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Chlorogenic acids from *Tithonia diversifolia* demonstrate better anti-inflammatory effect than indomethacin and its sesquiterpene lactones

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ABSTRACT

Ethnopharmacological relevance: T. diversifolia (Hemsl.) A. Gray (Asteraceae) has been used in the traditional medicine in several countries as anti-inflammatory and against other illnesses. It is important to evaluate the anti-inflammatory activity of extracts from the leaves of this species, including an infusion, to identify the main constituents of the extracts, observe their effects and correlate them with the anti-inflammatory activity.
Materials and methods: An infusion, a leaf rinse extract (LRE) and a polar extract from the rinsed leaves (PE) were obtained and analysed by HPLC-UV-DAD and infrared spectroscopy. The major compounds of these extracts were quantified. The three obtained extracts were evaluated for their anti-inflammatory activities using the paw oedema and croton oil ear oedema assays in mice. Furthermore, neutrophil migration was measured by evaluating myeloperoxidase activity.
Results: The PE consists primarily of chlorogenic acids (CAs) and lacks sesquiterpene lactones (STLs). The LRE is rich in STLs and includes a few flavonoids. The infusion is chemically similar to the PE but also contains very low amounts of STLs. The PE and LRE have better mechanisms of action than non-steroidal anti-inflammatory drugs (NSAIDs). Unlike NSAIDs, both the PE and LRE inhibit oedema and neutrophil migration. The pool of CAs from the PE of *T. diversifolia* has an additional mechanism of action, and its anti-inflammatory effect was greater than what is described in the literature for this class of compounds using the same evaluation models. The similar chemical compositions observed for the infusion and the PE, contrasted with the different activities observed, suggests the presence of antagonist compounds produced during the extraction procedure (infusion); the infusion did not inhibit oedema, however it inhibited neutrophil migration. It suggests that although the great majority of plants present CAs, the category of anti-inflammatory effect of their extracts depends on a suitable pool of compounds and an absence of antagonists, among other factors.
Conclusions: CAs from *T. diversifolia* comprise a good pool of anti-inflammatory compounds with better activity mechanisms than NSAIDs, other active compounds from the leaf extracts (STLs and flavonoids) and CAs from other plant sources. Thus, the PE of *T. diversifolia* has high potential for the development of new anti-inflammatory phytomedicines. The infusion probably contains antagonists, and therefore it can be useful to treat inflammation processes where neutrophil recruitment is involved and oedema is not.

1. Introduction

Tithonia diversifolia (Hemsl.) A. Gray (Asteraceae) is native to the lowlands of Southeastern Mexico and Central America (La Duke, 1982). Nowadays, it is spread all over the world; it is found in Central and South America as well as in Asia, Africa and Australia (Ambrosio et al., 2008). *T. diversifolia* has been exhaustively studied

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and possesses several biological activities including the following: anti-inflammatory, analgesic, antimalarial, antiviral, antidiabetic, antidiarrhoeal and antimicrobial, among others (Cos et al., 2002; Owoyele et al., 2004; Kuroda et al., 2007). Besides these numerous, documented biological activities, this plant is important due to its substantial use within traditional medicine in several countries (Frei et al., 1998; Heinrich et al., 1998; Játem-Lásser et al., 1998).

The biological activities presented by *T. diversifolia* have been attributed to the presence of sesquiterpene lactones (STLs) because these compounds occur in large amounts (>2% dry weight of leaves) in Asteraceae and comprise an important class of secondary metabolites responsible for several pharmacological or toxic activities (Heinrich et al., 1998). The investigated active compounds from *T. diversifolia* extracts are almost exclusively STLs, but include a few flavonoids and the diterpene kaurenoic acid. Polar extracts have also displayed biological activities (Cos et al., 2002; Owoyele et al., 2004), but their chemical compositions have not yet been studied in detail. In summary, although many pharmacological activities have been reported for compounds isolated from *T. diversifolia*, there is still a lack of extract standardization and high-quality phytochemical studies.

