Pharm Res (2011) 28:331–336
DOI 10.1007/s11095-010-0279-3

RESEARCH PAPER

Evidence of Deep Percutaneous Penetration Associated with Anti-Inflammatory Activity of Topically Applied *Helicteres gardneriana* Extract: A Photoacoustic Spectroscopy Study

Juliana Oliveira de Melo • Franciana Pedrochi • Mauro L. Baesso • Luzmarina Hernandes • Maria Conceição T. Truiti • Silmara Baroni • Ciomar A. Bersani-Amado

Received: 4 August 2010 / Accepted: 13 September 2010 / Published online: 22 September 2010
© Springer Science+Business Media, LLC 2010

ABSTRACT

Purpose To apply the photoacoustic spectroscopy technique to investigate the penetration rate of topically applied *Helicteres gardneriana* extract used as anti-inflammatory agent.

Methods Experiments were performed *ex vivo* in a well-controlled group of mice. The crude extract was obtained from leaves of the plant *Helicteres gardneriana.* Croton oil was applied into the ventral surface of the mouse's right and left auricles in order to induce an inflammatory response. The left auricle was treated with crude extract, while the right one served as the control. After 6 h, the auricles were sectioned for measurements of edema intensity, myeloperoxidase activity and the formulation penetration rate.

Results Croton oil induced inflammatory response in both auricles. The application of *Helicteres gardneriana* extract reduced significantly the edema of the auricle and inhibited the activity of the myeloperoxidase enzyme. The photoacoustic data showed that the propagation of the formulation was efficient to reach the deep region of the auricle, crossing the cartilage. The strong anti-inflammatory effect was associated with the observed deep penetration of the formulation.

Conclusion This pre-clinical study showed the anti-inflammatory effect of *Helicteres gardneriana* extract. The photoacoustic technique was useful to demonstrate that this anti-inflammatory activity was associated with deep percutaneous penetration.

KEY WORDS anti-inflammatory agent · *Helicteres gardneriana* extract · percutaneous penetration · photoacoustic spectroscopy

J. Oliveira de Melo · M. C. T. Truiti · S. Baroni ·
C. A. Bersani-Amado (✉)
Departamento de Farmácia e Farmacologia
Universidade Estadual de Maringá
Avenida Colombo 5790
87020-900 Maringá, Pr, Brazil
e-mail: cabamado@uem.br

F. Pedrochi · M. L. Baesso (✉)
Departamento de Física
Universidade Estadual de Maringá
Avenida Colombo 5790
87020-900 Maringá, Pr, Brazil
e-mail: mlbaesso@uem.br

L. Hernandes
Departamento de Ciências Morfológicas
Universidade Estadual de Maringá
Avenida Colombo 5790
87020-900 Maringá, Pr, Brazil

INTRODUCTION

Anti-inflammatories are nowadays among the most prescribed drugs in the world (1,2). The side effects induced by the available oral formulations have continuously demanded the search for non-toxic compounds. Previous studies have shown that the use of topical application can reduce the toxic effects by decreasing systemic distribution of the drugs (3,4). In addition, topical formulations can deliver higher concentration of the compounds at the target area (4,5).

Natural compounds are widely studied with the aim to obtain non-toxic anti-inflammatory formulations. Exploring the popular knowledge, several plant species have been successfully investigated in order to identify the active principles and their pharmaceutical properties (6–12). Recently, we have shown that the crude extract of the *Helicteres gardneriana* (EEHg) reduces vascular permeability and cellular migration

induced by phlogistic agents (13). This natural compound can be obtained at low cost and has been shown to be effective and safe for treatment of inflammation (14,15).

On the other hand, it is recognized that the compound's penetration rates to achieve the deep regions where the inflammatory process takes place are crucial to their anti-inflammatory efficacy. Then, the topical treatment effects depend on the formulation penetration characteristics, which have been previously shown to depend on the used vehicle and the skin conditions, such as its moisture degree (3,4).

