 200X

A3 and B3) are similar between them relative to the number of inflammatory cellules, although PE at 8% induced the best wound healing process in relationship to the deposition of collagen, presenting a significative increase in all times evaluated (Fig. 3 B3 and Fig. 4). Furthermore, PE at 8% (PE8%.E group) is significantly better concerning the collagen deposition on the 28th days of analysis compared to CE at 8% (CE8%.E group).

The comparative analysis (Fig. 4) demonstrated that the wounds treated with prepared comfrey emulsion at 8% (PE8%.E group) showed a more efficient wound healing process, when compared to the untreated group (C group), allantoin group (AE group) and EC a 8% (CE8%.E group), characterized by the lower amount of inflammatory cells (Fig. 4 A, C, E and G) and increase of collagen deposition (Fig. 4 B, D, F and H) and this could be

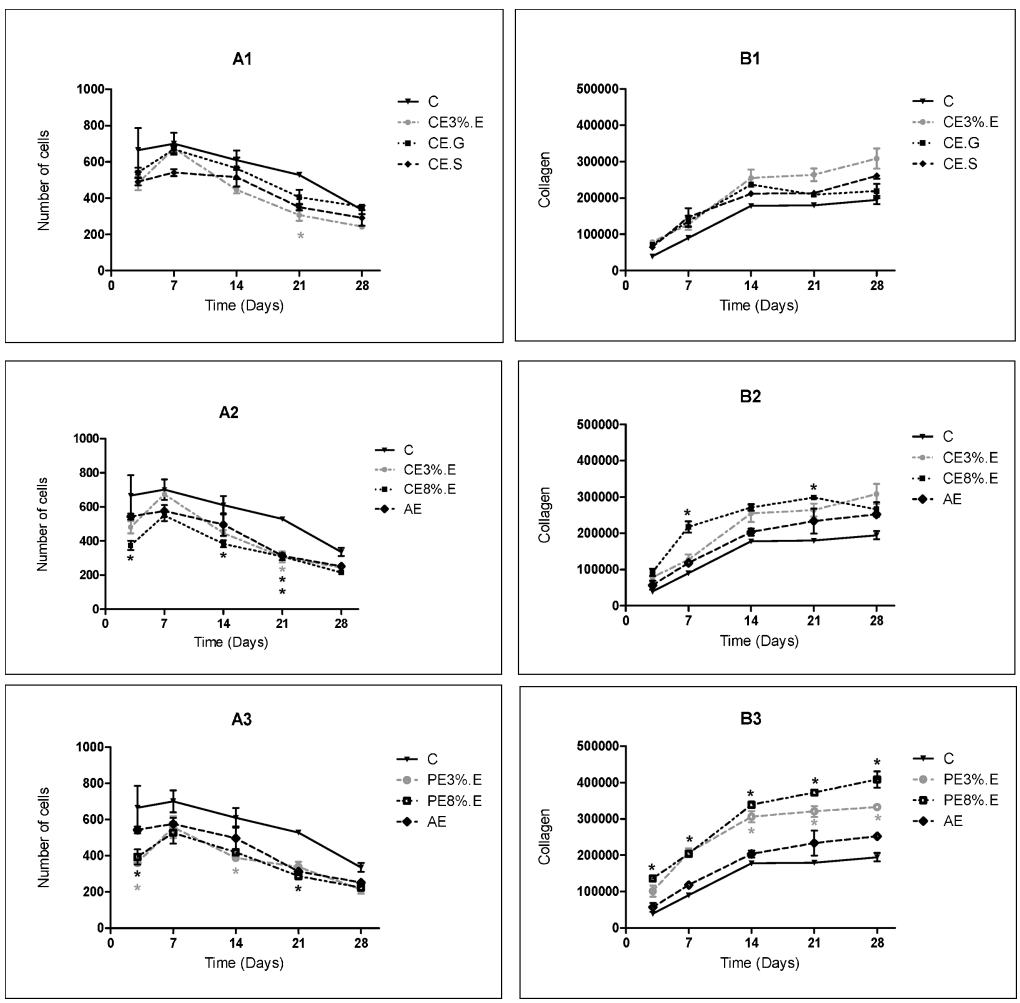

Fig. 3: Inflammatory process (A1, A2, A3) and collagen deposition (B1, B2, B3) of rat skin. C – Control (untreated); CE3%.E – O/W emulsion containing CE3%; CE.G– Carbomer gel containing CE 3%; CE.S – Glycero-alcoholic solution containing CE 3%; CE8%.E – O/W emulsion containing CE 8%; PE3%.E – O/W emulsion containing PE 3%; PE8%.E – O/W emulsion containing PE 8%; AE – O/W emulsion containing allantoin 5%. Results are presented as means ± SEM. Statistically significant data are given as *P< 0.05. Total area covered equal to 1.5×10^6 μm^2