The oedema and cell recruitment are important effects in the inflammatory process. There are animal models that can be used to evaluate anti-oedematogenic effect and inhibition of cell recruitment. The carrageenan-induced paw oedema and croton oil ear oedema are good models that can evaluate both effects and are suitable for p.o. and topical evaluations, respectively (Winter et al., 1962; Tubaro et al., 1985). These models are well known and several inflammatory mediators are involved in the oedematogenic effect; nevertheless, oedema is principally related to the release of prostaglandin via cyclooxygenase (COX), an enzyme that is inhibited by non-steroidal anti-inflammatory drugs (NSAIDs) (Di Rosa, 1972; Sánchez and Moreno, 1999; Wang et al., 2001; Ueno and Oh-ishi, 2002). Cell recruitment cannot be inhibited by common anti-inflammatory drugs; thus, the NSAIDs have limited effect in some categories of inflammatory processes and present some side effects, as gastric damage (Ferreira and Vane, 1974; Parente, 2001). Therefore it is important to find anti-oedematogenic treatments that can also inhibit cell recruitment (Witko-Sarsat et al., 2000; Parente, 2001; Venkatesha et al., 2011).

Considering the possibility that polar compounds display anti-inflammatory activities without the side effects associated with STLs, the main aim of the present study was to evaluate the anti-inflammatory activity of three different extracts from the leaves of *T. diversifolia* when administered orally or topically in suitable animal models. Of the three extracts described, one is rich in STLs, another is rich in polar compounds, and the third is an infusion. We also propose to obtain the chemical profile of all extracts by HPLC-UV-DAD to identify the main chemical constituents as well as to quantify the major compounds. Finally, we propose to correlate the observed biological activities with the main metabolite groups present in each extract.

2. Materials and methods

2.1. Plant material

Leaves from *T. diversifolia* were collected by D.A. Chagas-Paula in March, 2008, in Ribeirão Preto, SP, Brazil (S 21° 10.681′; W 047° 51.541′; altitude 538 m). A voucher specimen (R.B. Oliveira 497) was deposited in the herbarium SPFR of the Departmento de Biologia, FFCLRP, USP, Ribeirão Preto, SP, Brazil. Whole leaves were air-dried at 40 °C for a week and kept in humidity and light-free conditions until the extraction process was initiated.

2.2. Extract preparation

2.2.1. Leaf rinse extract (LRE)

The LRE was obtained from 40 g of whole, dried leaves individually rinsed for 20 min with 500 ml of acetone. The obtained extract was filtered through common filter paper, and after solvent evaporation under reduced pressure, the dry residue was re-suspended in 10 ml of MeOH/H_2O (7:3, v/v) to precipitate lipophilic material. The precipitate was discarded, and the solvent from the supernatant was evaporated under reduced pressure. After evaporation, the supernatant was lyophilized and kept at −20 °C until use. This procedure promotes preferential extraction of compounds stored in glandular trichomes on the abaxial leaf surface. As the main constituents of such trichomes are STLs (Ambrosio et al., 2008), the obtained extract (453 mg) was presumed to be very rich in this class of compounds.

2.2.2. Polar extract (PE)

The polar extract (PE) was prepared using 8 g of dried leaves previously rinsed with acetone (see above) that were further macerated at room temperature with MeOH/H_2O (7:3, v/v) for three days; the solvent (40 ml) was changed every 24 h. The obtained extract was filtered through common filter paper and further partitioned three times with *n*-hexane. The hydromethanolic fraction had the solvent evaporated under reduced pressure, and was then lyophilized and kept at −20 °C until use. Using leaves previously rinsed with acetone, the extract (1.0 g) was expected to be very poor in STLs, or lack them completely, and very rich in polar compounds.

2.2.3. Infusion

The infusion was prepared from 8 g of powdered dried leaves mixed in a capped container with boiling distilled water (1:10, w/v), for 20 min. After filtration through common filter paper, the infusion was lyophilized and yielded 544.4 mg of crude material. The material was stored at −20 °C until use.