One of the limitations in the pharmaceutical area is the measurement of the formulation penetration rate through the adjacent tissue where the formulation is topically applied, especially because of the lack of suitable techniques with ability to perform depth profile analysis. Photoacoustic Spectroscopy (PAS) has already been shown to be a valuable tool to measure the penetration and distribution of substances through skin *in vitro, ex vivo* and *in vivo* (10–12,16–18). The usefulness of PAS in dermatological research has been previously demonstrated in several studies of our group (10–12,17). For example, the method was efficient to measure the behavior of the penetration rate of a bee propolis formulation during different stages of wound healing (11).

The aim of this study was, therefore, to use the photoacoustic spectroscopy to determine the penetration profile of a topically applied formulation of *Helicteres gardneriana* crude extract used as an anti-inflammatory agent in inflamed mouse auricles.

MATERIALS AND METHODS

Plant Material

Leaves of the plant *Helicteres gardneriana* were collected in March 2000 in the floodplain of the Upper Paraná River, Municipality of Taquaruçú, state of Mato Grosso do Sul. The material was appropriately preserved in the Nupelia Herbarium at the Universidade Estadual de Maringá, Paraná (HNUP nº 2844). The plant material was dried in an air-circulating oven at 40°C and then ground in a cutting mill. The *Helicteres gardneriana* extract (EEHg) was obtained by extraction with absolute ethanol at room temperature. The solvent was then removed in a rotating evaporator, resulting in ethanolic extract (13). Part of the crude extract was dissolved in MeOH:H_2O (1:1) and extracted by liquid-liquid partitioning with solvents of different polarities, resulting in the following fractions: hexane fraction (HF), hexane-acetate fraction (HAF), acetate fraction (AF), acetate-hydromethanol fraction (AMF) and hydromethanol fraction (MF). For the trials with edema of the auricle, 5 mg of EEHg and the acetate, acetate-hydromethanol and hydromethanol fractions were diluted in a solution of 20 µl of acetone/water (1:1). The hexane and hexane-acetate fractions were diluted in a solution of chloroform/water (7:3).

Croton Oil-Induced Auricle Edema in Mice

The edema was induced by application of 20 µl of croton oil (200 µg) diluted in a solution of acetone/water (7:3) or a solution of chloroform/water (7:3) to the ventral surface of the mouse's left auricle (male, 25–30 g, $n=8$). The right auricle received only the vehicle, 20 µl of acetone or chloroform, adapted from the procedure described in reference (19). Immediately after application of the phlogistic agent in the groups of treated animals, 20 µl of the crude extract (2.5, 5.0 and 7.5 mg), the fractions of EEHg (5.0 mg) or dexamethasone (0.1 mg) as positive control, were applied onto the left auricle. In the control group, 20 µl of the vehicle was applied onto the left auricle.

After 6 h, the animals were killed, and the auricles were sectioned in discs of 6.0 mm in diameter and weighed (mg) in an analytical balance with resolution of 0.0001 g, following the procedure described in ref. (20). The percentage of inhibition of edema was determined by comparing the differences in the weights of sectioned discs of control and treated auricles. In the case of the photoacoustic readings, the experiments were performed at 3 and 6 h after the edema induction in the control samples and in those treated with 5.0 mg of EEHg. The experimental protocol was approved by the Ethics Committee for Animals of the Universidade Estadual de Maringá (nº 024/2006)

Activity of Myeloperoxidase (MPO)

The MPO activity was evaluated in the supernatant of homogenates of the auricle sections (controls and those treated with crude extract: 5.0 mg; fractions: 5.0 mg; or dexamethasone: 0.1 mg), according to the technique described by Bradley *et al.* (21). The auricle tissue was placed in 50 mM potassium phosphate buffer, pH 6.0, containing 0.5% hexadeciltrimethyl ammonium bromide (HDTMA) (Sigma, 1 ml/50 mg of tissue) in a Potter homogenizer. The homogenate was vortex-mixed and centrifuged for 5.0 min at 2500 rpm. Then, 10 µl of the supernatant thus obtained was added to a 96-well microplate, in triplicate, followed by addition of 200 µl of a buffer solution containing O-dianisidine dihydrochloride (Sigma, 16.7 mg), bidistilled water (90 ml), potassium phosphate buffer (10 ml) and 1% of H_2O_2 (50 µl). The enzyme activity was determined by measuring the optical absorbance (460 nm).