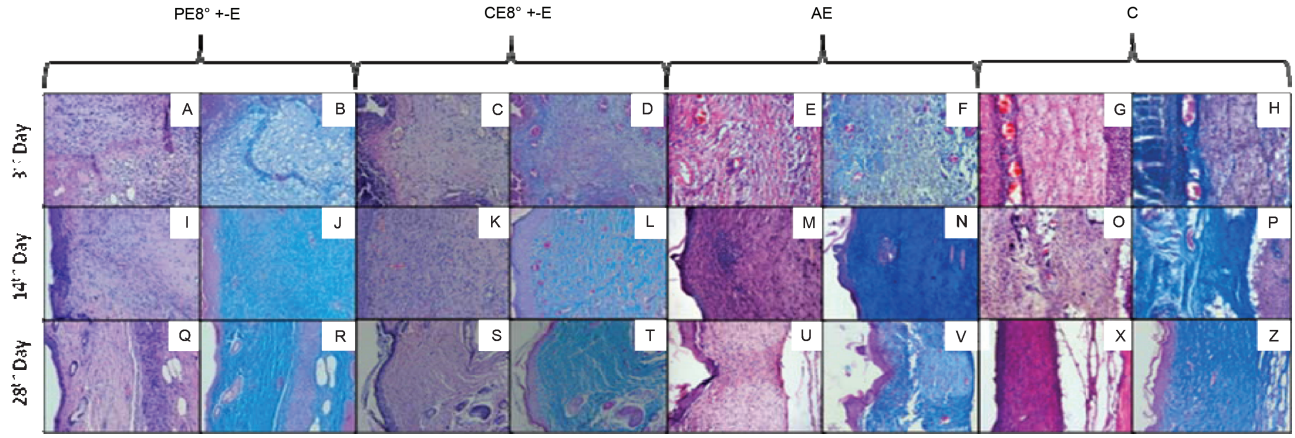

Fig. 4: Photomicrographs of rat skin showing comparative analysis of wound healing process induced by extracts and allantoin. PE8%.E - O/W emulsion containing PE 8%; CE8%.E - O/W emulsion containing CE 8%; AE - O/W emulsion containing 5% of allantoin; C - Control (untreated). Days: 3rd (A, B, C, D, E, F, G, H); 14th (I, J, K, L, M, N, O, P); 28th (Q, R, S, T, U, V, X, Z). Hematoxylin-Eosin (A, C, E, G, I, K, M, O, Q, S, U, X) and Masson Trichrome (B, D, F, H, J, L, N, P, R, T, V, Z), 200X

observed from the 3rd day of treatment. Thus, on the 14th day (Fig. 4 I, J, K, L, M, N, O and P) it was possible to observe for PE8%.E group a more organized scar tissue (Fig. 4 A, B, I, J, Q and R), and on the 28th day (Fig. 4 Q and R) the scar tissue was very close to the normal tissue, presenting a great tissue organization and presence of skin appendages, indicating the better efficacy of this treatment.

3. Discussion

The data of the physico-chemical parameters suggest that both extracts were in accordance to the pharmacopeical specifications. The HPLC chromatogram (Fig. 1) indicates a similarity of chemical composition profile, however with marked differences in number and intensity of some peaks. Allantoin, reported to be one of the main compounds responsible for the wound healing properties of the comfrey extract (Saito and Oliveira 1986; Cunha et al. 2003; Carvalho 2004) was found only in the PE extract.

Wound healing is a physical, chemical and biological process which starts immediately after a tissue injury in order to repair the damaged tissue. It is a complex phenomenon and of great importance in skin regeneration but, unfortunately, still unclear in detail (Sanchez Neto et al. 1993).

In the present work, leave extracts of *S. officinale* L. presented wound healing activity, thereby justifying their extensive use in Brazil folk medicine, as already observed by different authors (Parente and Rosa 2001; Luz 2001; Ritter et al. 2002; Champs et al. 2003; Souza and Felfili 2006) and in clinical studies conducted in other countries (Kucera et al. 2000; Koll et al. 2004; Kucera et al. 2004, Predel et al. 2005; Kucera et al. 2005; Staiger 2007; Giannetti et al. 2010). The inflammatory response is an important step of the wound healing process as it prepares the environment of the wound repair itself. However, this stage should not be extremely intense, because an excessive inflammatory response could cause delay in wound healing favoring the disturbance between synthesis and degradation of collagen, and promoting degradation of the matrix (Ashcroft et al. 2002). Our results suggest that the comfrey extract modulates the inflammatory response possibly by inhibiting the chemotaxis of inflammatory cells to the site of the wound, thus preventing the release of reactive species responsible for the oxidative stress and tissue damage, as described by Bradbury et al. (1993) in a study of pathogenesis of vascular diseases. Moreover, the well formed collagen fibers observed in PE3%.E and PE8%.E (prepared comfrey extract at 3% and 8%, respectively) support the effectiveness of comfrey in fibroblastic proliferation and synthesis of extracellular matrix during wound healing.