2.3. Phytochemical analysis and quantification of the major compounds

The three extracts were analysed by infrared (IR) spectroscopy and HPLC-UV-DAD. The IR measurements were carried out in a Perkin Elmer RX-ISTIR System spectrometer, and the samples were analysed in discs of KBr. The identification of the main constituents was based on UV data and comparison of their retention times in HPLC with authentic standards available in the laboratory (Ambrosio et al., 2008; Gobbo-Neto and Lopes, 2008). The HPLC-UV-DAD profiling of the three extracts was performed on a Shimadzu liquid chromatograph (LC-10 Avp pumps, SCL-10 Avp controller, SPD-10 Avp diode array detector – DAD, and software Class VP, version 5.02) using two C-18 Onix monolithic columns (3 mm × 100 mm; Phenomenex) coupled in series, with a flow rate of 1.2 ml/min and the following gradient elution: MeCN 0.1% AcOH (B)/H_2O(A) 0.1% AcOH; 0–35 min 0–25% B (linear gradient), 35–60 min 25% B (isocratic), 5 min 100% B. The samples were solubilized in 1:1 A/B to give a concentration of 2 mg/ml then filtered through a 0.45 μm PTFE membrane (Millipore). The volume injected was 20 μl. The UV-DAD detector was set to record between 210 and 600 nm, and UV chromatograms were recorded at 215, 254 and 325 nm.

The quantification was carried out using analytical curves plotted with data of standard compounds which were previously isolated in our laboratory. These compounds were solubilised in a stock solution of 1:1 MeCN–H_2O containing flavone as internal standard. The analyses were carried out in duplicate and in serial concentrations: tagitinin C from 2000 to 1 μg/mL, 5-O-(*E*)-caffeoylquinic acid from 500 to 0.25 μg/mL and luteolin from 250 to

0.5 μg/mL. The standard compounds comprise a STL, a chlorogenic acid (CA) and a flavone, respectively. Tagitinin C and luteolin were analysed at 254 nm and 5-O-(*E*)-caffeoylquinic acid at 325 nm. The chromatographic conditions are the same described in the paragraph above.

2.4. Animals

Male BALB/c mice, 25–30 g, were obtained from the animal facilities of the FCFRP, USP, Ribeirão Preto, SP, Brazil, and maintained under standard laboratory conditions (21–24 °C at 40–60% relative humidity on a 12 h light-dark cycle). The experiments were approved by the Institutional Ethical Animal Committee of the Universidade de São Paulo (protocol number 08.1.464.53.7), which followed the rules of the Brazilian Committee to Animal Care (COBEA).

2.5. In vivo anti-inflammatory assays

2.5.1. Mice paw oedema

The previously described method of carrageenan-induced paw oedema in mice (Winter et al., 1962) was used in this work. Paw oedema was induced in the hind paws of mice by the intraplantar injection of 50 μL of lambda carrageenan (Sigma–Aldrich), 1% w/v in phosphate buffered saline (PBS). The contralateral paw was used as a control, being injected with the same volume of the vehicle (PBS). The course of the oedema was monitored by measuring the thickness of footpad swelling at 0, 1, 2, 3, 4, 5 and 6 h after carrageenan injection using a dial thickness gauge calliper Mitutoyo (Kawasaki). Animals ($n = 6$) received either vehicle (control animals), or extracts at concentrations of 10, 50, 100 or 150 mg/kg, p.o., 30 min before the carrageenan administration. A tube-feeding was used as p.o. administration and the volume administrated was 10 mL/kg. Indomethacin (Sigma–Aldrich), 10 mg/kg, was used as the positive control. The vehicle for PE, infusion and indomethacin was distilled water, and for LRE it was composed of 10% Cremophor® EL in distilled water. The different vehicles did not differ significantly in their effects; in order to simplify, only one of them is shown here. The assay was double-blinded and always initiated in the same period of the day (between 9:00 am and 12:00 pm) to avoid interference with circadian variation in the level of corticosteroids (Soliman et al., 1983). The data were expressed as mean ± SEM and analysed by two-way ANOVA following the application of Bonferroni's test. The inhibition percentages were calculated according to the following formula: [(mean of values of negative control group − mean of values of treated group)/mean of values of negative control group] × 100.

2.5.2. Croton oil ear oedema

The previously described method of the croton oil ear oedema assay in mice (Tubaro et al., 1985) was used in this work with groups of eight animals. The extracts (0.05, 0.5, 1, 2 and 3 mg/ear) and vehicle (control animals) were applied topically 30 min after the application of 20 μl croton oil (Sigma–Aldrich), 5% v/v in acetone, in the inner surface of each left ear. Indomethacin (0.45 mg/ear) was used as positive control. The vehicle for PE, indomethacin and infusion was 20% distilled water in glycerine; for LRE it was 20% acetone in glycerine. The different vehicles did not differ significantly in their effects and only one is shown here. The oedema was measured 6 h after starting the experiment as the weight difference between 6 mm plugs taken from the left and right ears. The assay was double-blinded and always initiated at the same time of day, as in the above experiment (Section 2.5.1). The data were expressed as mean ± SEM and analysed by one-way ANOVA following the use of Dunnett's multiple comparison test. The inhibition percentages were calculated according to the same formula given in the previous section.