Penetration Rate Evaluated by Photoacoustic Spectroscopy

The photoacoustic (PA) measurements were performed as a function of time, at 3 and 6 h after the application of the croton oil in the control and EEHg (5 mg)-treated auricles. The samples presented thicknesses varying between 100 and 300 μm. In this part of the work we selected the formulation with 5 mg of EEHg. The experiments were carried out about 20 min after killing the animals, the necessary time interval to prepare the samples with discs of about 6 mm in diameter. The measurements were performed using an experimental setup as shown in Fig. 1A. The monochromatic light was obtained from a 1000 Watts Xenon arc lamp with a stable power supply (Oriel Corporation 68820). The used monochromator was also from the Oriel Instruments, model 77250, with wavelength resolution of 6 nm. The light beam was modulated with a mechanical chopper, Stanford Research Systems SR540.

The photoacoustic cell was home-made and projected to have a minimal volume. It was made of aluminum block, machined to hold samples with maximum dimensions of about 6 mm in diameter and 1 mm thick, which allows light to enter through a high transparent quartz window of 8 mm in diameter and 2 mm thick. The microphone chamber was 15 mm away and connected to the sample holder chamber by means of a 1 mm diameter duct. The used capacitive microphone is a very sensitive 12 mm diameter Bruel&Kjaer model 2639, which presents high gain of 50 mV/Pa and flat performance for frequency response from 1 Hz to 10 kHz. The Lock-in amplifier was from EG & G Instruments, model 5110. The photoacoustic spectra were obtained for different light modulation frequencies from 13 to 60 Hz and recorded between 250 and 500 nm.

The data acquisition was performed by a personal computer, and the sample PAS spectra were normalized with respect to the carbon black signal as a function of the incident radiation wavelengths. In the photoacoustic measurements, the thermal diffusion length (μ_s) defines the sample skin depth which contributes to the signal (18,22). This parameter is defined as $\mu_s = (d/\pi f)^{1/2}$, in which d is the sample thermal diffusivity and f the light modulation frequency. With low frequencies, one can make inspection on long depth beneath skin surface, while higher frequencies probe skin surface. This is the well-known characteristic of this technique widely used to perform depth profile analysis (23). Taking f in the interval between 13 and 60 Hz and the thermal diffusivity of the skin measured before as $d = 4.0 \times 10^{-4}$ cm^2 s^{-1} (18), the value of μ_s in our measurements varied approximately from μ_s ~15 and 30 μm.

Since the studied samples presented thicknesses varying between 200 to 300 μm, and therefore were thicker than the technique probe layer, in order to guarantee the detection of the active substances that propagated through the skin, we adopted the same procedure as that described in references (10–12). The samples were excited first onto the edema side (in the ventral side of the auricle), as shown in Fig. 1B, then turned upside down to impinge the light into the dorsal side. In this way, the detection of the EEHg optical absorption bands at the dorsal side of the auricle means that the applied substances propagated through the auricle, crossing the inflamed area.

