

Development and stability study of *Kalanchoe crenata* semi-solid formulation and its positive effect on the inflammatory response induced by *Leishmania braziliensis* in mice.

Desenvolvimento e estudo de estabilidade de formulação semi-sólida de *Kalanchoe crenata* e seu efeito positivo sobre a resposta inflamatória induzida pela *Leishmania braziliensis* em camundongos.

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ABSTRACT

Kalanchoe crenata (Andrews) Haworth (syn. *K. brasiliensis*) is used as an anti-inflammatory in traditional medicine of South America and Africa. This plant has demonstrated immunomodulatory and anti-inflammatory activities in different models studied. The anti-leishmania effect was verified and these activities are related to flavonoids, mainly quercitrin. The aim of this work was to develop semi-solid formulations and liposomes with a standardized quercitrin extract and fraction, to evaluate the long-term stability of the formulation and the anti-inflammatory activity in an animal model of leishmaniasis. The study was developed using pharmaceutical techniques of development, HPLC analysis of the extract, fraction and formulations and anti-inflammatory evaluation in mice leishmaniasis. Formulations were obtained using a gel base, and chemical and physical stability were observed during the 180 days study. The use of the formulations reduced the inflammatory response induced by *Leishmania braziliensis* in mice. The use of liposomes did not increase the activity of the *Kalanchoe crenata* extract or concentrated fraction.

KEYWORDS

Kalanchoe crenata, formulations, liposomes, quercitrin, leishmaniasis, anti-inflammatory.

RESUMO

Kalanchoe crenata (Andrews) Haworth (syn. *K. brasiliensis*) é usada como um anti-inflamatório na medicina tradicional da América do Sul e África. Esta planta tem demonstrado atividade imunomoduladora e anti-inflamatória em diferentes modelos de estudo. O efeito anti-leishmania também foi verificado e essas atividades estão relacionadas com flavonóides, principalmente a quercitrina. O objetivo deste trabalho foi desenvolver uma formulações semi-sólida e o lipossoma contendo extrato e fração de *K. crenata* padronizados em quercitrina, avaliar a estabilidade a longo prazo das formulações e a atividade anti-inflamatória em modelo animal da leishmaniose. O estudo foi desenvolvido usando técnicas farmacêuticas de desenvolvimento, análise por HPLC do extrato, fração e formulações e avaliação anti-inflamatória em camundongos BALB/c inoculados com *L. braziliensis*. As formulações foram obtidas utilizando uma base de gel, e foi observada a estabilidade química e física durante 180 dias. O uso das formulações reduziu a resposta inflamatória induzida por *L. braziliensis*, e a utilização de lipossoma não aumentou a atividade do extrato ou fração concentrada de *K. crenata*.

PALAVRAS-CHAVE

Kalanchoe crenata, lipossomas, quercitrina, leishmaniose, anti-inflamatório.

1. INTRODUCTION

Leishmaniasis is an infectious disease caused by protozoan parasites of the genus *Leishmania* and presents endemic occurrence in tropical and subtropical regions. In different countries of the world this disease has great importance in public health, due to high morbidity and mortality. Leishmaniasis is one of the most neglected tropical diseases, and more than 12 million people worldwide are currently infected, with an incidence of two million new cases each year (WHO, 2010). An increase in the incidence of leishmaniasis can be associated with urban development, deforestation, environmental changes and migrations of people to areas where the disease is endemic (Rocha, 2005). Different clinical manifestations of the disease are observed, depending on the *Leishmania* species involved and the genetic makeup or immunological status of the host (Moura, 2005). This disease has a clinical spectrum that includes asymptomatic infection and three main clinical syndromes: visceral leishmaniasis (also known as 'kala-azar'), cutaneous leishmaniasis, and mucosal leishmaniasis (Pavli, 2010).

Based on geographical distribution, leishmaniasis is divided into Old and New World leishmaniasis. New World cutaneous leishmaniasis caused by *Leishmania braziliensis* is distinguished from other leishmaniases by its chronicity, latency, and tendency to metastasize in the human host (Moura, 2005). New World cutaneous leishmaniasis has variable clinical manifestations, ranging from ulcerative skin lesions to destructive mucosal inflammation, and it is often accompanied by local lymphadenopathy (Schwartz, 2006). The parasite infection is followed by a complicated host-parasite interaction, which involves the immune response mediated by cells, and parasite-macrophage interactions, the survival of the parasite within macrophages is crucial, which is ensured by deviously manipulating the macrophage related immune functions. Different species of *Leishmania* modulate macrophage signaling pathways and metabolism to different extents (Naderer, 2008). The role of the Th1 cellular immune response has been associated to the control of infection in human leishmaniasis. The key event for the immune healing response is an efficient activation of cells capable of cytokines production that leads to the activation of macrophages, resulting in nitrogen intermediates and reactive oxygen synthesis and consequently death of intracellular parasites (Reis, 2006).

Pentavalent antimonials, amphotericin B and pentamidine were respectively first and second-line drugs used for treatment of the different forms of leishmaniasis, they are expensive and show toxicity together with numerous side effects (Barret, 2002). The most

frequently reported clinical adverse effects of pentavalent antimonials and pentamidine are musculoskeletal pain, gastrointestinal disturbances, and mild to moderate headache. Patients treated with liposomal amphotericin B had mild dyspnea and erythema (Oliveira, 2011). The small number of drugs available and the wide variability in the therapeutic regimens is also reported as a problem to be considered in the treatment of leishmaniasis.