Allantoin is widely used in pharmaceutical and cosmetics preparations due to its wound healing properties as anti-irritant, moisturizer and necrotic tissue remover (Saito and Oliveira 1986; Oliveira et al. 2008). Due to these related properties, allantoin was use as a positive control in this work. The results obtained here showed that allantoin at 5% in soft lotion O/W emulsion has a wound healing effect when compared with the controls groups, however its activity was lower compared to the extracts. Thus, the differences in healing effects observed in this work between PE and CE at 8% should not be attributed only to allantoin, better evidenced in PE extract (Fig. 1). Araújo et al. (2010) showed, for the first time, the histological wound healing profile induced by allantoin in rats and demonstrated that it is able to ameliorate and fasten the reestablishment of the normal skin.

From Table 2 it can be observed that healing estimated as collagen synthesis is dose dependent and it was maintained up to complete healing. It is also dependent of the extract preparation method, different collection location or probably from seasonal influences of comfrey leave collection. It can be evidenced that PE has a stronger effect in both parameters of healing during the experimental period of observation. Cell migration to wound tissue was increased especially in days 7–14 post injury. At the same days stronger effects of *S. officinale* extracts were also observed, suggesting modulation of the inflammatory processes. These results about the wound healing activity of comfrey extract related to its anti-inflammatory effect are in agreement with the clinical studies results described by Kucera et al. (2000); Koll et al. (2004), Kucera et al. (2004), Kucera et al. (2005), Barna et al. (2007).

In particular, the results obtained in this work corroborate with the clinical study of Barna et al. (2007), that also used a topically applied preparation (Traumaplant®) containing 10% active ingredient from the aerial parts of medicinal comfrey (*Symphytum* × *uplandicum* NYMAN). Furthermore, in this study the patients did not reported adverse reactions or tolerability problems, thus indicating the safety of the topical use of aerial parts extracts.

Aditionally, our results confirm the anti-inflammatory properties of the topical comfrey products described by Kucera et al. (2004) in a randomized double blind study in patients with acute ankle distortions.

The present work demonstrates that wound healing processes can be induced by comfrey extract, which modulates the inflammatory process and stimulates the production of collagen, evidenced by morphometric analysis. Furthermore, the O/W emulsion showed to be the vehicle most effective to induce healing activity, particularly with prepared extract obtained from

comfrey leaves collected in Minas Gerais state in Brazil. It shows the best efficacy to control the inflammatory process and to induce collagen deposition at 8% concentration.

It is clear that the higher concentration of *Symphytum* active ingredients in the PE8%.E was responsible for the best control of the inflammatory process and collagen deposition. These results are in agreement with the results found by Kucera et al. (2004; 2005), where the cream with the highest concentration of extract showed better results.

4. Experimental

4.1. Plant material

Leaves of *Symphytum officinale* L. were collected in Betim, Minas Gerais, Brazil, in April 2007. The voucher specimen (OUPR 21806) was identified and deposited at the Herbarium of the Instituto de Ciências Exatas e Biológicas, Universidade Federal de Ouro Preto (UFOP), Ouro Preto, Minas Gerais, Brazil.

4.2. Preparation of extract

Leaves were dried at 37 °C for two days, reduced to powder (255.0 g) and extracted by maceration with a mixture of water and propyleneglycol (60:40) for 48 h before filtration (Brasil 1959). This extract was prepared at the same concentration of the commercial glycolic extract.

A commercial glycolic extract from leaves of *S. officinale* L. containing 20% w/v from dry plant was obtained from a Brazilian manufacturer (Natural Pharma® Brazil).

The reagents used were analytical grade and the MilliQ water was purified by the Symplicity 185 System (Millipore®, Brazil). Allantoin was purchased from Sigma-Aldrich® (Brazil). The reagents used were analytical grade and the MilliQ water was purified by the Symplicity 185 System (Millipore®, Brazil).