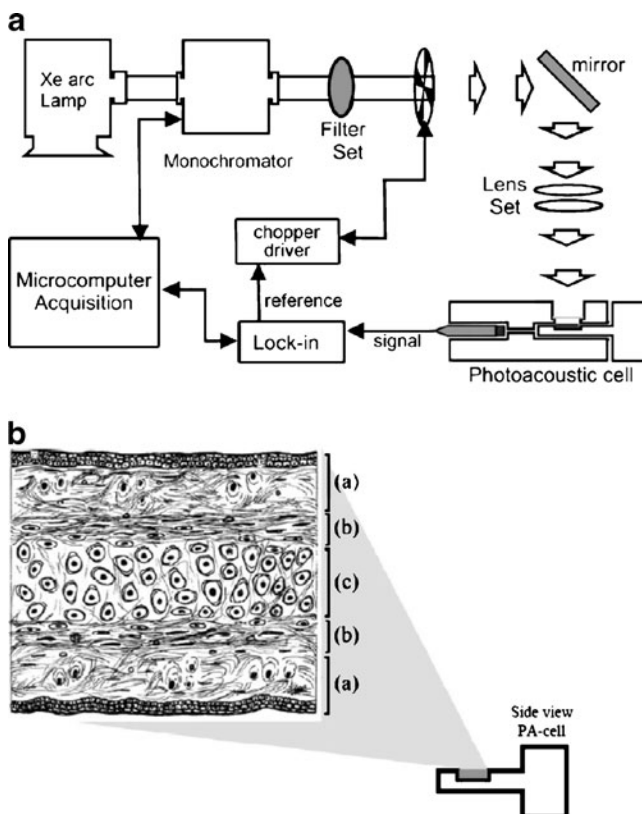

Fig. 1 (**a**): Experimental set up of the Photoacoustic Spectroscopy; (**b**): Diagram of the histological structure of the mice auricule. a) skin, b) perichondrium, c) elastic cartilage.

Statistical Analysis

The sectioned discs weights and myeloperoxidase activity results are presented as means±SD. Analysis of the data was performed using analysis of variance (ANOVA), followed by Tukey's test. $P < 0.05$ was considered as the significance level.

RESULTS AND DISCUSSION

Effect of the Crude Extract and the Fractions from *Helicteres gardneriana* on Edema of the Auricle

Application of croton oil to the left auricle of the mice induced a very evident inflammatory response by hour 6.

The weight of the auricle doubled compared to the right auricle (with no croton oil applied). The EEHg at doses 5.0 and 7.5 mg significantly reduced the intensity of edema ($EEHg_{5.0}=61\%$, $EEHg_{7.5}=72\%$; $P<0.001$), and there is no statistical difference between these groups (Fig. 2). At the dose of 2.5 mg, the EEHg did not alter the development of the edema. Additionally, application of the fractions HF, HAF, and AF at a dose of 5.0 mg also significantly reduced the edema of the auricle (93%, 64%, 69%; $P<0.001$, respectively). The fractions AMF and MF changed only slightly the response (Fig. 2). Treatment with dexamethasone, the positive control, caused an accentuated inhibition of the inflammatory response which did not differ significantly from the treatment with EEHg (5.0 and 7.5 mg) and fractions (HF and AF).

Effect of the Crude Extract of *Helicteres gardneriana* on the Activity of Myeloperoxidase (MPO)

MPO is an enzyme present in the intracellular granules of neutrophils and can be used as a marker for the influx of polymorphonuclear leucocytes into inflamed tissues. Application of croton oil induced an increase in MPO activity on the order of 20-fold at hour 6 after application of the stimulus. The EEHg (5.0 mg) and dexamethasone (0.1 mg) significantly inhibited the activity of the enzyme (72% and 78%, $P<0.001$, respectively). These results are showed in Fig. 3. Treatment of animals with the fractions reduced significantly the activity of MPO (HF=96%, $P<0.001$; HAF=88%, $P<0.001$; AF=87%, $P<0.01$; AMF=63%, $P<0.05$). The application of fraction MF did not alter the MPO activity significantly as shown in Fig. 3.

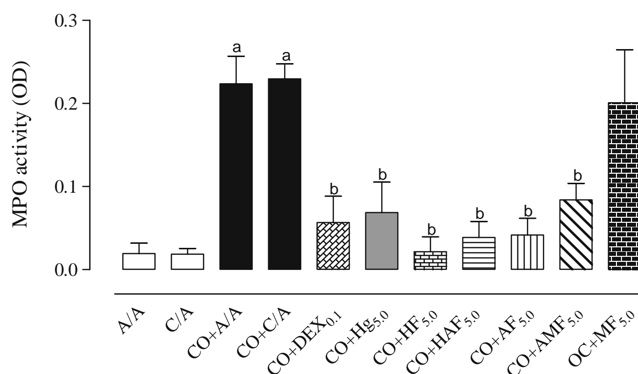

Fig. 3 Effect of the crude extract of *Helicteres gardneriana* (EEHg) and the fractions: hexane (HF), hexane-acetato (HAF), ethyl-acetate (AF), acetate-hidromethanol (AMF) and hidromethanol (MF) on MPO activity in auricle tissue from male Swiss mice (25–35 g). The animals ($n=8$ for each group) were treated topically with the extract (5.0 mg) and the fractions (5.0 mg) immediately after application of 200 μg of croton oil (CO) to the left auricle. The dexamethasone (Dex) at dose of 0.1 mg was used as the reference anti-inflammatory. A/A e C/A = auricles which received only an application of the vehicles acetone 70% or clorophorm 70%, respectively. Each column represents the mean OD (optical density) ± SD of the 5-6 auricles, 6 h after application of the croton oil. $^{a}P<0.001$ compared to the groups A/A and C/A, $^{b}P<0.001$ compared to the groups CO+A/A and CO+C/A (ANOVA, Tukey's test).