The treatment of leishmaniasis is difficult because of the intra-macrophagic parasite localization, which is not efficiently eliminated by the host immune system (Rocha, 2005). New anti-leishmanial drugs should focus on their direct parasitocidal and/or indirect immunomodulatory activity, achieved via restoration of impaired host signaling pathways (Saha, 2011). There is a worldwide effort to discover new lead compounds for infectious diseases, and several research groups screen plant extracts to detect secondary metabolites with relevant biological activities (Weniger, 2006). Recently, a review identified 101 plants and 288 isolated compounds from higher plants and microorganisms with antileishmanial activity (Rocha, 2005). This study presented a range of plant extracts with interesting antileishmanial properties *in vitro* and potent leishmanicidal activities of certain chemically defined molecules isolated from natural origins.

Plants of the genus *Kalanchoe* (syn. *Bryophyllum*) (Crassulaceae) are cited in ethnopharmacological surveys and *in vivo* studies regarding their positive effect against leishmaniasis. *Kalanchoe crenata* (Andrews) Haworth (syn. *K. brasiliensis*) (Zappi, 2012) is traditionally used for inflammations (Nguelefack, 2006) and anti-inflammatory and immunosuppressive effects were investigated (Ibrahim, 2002). The leishmanicidal effect of *Kalanchoe pinnata* (Lamarck) Persoon was evaluated in animal models (Da Silva, 1995), and this activity is linked to the quercitrin (Muzitano, 2006a).

In order to verify the gel semisolid formulation activity containing *Kalanchoe crenata* extract, it was performed: (i) obtainment of the *K. crenata* crude extract and fraction; (ii) quercitrin quantification; (iii) development and stability study of semi-solid formulation with *K. crenata*; and (iv) evaluation of anti-inflammatory effect in animal models of inflammation induced by *Leishmania*.

2. MATERIALS AND METHODS

2.1. Chemicals

Dichloromethane (Biotec, Brazil), ethyl acetate (F. Maia, Brazil) and hydrochloric acid (Labsynth, Brazil), sodium hydroxide (All Chemistry, Brazil) were used for the extraction process. The raw materials (Carbopol 940, EDTA and Methylparaben) used for the formulations were purchased from M. Cassab (Brazil), propylene glycol and Optiphen[®] (phenoxyethanol and caprylyl glycol) (DEG-Brazil), phosphatidylcholine (Pro-Lipo DUO[®] - Lucas Meyer Cosmetics, France), and glycerin (Vetec, Brazil) and sodium hydroxide (All Chemistry, Brazil). High-performance liquid chromatography (HPLC) grade trichloroacetic acid (Vetec, Brazil) and acetonitrile (Carlo Erba, Italy) were used. Quercitrin ($\geq 95\%$) was purchased from Sigma-Aldrich (St Louis, Missouri).

2.2 Extract and formulations

Kalanchoe crenata (Andrews) Haworth leaves - (Crassulaceae) - (Herbarium do Depto. de Botânica, Universidade Federal do Paraná, UPCB - n^o 72.035) were collected in April-2010. The cultivation is located in Tangará (SC/Brazil) at an altitude of 724 m (S 27°5'30'' - W 51°12'24''). Fresh leaves were used to obtain the crude extract (CE), they were extracted with water (1:5 w/v – 30 min. / 50.0±5.0 °C) in reactor tank, subsequently it was centrifuged and concentrated (20% v/v) in falling film evaporator (50.0±1.0 °C / -600 to -700 mmHg). Crude extract was partitioned with dichloromethane at pH 2.0 (conc. HCl) and then pH 11.0 (10 N NaOH), and the residual aqueous phase was neutralized and partitioned with ethyl acetate (Muzitano, 2006) for the concentrated fraction (CF).

Formulations were developed using carbopol gel (GC) shown in Table 1, using the crude extract (GCE), concentrated fraction (GCF), liposome extract (LGCE) and liposome concentrated fraction (LGCF). Liposomes were prepared using phosphatidylcholine and incorporated in the gel as instructed by the manufacturer.

Table 1. Composition percent of the *Kalanchoe crenata* formulations (w/w). Formulations were developed using carbopol gel (GC) crude extract (GCE), concentrated fraction (GCF), liposome extract (LGCE) and liposome concentrated fraction (LGCF).

Components	GC	GCE	GCF	LGCE	LGCF
Carbopol 940	4.00	4.00	4.00	4.00	4.00
Propylene glycol	3.00	3.00	3.00	3.00	3.00
Optiphen®	0.50	0.50	0.50	0.50	0.50
EDTA	0.12	0.12	0.12	0.12	0.12
Methylparaben	0.10	0.10	0.10	0.10	0.10
Glycerin	-	-	-	-	16.67
Crude Extract	-	60.00	-	20.00	-
Concentrated Fraction	-	-	0.10	-	0.10
Phosphatidylcholine	-	-	-	4.00	5.00
Water	99.28	32.28	92.18	68.28	70.52

Sodium hydroxide q.s.p. pH 7.0

2.3. Stability study

Formulations were packaged in three different containers (aluminum tube, plastic tube and plastic pot) and stored in climatic chamber at 40 ± 2 °C / $75 \pm 5\%$ RH (Zone IV). Samples were evaluated after 90, and 180 days using the following methods: organoleptic test, pH measurements, viscosity, microbiological analysis and chromatographic fingerprint. The procedures were performed in accordance with the stability test of international pharmaceuticals guideline (ICH, 2003).