4.3. Characterization of the extracts

The extracts were evaluated considering physical appearance, smell, color, pH (pH 300M Analyzer, Brazil) and solubility. The chromatographic profiles were obtained on a HPLC system Waters 2695 Alliance System (Waters Corporation, Milford, EUA) using a 2996 PhotoDiodo Array Detector. The chromatographic profiles of extracts were achieved by using an ODS2 column (Spherisorb Waters 4.6 × 150 mm, 5 μm) and Phenomenex guard column (C18 4.0 × 3.0 mm). The mobile phase were: solvent A, HPLC grade acetonitrile (Tedia, Brazil) filtered using 0.45 μm Millipore® membrane and solvent B, MilliQ water. The gradient profile was: 0, 5, 10, 15 and 20 min, A%: 1, 1, 100, 100 and 1, respectively. Chromatograms were obtained at 25 °C at 210 nm wavelength. Eluted mobile phase was monitored at 200 – 800 nm. The flow rate was 1 ml/min and the injection volume was 50 μl of diluted extract samples (extract/water, 1:1), which were filtered through a 0.20 μm filter (Syringe Filter, Nalgene).

4.4. Preparation of pharmaceutical formulations

Three formulations containing *Symphytum officinale* L extract were evaluated: carbomer® gel (1.5 g carbopol® 940, 10 ml glycerin, 0.5 ml triethanolamine, 0.15 g methylparaben, 0.10 g propylparaben, 87.75 ml water) glycero-alcoholic solution (40 ml glycerin, 1 ml dimethylsulfoxide, 59 ml alcohol 60 ° GL) and a oil/water (O/W) emulsion (soft lotion) (3.6 g cetylestearyl alcohol, 0.4 g sodium cetylestearyl sulfate, 4 ml mineral oil, 5 ml sorbitol solution 70% w/v, 0.15 g methylparaben, 0.10 g propylparaben, 86.75 ml water). The comfrey commercial glycolic extract (CE) at 3% w/w was incorporated. After this first evaluation the two comfrey glycolic extracts, commercial (CE) and prepared (PE), were incorporated into emulsion, at 3 and/or 8% w/w. A positive control with allantoin 5% in the same preparation was used for comparison.

4.5. Animals

Female Wistar rats (180–200 g) were used and housed individually, on a 12 hours light/dark cycle with a standard pellet diet (Labcil Petilizado-Socil, Brazil) and water *ad libitum*. The experimental protocol was approved by the Ethical Committee of UFOP (number 2007/98) and was in accordance to the Guide for the Care and Use of Laboratory Animals, published by the US National Institute of Health (NIH Publication, revised in 1985).

4.6. Wound healing evaluation

The animals were anesthetized by intraperitoneal with sodium pentobarbital (50 mg/kg). The hair of the dorsal back of each animal was removed and an excision wound (1 cm^2) was made by removing a full thickness piece of the skin (Sekine et al. 1998). The animals were randomly distributed in 13 groups (n = 4): control untreated (C), carbomer gel excipients (G), carbomer gel with CE 3% (CE.G), carbomer gel with allantoin 5% (AG); glycero-alcoholic solution excipients (S); glycero-alcoholic solution with CE 3% (CE.S), glycero-alcoholic solution with allantoin 5% (AS); O/W emulsion excipients (E); O/W emulsion with CE 3% (CE3%.E); O/W emulsion with CE 8% (CE8%.E); O/W emulsion with PE 3% (PE3%.E); O/W emulsion with PE 8% (PE8%.E) and O/W emulsion with allantoin 5% (AE). The weighed topical formulations (0.25 g) were administered daily during 14 days (Goldman et al. 1985).

4.7. Histopathological analysis

After sacrifice (sodium pentobarbital, 100 mg/kg) the wounds of animals were excised on the 3rd, 7th, 14th, 21st and 28th days after the surgery, containing a margin of normal skin around the wound. The tissues were preserved in 10% buffered formalin. Four μm thickness sections were stained with hematoxylin-eosin and Masson Trichrome (Beçak and Paulete 1976; Behmer et al. 1976). For the qualitative analysis, the specimens were assessed under a light microscope (Olympus CH30, Japan) in order to analyze the new epithelium, inflammation, vascular responses and the collagen formation.

The quantitative analysis (morphometry) was performed using scans of the tissues for determination of intensity of the inflammation and collagen deposition. Cellular nucleus and the collagen present in the skin fragments were quantified in 20 randomly fields (total area covered equal to 1.5×10^6 μm^2). The images were amplified, acquired by a Microcamera Leica and the programe DM5000B Leica Application Suite (Version 2.4.0 R1 Leica Microsystems, Switzerland Ltd) and analyzed by Leica programe QWin V3 (Leica Microsystems, Switzerland Ltd). The tissues were analyzed microscopically, qualitative and quantitatively, by the same pathologist without prior knowledge of the identity of the groups.

4.8. Statistical analysis

The results of stability were expressed as means ± SD, and statistical evaluation was performed by Kruskal-Wallis and Dunns post-test. The data of histological analysis were expressed as means ± SEM, and statistical evaluation was performed by ANOVA and Tukey post-test. Values lower than $P < 0.05$ were considered significant.

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