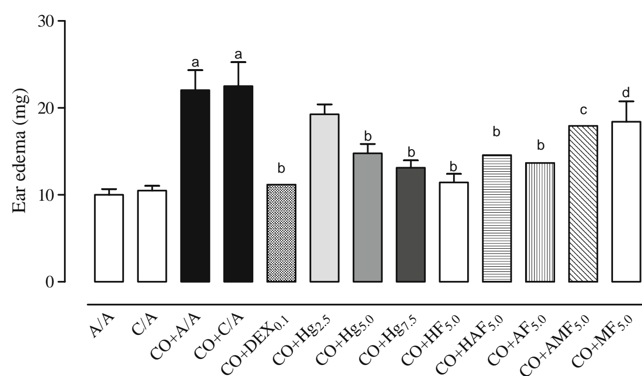

Fig. 2 Effect of the crude extract of *Helicteres gardneriana* (EEHg) and the fractions: hexane (HF), hexane-acetato (HAF), ethyl-acetate (AF), acetate-hidromethanol (AMF) and hidromethanol (MF) on edema of the auricle induced by croton oil (CO) in male Swiss mice (25–35 g). The animals ($n=8$ for each group) were treated topically with different doses of the extract (2.5, 5.0 and 7.5 mg) and the fractions (5.0 mg) immediately after application of croton oil (200 μg). The dexamethasone (Dex) at dose of 0.1 mg was used as the reference anti-inflammatory. A/A and C/A = auricles which received only an application of the vehicles acetone 70% or clorophorm 70%, respectively. Each column represents the mean weight ± SD of the 5-6 auricles, 6 h after application of the croton oil. $^{a}P<0.001$ compared to the groups A/A and C/A, $^{b}P<0.001$, $^{c}P<0.01$ an $^{d}P<0.05$ compared to the groups CO +A/A and CO+C/A (ANOVA, Tukey's test).

Penetration Rates of the Formulations

The first step in the permeation study was to apply the photoacoustic spectroscopy to measure the optical absorption spectra of the skin, the croton oil, the EEHg and the skin after application of the control oil. Their spectral responses are shown in Fig. 4. It can be observed that the

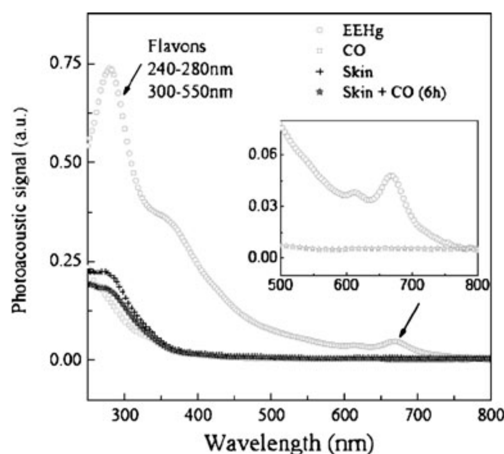

Fig. 4 Photoacoustic spectra of: EEHg; Croton oil; Skin; Skin+croton oil.

two optical absorption bands in the spectral range between 600 and 680 nm are present only in the EEHg formulation. Therefore, we assumed that the detection of the two bands along the skin thickness would be a marker of the presence of EEHg extract. The next step was the readings of the samples with the topically applied formulations, after 3 and 6 h of treatment.