2.3.1. Organoleptic test

The organoleptic features (appearance, phase separation, color and smell, homogeneity and consistency) of the samples were examined at the same temperature, lighting, and packaging conditions. The test was performed by visual inspection and direct perception of the sample, with verification of modifications over the standard (Time: 0 day). Photographs of the samples were used to facilitate comparison of the color.

2.3.2. *pH measurements*

One gram of each formulation was weighed and diluted with distilled water to a volume of 10mL. After homogenization, the pH of the samples was measured using a digital pH meter (Gehaka) and samples were run in triplicate.

2.3.3. *Weight variation*

The weight variation determination was obtained by the sample weight difference between the beginning and the end time of the stability study.

2.3.4. *Viscosity*

The viscosity was determined using a rotational viscometer (Brookfield, LVT model), with a sample of 50 g that was run in triplicate (5 min.). The temperature was adjusted to 25.0 ± 1.0 °C and a speed of 0.3 rpm. The viscosity was determined in centipoises (cP).

2.3.5. *Microbiological analysis*

Microbiological analysis was assessed by counting the total viable counts microorganisms (bacteria and fungi) and search for specific pathogens (*Pseudomonas aeruginosa*, *Staphylococcus aureus*). The methodology and acceptance criteria followed the specifications of American Pharmacopoeia (USP 32, 2009), recommended for non-sterile pharmaceutical products.

2.3.6. *Chromatographic fingerprint*

Samples of the gels were dissolved in hydrochloric acid (10%), added acetonitrile (1:1) and filtered with 0.45 µm cellulose filters. and were evaluated using chromatographic method (HPLC) described below.

2.4. *HPLC analysis*

For the quantification of quercitrin in the crude extract (CE) and concentrated fraction (CF) it was used a Shimadzu liquid chromatograph (Shimadzu Corporation, Kyoto, Japan),

equipped with a SCL-10 ATvp, solvent pump unit LC-10ATvp, and SPD-10ATvp UV-VIS detector operating at 254 nm. Samples (10 µl) were injected with SIL-10AFvp injector. The flow rate was 0.8mL/min at a constant temperature (25° C). A C18 (Kinetex 4.6 x 150 mm, 2.6 µm) chromatographic column previously stabilized was used to separate the components.

Samples of **CE** (1:2 v/v) and **CF** (1:1 w/v) were diluted in solvent (water:acetonitrile 1:1 v/v) and filtered with 0.45 µm cellulose filters. Chromatographic solvent system consisted of (A) acidulated water purified by the Milli-Q system (Milipore Milford, MA), with 0.1% trichloroacetic acid, and (B) acetonitrile. The solvents were run using the following gradient: 0% B to 60% B for 27 min, 60% B to 0% B for 1 min, 0% B for 7 min, totaling 36 min. All samples were run in duplicate. Chromatographic peaks were identified by comparing retention times with those of quercitrin standards recorded in the same conditions. Calibration curves were obtained with the mentioned standards after dilution in the mobile phase. Linearity was determined by regression and precision and accuracy was determined using the variation coefficient (CV < 3%). The determination coefficients obtained were $R^2 = 0.9869$. The content of quercitrin in crude extract (mg/ml) and concentrated fraction (mg/g) were obtained by correcting the concentration of the samples.

2.5. Efficacy of the topical formulation

2.5.1. Animals

In vivo experiments were performed on 3-month old (20-25 g) BALB/c female mice (*Mus musculus*). The animals were housed in a temperature-controlled room (22 ± 2 °C), with constant 12 h light/dark cycle and access to water and food *ad libitum* until use. All the experimental procedures adopted in this study were previously approved by the Institutional Ethics Committee (Universidade Paranaense – protocol n° 18.595/2010).

2.5.2. Inflammation and ulceration induction

The model was based on the methodology of Moura (2005), which defined the use of BALB/c female mice inoculated with *Leishmania braziliensis* to inflammation and ulcerated lesion. *L. braziliensis* strain MHOM/BR/01/BA788 was isolated from a patient with cutaneous leishmaniasis. This isolate was identified as *L. braziliensis* by using PCR and

monoclonal antibodies. The BALB/c mice ear dermis was inoculated with *Leishmania* promastigotes (10^6 parasites/15 μ L of saline). Stationary-phase promastigotes were inoculated into the posterior left ear dermis of each mouse using a BD ultra-fine II syringe.

2.5.3. Experimental protocol

Animals were divided into two control groups (n = 4) and four test groups (n = 8). The first group remained untreated (naive group), while the other groups were inoculated with *L. braziliensis*. One group was treated with vehicle only (control group). The positive controls received glucantime (100 mg/kg; i.p.). The other groups received topical application of different formulations containing crude extract (**GCE**), liposome crude extract (**LGCE**), concentrated fraction (**GCF**) and liposome concentrated fraction group (**LGCF**).

Subsequent to inoculation the mice were observed for 15 days to assess the inflammation development. The ears inflammation (mm) was measured with a digital caliper (Mitotoyo) after 15 days of intradermal inoculation and every 5 days for 15 days period. Treatment consisted in topical application of 20 to 30 mg of each formulation, with the aid of a syringe without a needle to the left ear of each animal of the four test groups. The ear inflammation (mm) was determined by Formula (1):

$$\text{Ear Inflammation (EI)} = \text{left ear thickness (LET)} - \text{right ear thickness (RET)} \quad (1)$$

2.6. Statistical analysis

The results are expressed as mean \pm standard error of mean (S.E.M) of 4-8 animals per group. Statistical analyses were performed using two-way analysis of variance (ANOVA) followed by Bonferroni's test or Student's t-test, when applicable. A *p* value less than 0.05 was considered statistically significant. The graphs were drawn and the statistical analyses were performed using GraphPad Prism version 5.0 for Windows (GraphPad Software, San Diego, CA, USA).

3. RESULTS

3.1. Extraction and concentration process

The aim of this study was to evaluate the activity of a semi-solid formulation of *Kalanchoe crenata*. At first we verified the efficiency of the extraction process by the presence of the main active compound. The use of external standard allows the identification of quercitrin at 15.366 in crude extract (CE) and 14.324 in concentrated fraction (CF) (Figure 1).

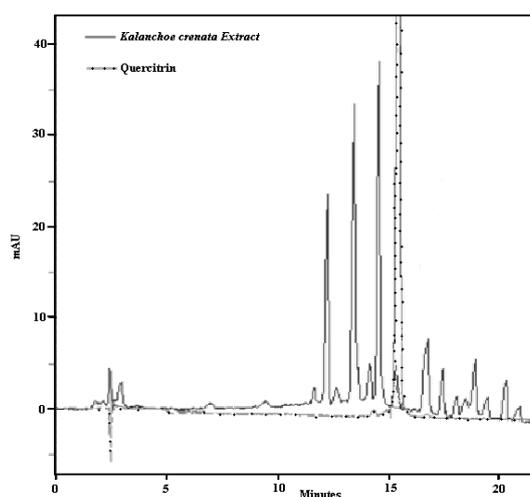


Figure 1. Representative HPLC-DAD chromatogram of *Kalanchoe crenata* extract and Quercitrin (254 nm). Chromatographic conditions are specified in materials and methods.

The content of quercitrin (quercetin 3-*O*- α -L-rhamnopyranoside) was evaluated in the crude extract (CE) and concentrated fraction (CF) from the extraction and analysis methodology described above. The HPLC chromatographic method allowed the quantification of the quercitrin in the extract and fractions, the quercitrin contents in the *Kalanchoe crenata* crude extract (CE) and concentrated fraction (CF) as 2.78 and 4.16 mg/g, respectively. Resulting in 0.0195 % yield on fresh leaves.

3.2. Development of topical formulations

A pre-formulation study was conducted to determine the compatibility between components. The carbopol base was used for a gel semi-solid formulation, with the addition

of the extract and fraction, beyond preparing the liposome formulation for both products. The samples were subjected to freezing cycles and subsequently evaluated for parameters: organoleptic test, pH measurement, viscosity and centrifugation assay (data not shown). Formulations contained in Table 1 were defined after preliminary tests. After the results of preliminary studies it was decided by the formulations (**GCE** and **GCF**) and liposome formulations (**LGCE** and **LGCF**) for the implantation of the stability study.

3.3. Stability Study

The stability study was conducted with the use of international parameters (ICH, 2003), storage conditions for Zone IV climatic regions were selected (40 ± 2 °C and 75 ± 5 % RH). Samples of the formulations (**GC**) containing crude extract (**GCE**) and concentrated fraction (**GCF**), liposomes formulations containing extract (**LGCE**) and fraction (**LGCF**) were placed in three different packs and analyzed in duplicate at each time (60, 90 and 180 days). The parameters evaluated, patterns and variations accepted in the stability study are described in Table 2. Regarding the organoleptic characteristics, small changes after 90 days (darkening) were observed in visual products contained in plastic containers, the other samples remained the standard of the initials organoleptic test. The pH values remained within the expected range for most formulations packaged in tubes. Only samples packaged in plastic pot had varying pH values above the level ($-5.83 \pm 0.37\%$) in the beginning of the stability study, with acidification. At the end of the stability study the samples that were packed in plastic pot presented weight variation above the limit established ($17.68 \pm 6.6\%$).

Table 2. Parameters evaluated in stability study for *Kalanchoe crenata* semisolid formulations for Zone IV climatic regions (40 ± 2 °C and 75 ± 5 % RH).

Parameters	Standard (accepted limit %) *		Alterations occurred after 180 days
	Gel Formulation	Gel Liposome Formulation	
Organoleptic test	Comparison with product standard - 0 day to color, odor visual appearance, consistency and texture. (Subjective evaluation by visual inspection and direct perception of the sample.)		Plastic Pot (Darkening)
pH *	7.0 ($\pm 5\%$)	7.0 ($\pm 5\%$)	Plastic Pot (-5.83 \pm 0.37%)
Weight variation (%)*	Initial value ($\leq 5\%$)	Initial value ($\leq 5\%$)	Plastic tube (17.68 \pm 6.6%)
Viscosity (cP) *	1.000 – 1.200 ($\leq 10\%$)	1.200 – 1.300 ($\leq 10\%$)	Plastic Pot (-23.18 \pm 6.73%) Aluminum tube (-14.99 \pm 3.92%) Plastic tube (-22.59 \pm 16.22%)
Microbiological	Total microorganisms: < 100 CFU / g Fungi: < 10 CFU / g Pathogens: Absence of <i>Staphylococcus aureus</i> and <i>Pseudomonas aeruginosa</i> / g		No
Fingerprint	Comparison with product standard - 0 day to HPLC chromatogram.		No

* ICH Working Group, 2003.