Figure 5 shows the results for the 3 h treated sample using excitation in the ventral side of the auricle (as illustrated in the diagram shown in Fig. 1B). For the ventral side, the light modulation frequency was 21 Hz, which means that the probe thickness of the auricle that contributes to the generation of the photoacoustic signal is of the order of 25 μm. The presence of the extract in the inflamed region was confirmed through the two optical absorption bands between 600 and 680 nm. For comparison, the EEHg spectrum is shown again. The curves obtained with excitation in the dorsal side using 13 and 21 Hz also showed the presence of the EEHg optical absorption bands, confirming the penetration of the extract through the auricle reaching the opposite side of the cartilage. However, the EEHg bands were not detected when the spectrum was measured with the light modulation frequency at 29 Hz, which corresponds to a probe layer of about 20 μm. This means that after 3 h, the formulations did not reach the epidermis region of the dorsal side of the auricle.

Figure 6 shows the results for the sample with 6 h of treatment, demonstrating that even for 61 Hz, about 15 μm of probe layer in the dorsal side, the EEHg optical absorption bands were detected. This shows that the formulation was spread through the auricle even after 6 h of treatment, the period of time in which the edema was significantly reduced, as observed in the results shown in

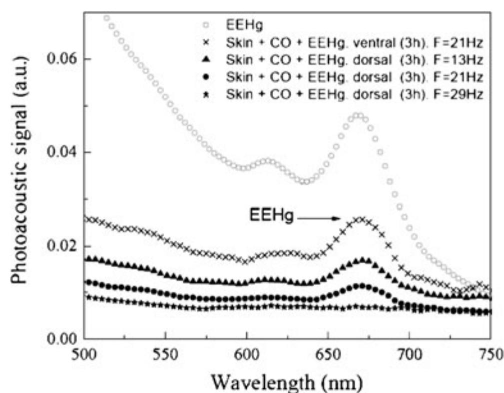

Fig. 5 Photoacoustic spectra obtained 3 h after the topical application in the ventral side of the auricle: EEHg; Skin+CO+EEHg (ventral, F=21 Hz); Skin+CO+EEHg (dorsal, F=13 Hz); Skin+CO+EEHg (dorsal, F=21 Hz); Skin+CO+EEHg (dorsal, F=29 Hz).

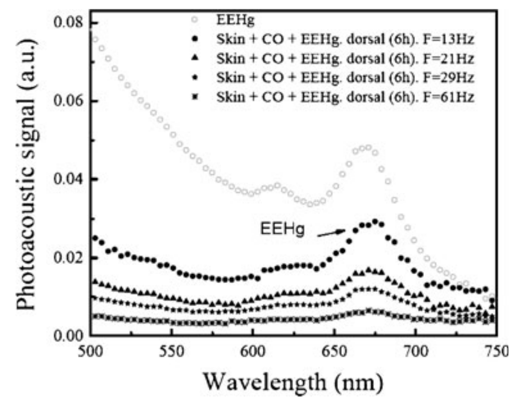

Fig. 6 Photoacoustic spectra obtained 6 h after the topical application in the ventral side of the auricle: EEHg; Skin+CO+EEHg (dorsal, F=13 Hz); Skin+CO+EEHg (dorsal, F=21 Hz); Skin+CO+EEHg (dorsal, F=29 Hz); Skin+CO+EEHg (dorsal, F=61 Hz).

Fig. 2. An estimation of the intensity of the optical absorption band center, around 670 nm, as a function of the light modulation frequency provided the values 22, 13, 7 and 4.5 for 13, 21, 29 and 61 Hz, respectively. This means that the decrease of the optical absorption band intensities approximately follows the single exponential decay dependence. This variation is practically the same as that expected for the decrease of the photoacoustic signal as a function of the light modulation frequency in homogenous samples (23). In other words, within the technique resolution, the spectra as a function of the used light modulation frequency, corresponding to a probe layer varying between approximately 15 and 30 μm, suggest that the EEHg extract is homogeneously distributed on the auricle dorsal side.

In previous works, we observed that one of the drawbacks of the photoacoustic method was the study of formulations with optical absorption bands close to those of the tested tissue (10–12). This demanded particular curve fittings of the optical absorption bands of the skin, injured tissue and formulations in order to detect the presence of the compounds in the target region where the active principle was supposed to reach. On the contrary, in the case of this work, with the optical absorption bands of the EEHg formulation in the visible spectral region, where the skin behaves like a transparent window, the tested compounds was easily detected along the whole auricle.