The viscosity values of the samples followed variation in relation to the initial time, with variation for samples packaged in plastic pot (-23.18 \pm 6.73%), aluminum tube (-14.99 \pm 3.92%) and plastic tube (-22.59 \pm 16.22%). The alterations found for organoleptic characteristics, pH and viscosity suggest that greater permeability to water vapor mainly in products in plastic pot, consequent to the modification of the parameters analyzed.

No significant results for microbiological analysis were found during the stability study. The microbiological analysis evaluation showed the efficiency of manipulation techniques and microbiological preservatives to preserve the formulations characteristics and prevent growth of microorganisms. The samples were subjected to HPLC analysis using the same methodology described above. Chromatographic fingerprint analysis revealed the presence of a degradation compound detected on the method used for quantification of quercitrin (Figure 2). This degradation compound was common to all formulations and packaging and it was unable to identify with the techniques used in this study. A comparison of samples (different times and packaging) of the baseline showed that there was no change in the chromatographic peak quercitrin.

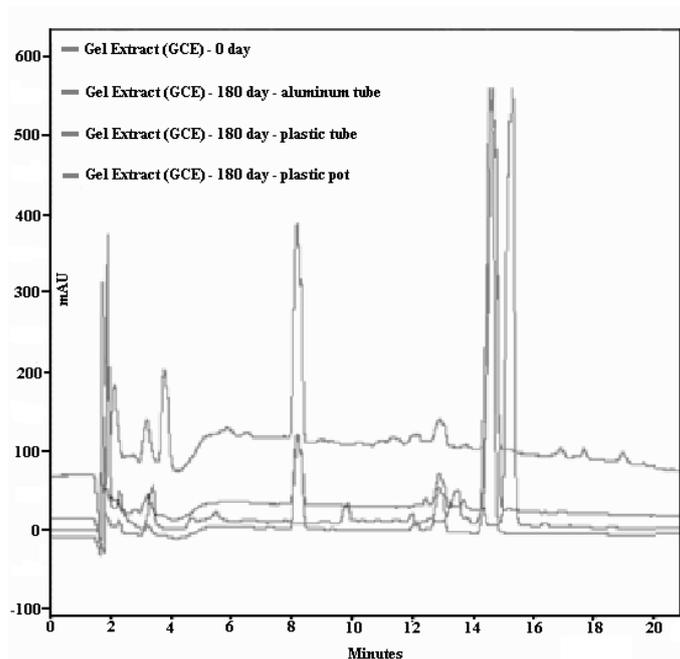


Figure 2. Representative HPLC-DAD fingerprint (254 nm) of *Kalanchoe crenata* formulation before and after stability study. Chromatographic conditions are specified in materials and methods.

3.4. Pharmacology activity

The anti-inflammatory effect of four formulations was tested against *Leishmania* in a mice model. The lesion area in mice ear infected with *L. braziliensis* was significantly increased after 15 days of initial contact with the parasite (Figure 3). The lesion size remains unaltered until the end of the experiment (30 days) in mice which not received treatment. Mice treated with the **GCE** and **LGCE** showed a significant decrease on lesion size on the 10th day of treatment, with an approximate reduction of 40% at the end of 15 days. In the same way, but only after 15 days of treatment, the mice treated with **GCF** and **LGCF** also showed a reduction of the ulcerated area of approximately 40%. The liposome formulation was not more effective than the extract or fraction in their original formulation. All tested extract or fraction showed similar effectiveness to the positive control (glucantime) at the end of experimental period.

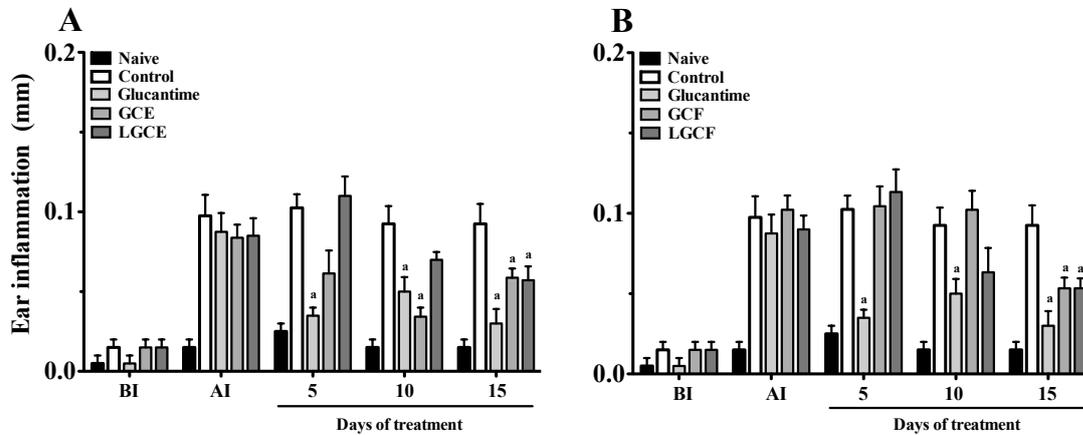


Figure 3. Effect of prolonged topic treatment with four semisolid formulations from *Kalanchoe crenata* on ear inflammation induced by *Leishmania braziliensis* in mice. After 15 days of infection the animals were treated (topically) once a day with crude extract (GCE), liposome crude extract (LGCE), concentrated fraction (GCF), liposome concentrated fraction (LGCF), or Glucantime (i.p.) and control group. The inflammation of the ears was measured before (BI) and after (AI) 15 days of intradermal inoculation and every 5 days for 15 days after the treatment. The ear inflammation (mm) was measured with a digital caliper. Each bar represents the mean of 4-8 animals and the vertical lines show the S.E.M. Asterisks denote the significance levels in comparison with the control group (two-way ANOVA followed by Bonferroni test) ($p < 0.05$).

4. DISCUSSION

The positive anti-leishmania effect of *Kalanchoe* has been reported by different authors (Da Silva, 1995; Rossi-Bergman, 1997; Da Silva, 1999; Ibraim, 2002). This pharmacological effect is associated to the flavonoids, mainly quercitrin (Muzitano, 2006). In this study we sought to verify the semisolid formulation activity containing *K. crenata* extract against *L. braziliensis* lesion in mice.

Different challenges are associated with the formulation development from natural products, including the botanical identification. Studies of leishmanicidal activity are normally associated to *K. pinnata* and *K. brasiliensis* species. The *K. brasiliensis* botanical name is a synonym for *K. crenata* (Zappi, 2012). *K. pinnata* (*B. pinnatum*) and *K. crenata* show close proximity in traditional usage, habitat, preparation and identification. The external

morphological features of *K. crenata* resemble that of *B. pinnatum*. This is attested by the same local name that is being used in Africa, and ethnobotanically, they are most often prepared and administered the same way (Akinsulire, 2007).

Flavonoids are chemical compounds present in plants of the *Kalanchoe* genus (Singab, 2011, Muzitano, 2011). The *Kalanchoe crenata* extract is rich in flavonoids including quercitrin (Rossi-Bergmann, 1997). This glycoside flavonoid was also isolated from *Kalanchoe pinnata* and proved to be a potent antileishmanial compound, with a low toxicity profile (Muzitano, 2006). Based on these studies it was defined a quercitrin standard formulation, and it was evaluated the concentration of this compound in the crude extract (CE) and fraction (CF). The yield of quercitrin in the extract and fraction was similar compared to previous work. Muzitano (2011) performed flavonoids quantification in *K. pinnata* in different seasons of the year. The variation in the content of flavonoids in different periods of the year was considerable. The values found for the autumn period are similar to those observed in our work to *K. crenata*. The environmental conditions of collection were not subject to verification in our work, but for *K. pinnata* the sunlight is attributed as the main factor correlated with increased levels of flavonoids.

Another challenge in developing formulations refers to chemical stability of the components related to the biological effect. Flavonoids are particularly sensitive to instability processes, which compromises the results of these pharmacological compounds. Quercitrin (quercetin 3-*O*- α -L-rhamnopyranoside) is a glycoside flavonoid known for its anti-inflammatory (Medina, 2002), antioxidant (Wagne, 2006; Ham, 2012) and leishmanicidal activity (Silva, 2012). To check the stability of this compound, after the incorporation of the extract or fraction it was performed a stability study. The study was conducted with four formulations using carbopol gel: extract (GCE), concentrated fraction (GCF), liposome extract (LGCE) and liposome concentrated fraction (LGCF), three different packaging (aluminum tube, plastic tube and plastic pot) and international guides criteria were used (ICH, 2003). The stability study showed that all of the formulations were physically stable under the investigated conditions. The formulations presented stability in their characteristics during the time and conditions of storage and the only alterations are related to the type of packaging. Comparison between chromatogram fingerprint at different times of the study show that there was no decrease in the content of quercitrin (Figure 2). The degradation compounds are probably not associated with quercitrin or other flavonoids present in the extract.

Aiming to verify a possible beneficial effect of the extract and fraction, formulations were developed with liposomes. Liposomes are spherical particles that encapsulate a fraction of the solvent, in which they freely diffuse (float) into their interior. Liposomes are able to enhance the performance of products by increasing ingredient solubility, improving ingredient bioavailability, enhanced intracellular uptake and altered pharmacokinetics and biodistribution (Xiao, 2002). Several studies have reported different strategies for modified release systems with several advantages compared with the isolated polymers or others pharmaceutical excipients (Dornelas, 2011). Liposomes are usually formed from phospholipids, and have been used to change the pharmacokinetics profile of, not only drugs, but also herbs, vitamins and enzymes (Ajazuddin, 2010). Works have shown positive effects in the use of liposomes to the cell penetration and activity on the intracellular parasite (Montanari, 2010). The formulations developed in which the extract (**LGCE**) and fraction (**LGCF**) were incorporated in liposome forms showed similar stability studies. The onset of degradation compounds was not related to decreased content of the marker, which was common to both formulations.

The evaluation of the pharmacological activity of *Kalanchoe crenata* extracts and fraction formulations was also the subject of this study. To verify the effectiveness of the formulations we used the induction model of leishmaniasis in BALB/c mice. This dermal model of infection with *L. braziliensis* is able to reproduce aspects of the natural infection, such as the presence of an ulcerated lesion, parasite dissemination to lymphoid areas, and the development of a Th1-type immune response (Moura, 2005). Significant differences were found for the effect of the formulations compared to control groups (Figure 3). The formulations developed with extract (**GCE** and **LGCE**) and fraction (**GCF** and **LGCF**) were very effective in reducing the inflammation area associated with infection by *L. braziliensis* after 15 days of treatment.