One possible explanation for the propagation of the formulation to the opposite side of the auricle in relation to the surface where it was applied may be the hydrophilic character of the cartilage which facilitates the propagation of compounds throughout its structure. As a final remark, it is important to highlight the observed efficacy of EEHg as an anti-inflammatory when topically applied.

CONCLUSION

We observed that the *Helicteres gardneriana* extract induced a significant reduction of the inflamed area as compared to the control group. The photoacoustic spectroscopy data provided a depth profile analysis showing that the applied substances propagated through the auricle, with evidence of percutaneous penetration associated with anti-inflammatory activity after topical application. Therefore, the results of this pre-clinical work reinforced the ability of the photoacoustic technique for drug diffusion measurements, and suggest future *in vivo* studies to correlate the dynamics of the substance diffusion rate and the mechanisms involved in the anti-inflammatory response.

ACKNOWLEDGMENTS

The authors are thankful to CAPES, CNPq and Fundação Araucária for the financial support of this work.

REFERENCES

1. Mathy FX, Lombry C, Verbeeck RK, Preat V. J Pharm Sci. 2005;94:144–52.
2. Lin J, Zhang W, Jones A, Doherty M. Br Med J. 2004;329:324–326B.
3. Lee CM, Maibach HI. J Pharmaceut Sci. 2006;95:1405–13.
4. Pereira HLA, Ribeiro SLE, Ciconelli RM. Rev Bras Reumatol. 2006;46:188–93.
5. Herkenne C, Alberti I, Naik A, Kalia YN, Mathy FX, Preat V, *et al.* Pharm Res. 2007;25:87–103.
6. Baricevic D, Sosa S, Loggia RD, Tubaro A, Simonovska B, Krasna A, *et al.* J Ethnopharm. 2001;75:125–32.
7. Kumar KTMS, Gorain B, Roy DK, Zothanpuia, Samanta SK, Pal M, *et al.* J Ethnopharmacol. 2008;120:7–12.
8. Cuellar MJ, Giner RM, Recio MC, Mañez S, Rıos JL. Fitoterapia. 2001;72:221–9.
9. Morteza-Semnani K, Saeedi M, Hamidian M. Fitoterapia. 2004;75:123–9.
10. Truite CVR, Philippsen GS, Ueda-Nakamura T, Natali MRM, Filho BPD, Bento AC, *et al.* Photochem Photobiol. 2007;83:1529–36.
11. Sehn E, Hernandes L, Franco SL, Gonçalves CCM, Baesso ML. Anal Chim Acta. 2009;635:115–20.
12. Rocha JCB, Pedrochi F, Hernandes L, de Mello JCP, Baesso ML. Anal Chim Acta. 2007;587:132–6.
13. Truiti MCT. Chemical study and evaluation of biological activities of naturally occurring species in the Brazilian stretch of the Upper Paraná River. Ph.D. Thesis, State University of Maringá, Maringá, PR, Brazil, 2004.
14. Castaldo S, Capasso F. Fititerapia. 2002;73:S1–6.
15. Menon GK, Bommannan DB, Elias PM. Skin Pharmacol. 1994;7:130–9.
16. Stolik S, Tomas SA, Ramon-Gallegos E, Cruz-Orea A, Sanches-Sinencio F. Rev Sci Instrum. 2003;74:374–6.
17. Sehn E, Silva KC, Retuci VS, Medina AN, Bento AC, Baesso ML, *et al.* Rev Sci Instrum. 2003;74:758–60.
18. Brown SM, Baesso ML, Shen J, Snook RD. Anal Chim Acta. 1993;282:711–9.
19. Dellinger A, Zhou Z, Lenk R, MacFarland D, Kepley CL. Exp Dermatol. 2009;18:1079–81.
20. Van Arman GC. Clin Pharmacol Ther. 1974;16:900–4.
21. Bradley PP, Priebat DA, Chiristensen RD, Rothstein G. J Invest Dermatol. 1982;78:206–9.
22. Bento AC, Dias DT, Olenka L, Medina AN, Baesso ML. Braz J Phys. 2002;32:483–94.
23. Rosencwaig A. Photoacoustics and Photoacoustic Spectroscopy. Vol. 54. John Wiley & Sons; 1980.