Despite their elevated effectiveness, there was no difference in activity between the formulations (**GCE** and **GCF**) and liposome formulations (**LGCE** and **LGCF**). One possible explanation for these results is related to the mechanism of action of the *Kalanchoe* extract and consequently its secondary metabolites. The leishmanicidal mechanism is related to modulation of the immunological response and not to a direct action on the parasite, and in this case, the flavonoid quercitrin may be involved in the modulation of the immune response (Cruz, 2008; Cruz, 2012). Several flavonoids against leishmania were assessed in *in vitro* studies (Weniger, 2004), and the most active compounds were the biflavonoids bilobetin,

ginkgetin and isoginkgetin. Quercetin derived compounds, among them the quercitrin, are presented as a multiple drug targets for the treatment of leishmaniasis (Silva, 2012). Although some flavonoids have a direct effect against intracellular amastigote form of leishmania, it seems that the activity of the extract of *Kalanchoe crenata* is not explained by this mechanism. Reinforcing above, the anti-inflammatory and immunosuppressive effect of the *Kalanchoe crenata* (*Kalanchoe brasiliensis*) extract was observed in previous work (Ibrahim, 2002) and this activity may explain the results found in our work for a formulation containing standardized extract.

Furthermore, other species of the genus *Kalanchoe* also had significant leishmanicidal effect. Oral treatment with *Kalanchoe pinnata* delayed onset of disease as compared to untreated mice or the ones treated by intravenous or topical routes. Later work demonstrated the effectiveness of the *Kalanchoe pinnata* extract for the oral treatment of human cutaneous leishmaniasis (Torres-Santos, 2003), leishmanicidal mechanisms involved (Da Silva, 1999) and main compounds responsible for this activity (Muzitano, 2006). Our results are not sufficient to indicate a leishmanicidal full effect, because this study was not to evaluate the survival of the parasites (Moura, 2005). However it is possible to assert a significant decrease in the inflammatory response using the topical formulation.

CONCLUSION

In this work we demonstrated the development of a *K. crenata* semi-solid formulation for topical use with quercitrin standardized content showed a positive effect on the inflammatory response associated with BALB/c mice infection by *L. braziliensis*.

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REFERENCES

Ajazuddin SS. Applications of novel drug delivery system for herbal formulations. *Fitoterapia*. 81(7): 680 – 689, 2010.

Akinsulire OR, Aibinu IE, Adenipekun T, Adelowotan T, Odugbemi T. In vitro antimicrobial activity of crude extracts from plants *Bryophyllum pinnatum* and *Kalanchoe crenata*. *Afr. J. Tradit. CAM*. 4(3): 338 – 344, 2007.

Barrett MP & Gilbert IH. Perspectives for new drugs against trypanosomiasis and leishmaniasis. *Curr. Top. Med. Chem*. 2(5): 471 – 482, 2002.

Cruz EA, Da-Silva SAG, Muzitano MF, Silva PMR, Costa SS, Rossi-Bergmann B. Immunomodulatory pretreatment with *Kalanchoe pinnata* extract and its quercitrin flavonoid effectively protects mice against fatal anaphylactic shock. *Int. Immunopharmacol*. 8(12): 1616 - 1621, 2008.

Cruz EA, Reuter S, Martin H, Dehzad N, Muzitano MF, Costa SS, Rossi-Bergmann B, Buhl R, Stassen M, Taube C. *Kalanchoe pinnata* inhibits mast cell activation and prevents allergic airway disease. *Phytomedicine*. 19(2): 115 – 121, 2012.

Da-Silva SAG, Costa SS, Mendonça SCF, Silva EM, Moraes VLG, Rossi-Bergmann B. Therapeutic effect of oral *Kalanchoe pinnata* leaf extract in murine leishmaniasis. *Acta Trop*. 60(3): 201 – 210, 1995.

Da-Silva SAG, Costa SS, Rossi-Bergmann B. The anti-leishmanial effect of *Kalanchoe* is mediated by nitric oxide intermediates. *Parasitology*. 118(6): 575 – 582, 1999.

Dornelas CB, da Silva AM, Dantas CB, Rodrigues CR, Coutinho SS, Sathler PC, Castro HC, Sousa Dias LR, Sousa VP, Cabral LM. Preparation and evaluation of a new nano pharmaceutical excipients and drug delivery system based in polyvinylpyrrolidone and silicates. *J. Pharm. Pharm. Sci*. 14(1): 17 – 35, 2011.

Fonseca YM, Catini CD, Vicentini FTMC, Cardoso JC, Albuquerque Junior RLC, Fonseca MJV. Efficacy of Marigold Extract-Loaded Formulations Against UV-induced Oxidative Stress. *J. Pharm. Sci.* 100(6): 2182 – 2193, 2011.

Ham YM, Yoon WJ, Park SY, Song GP, Jung YH, Jeon YJ, Kang SM, Kim KN. Quercitrin protects against oxidative stress-induced injury in lung fibroblast cells via up-regulation of Bcl-xL. *J. Funct. Foods.* 4(1): 253 – 262, 2012.

Ibrahim T, Cunha JMT, Madi K, Fonseca LMB, Costa SS, Koatz VLG. Immunomodulatory and anti-inflammatory effects of *Kalanchoe brasiliensis*. *Int. Immunopharmacol.* 2(7): 875 – 883, 2002.

ICH Working Group. Stability Testing of New Drug Substances and Products Q1A(R2), 6 February, 2003.

Medina FS, Vera B, Gálvez J, Zarzuelo A. Effect of quercitrin on the early stages of hapten induced colonic inflammation in the rat. *Life Sci.* 70(26): 3097 – 3108, 2002.

Montanari J, Maidana C, Esteva MI, Salomon C, Morilla MJ, Romero EL. Sunlight triggered photodynamic ultradeformable liposomes against *Leishmania braziliensis* are also leishmanicidal in the dark. *J. Control Release.* 147(3): 368 – 376, 2010.

Moura TR, Novais FO, Oliveira F, Clarêncio J, Noronha A, Barral A, Brodskyn C, Oliveira CI. Toward a novel experimental model of infection to study American cutaneous leishmaniasis caused by *Leishmania braziliensis*. *Infect Immun.* 73(9): 5827 – 5834, 2005.

Muzitano MF, Cruz EA, Almeida AP, Da Silva SAG, Kaiser CR, Guette C, Rossi-Bergmann B, Costa SS. Quercitrin: an antileishmanial flavonoid glycoside from *Kalanchoe pinnata*. *Planta Med.* 72(1): 81 – 83, 2006a

Muzitano MF, Tinoco LW, Guette C, Kaiser CR, Rossi-Bergmann B, Costa SS. The antileishmanial activity assessment of unusual flavonoids from *Kalanchoe pinnata*. *Phytochemistry.* 67(18): 2071 – 2077, 2006b.

Muzitano MF, Bergonzi MC, De Melo GO, Lage CLS, Bilia AR, Vincieri FF, Rossi-Bergmann B, Costa SS. Influence of cultivation conditions, season of collection and extraction method on the content of antileishmanial flavonoids from *Kalanchoe pinnata*. *J. Ethnopharmacol.* 133(1): 132 – 137, 2011.

Naderer T & McConville MJ. The *Leishmania* – macrophage interaction: a metabolic perspective. *Cell Microbiol.* 10(2): 301 – 308, 2008.

Nguelefack TB, Nana P, Atsamo AD, Dimo T, Watcho P, Dongmo AB, Tapondjou LA, Njamen D, Wansi SL, Kamanyi A. Analgesic and anticonvulsant effects of extracts from the leaves of *Kalanchoe crenata* (Andrews) Haworth (*Crassulaceae*). *J. Ethnopharmacol.* 106(1): 70 – 75, 2006.

Oliveira LF, Schubach AO, Martins MM, Passos SL, Oliveira RV, Marzochi MC, Andrade CA. Systematic review of the adverse effects of cutaneous leishmaniasis treatment in the New World. *Acta Trop.* 118(2): 87 – 96, 2011.

Pavli A, Maltezou HC. Leishmaniasis, an emerging infection in travelers. *Int. J. Infect Dis.* 14(12): e1032 – e1039, 2010.

Reis LC, Brito MEF, Souza MA, Pereira VRA. Mecanismos imunológicos na resposta celular e humoral na leishmaniose tegumentar americana. *Rev. Pat. Trop.* 35(2): 103 – 115, 2006.

Rocha LG, Almeida JRGS, Macêdo RO, Barbosa-Filho JM. A review of natural products with antileishmanial activity. *Phytomedicine.* 12(6-7): 514 – 535, 2005.

Rossi-Bergmann B. Brazilian medicinal plants: A rich source of immunomodulatory substances. *Ci. Cult.* 49(5-6): 395 – 401, 1997.

Saha P, Mukhopadhyay D, Chatterjee M. Immunomodulation by chemotherapeutic agents against Leishmaniasis. *Int Immunopharmacol.* 11(11): 1668 – 1679, 2011.

Schwartz E, Hatz C, Blum J. New World cutaneous leishmaniasis in travellers. *Lancet Infect Dis.* 6(6): 342 – 349, 2006.

Silva ER, Maquiaveli CC, Magalhães PP. The leishmanicidal flavonols quercetin and quercitrin target *Leishmania (Leishmania) amazonensis* arginase. *Exp. Parasitol.* 130(3): 183 – 188, 2012.

Singab ANB, El-Ahmady SH, Labib RM, Fekry SS. Phenolics from *Kalanchoe marmorata* Baker, Family Crassulaceae. *B. Fac. Pharm. - Cairo University.* 49(1): 1 – 5, 2011.

Torres-Santos EC, Da-Silva SAG, Costa SS, Santos APPT, Almeida AP, Rossi-Bergmann B. Toxicological analysis and effectiveness of oral *Kalanchoe pinnata* on a human case of cutaneous leishmaniasis. *Phytother. Res.* 17(7): 801 – 803, 2003.

Xiao YL & Li B. Drug-loaded nanoparticle and TCM modernization. *Chinese Trad. Herb. Drugs.* 33(5): 385 – 388, 2002.

Weniger B, Vonthron-Sénécheau C, Kaiser M, Brun R, Anton R. Comparative antiplasmodial, leishmanicidal and antitrypanosomal activities of several biflavonoids. *Phytomedicine.* 13(3): 176 – 180, 2006.

Wagner C, Fachinetto R, Corte CLD, Brito VB, Severo D, Dias GOC, Morel AF, Nogueira CW, Rocha JBT. Quercitrin, a glycoside form of quercetin, prevents lipid peroxidation *in vitro*. *Brain Res.* 1107(1): 192 – 198, 2006.

World Health Organization (WHO), 2010. WHA60.13 - Control of leishmaniasis. Disponível em: <http://www.who.int/neglected_diseases/mediacentre/WHA_60.13_Eng.pdf>. Acesso em 21 de fevereiro de 2013.

Zappi, D. 2012. *Crassulaceae* in Lista de Espécies da Flora do Brasil. Jardim Botânico do Rio de Janeiro. Disponível em: < <http://floradobrasil.jbrj.gov.br/2012/FB025394>>. Acesso em: 25 de março de 2013.