2.5.3. Measurement of neutrophil migration

The plugs of the left ears obtained in the assay described above (Section 2.5.2) were kept in 200 μl of NaEDTA/NaCl buffer (pH 4.7) at −20 °C until analysis. Samples were homogenised using a Polytron homogeniser (13,000 rpm, 3×), and centrifuged at 10,000 × *g* for 15 min, at 4 °C. The pellet was re-suspended in 200 μl 0.5% hexadecyltrimethyl ammonium bromide (Sigma–Aldrich) in buffer at pH 5.4, and homogenised one more time. The samples were re-centrifuged and 20 μL of the supernatant was used to measure myeloperoxidase (MPO) activity. The 20 μL sample was mixed with 30 μl of 0.08 M $NaPO_4$. The enzymatic reaction was carried out with 50 μL of substrate reagent (tetramethylbenzidine and hydrogen peroxide; BD-OptEIA™). After 10 min the reaction was stopped with 2.5 M H_2SO_4. The absorbance was measured at 450 nm, and the results were expressed as MPO activity (number of neutrophils 10^4/ear). The mean ± SEM was analysed by one-way ANOVA following Dunnett's multiple comparison test. The inhibition percentages were calculated according to the same formula given in the previous sections.

3. Results

3.1. Phytochemical analysis and quantification of the major compounds

The way the extracts were prepared fulfilled the objective of obtaining one extract rich in STLs, another lacking this class of compounds and a third containing polar compounds as well as STLs. The LRE is rich in STLs, as shown by the typically strong band of carbonyl stretching of the γ-lactones at 1760 cm^{-1} in the IR spectrum and UV absorption between 210 and 220 nm in the UV spectrum from the HPLC-UV-DAD analysis (Figs. 1–3; Fischer et al., 1979; Ambrosio et al., 2008). Moreover, the quantification procedure showed 479.39 μg of tagitinin C (peak 5, Fig. 3) per milligram of LRE ($y = 0.0148x + 0.0636$, $r^2 = 0.9999$). Some STLs, such as tagitinin C, show UV absorption around 250 nm because of the two conjugation extensions in the 10-membered ring. Some flavonoids are also present in the LRE (peaks 1, 3, 4 and 6—Fig. 3), as shown by their typical UV spectra (Andersen and Markhan, 2006; Gobbo-Neto and Lopes, 2008) as well as the quantification procedure: 5.10 μg of luteolin (peak 3) per milligram of LRE ($y = 0.0298x + 0.0726$, $r^2 = 0.9974$).

Conversely, the PE lacked STLs, or their quantities were undetectable in both the IR and HPLC-UV-DAD analysis (Figs. 1–3). This extract has polar compounds (39.08 μg of 5-O-(*E*)-caffeoylquinic acid – peak 7 – per milligram of PE; $y = 0.0608x - 0.2887$, $r^2 = 0.9961$) whose peaks appear in the beginning of the chromatogram until 30 min, with UV spectra characteristic of CAs (UVmax at approximately 298 and 325 nm; Gobbo-Neto and Lopes, 2008).

The infusion has a chemical profile very similar to the PE (8.02 μg of 5-O-(*E*)-caffeoylquinic acid per milligram of infusion), except for the presence of STLs at very low concentrations (3.13 μg of tagitinin C per milligram of infusion, Fig. 2C and D), which can be observed by the low-intensity peaks on the chromatogram (Fig. 3). Curiously, the IR spectrum of the infusion did not show the typical carbonyl-associated bands at 1760 cm^{-1} (Fig. 1).

3.2. In vivo anti-inflammatory assays

3.2.1. Mice Paw oedema

In carrageenan-induced mice paw oedema, the LRE and PE, whose chemical constitutions are quite different (Fig. 2B and D),

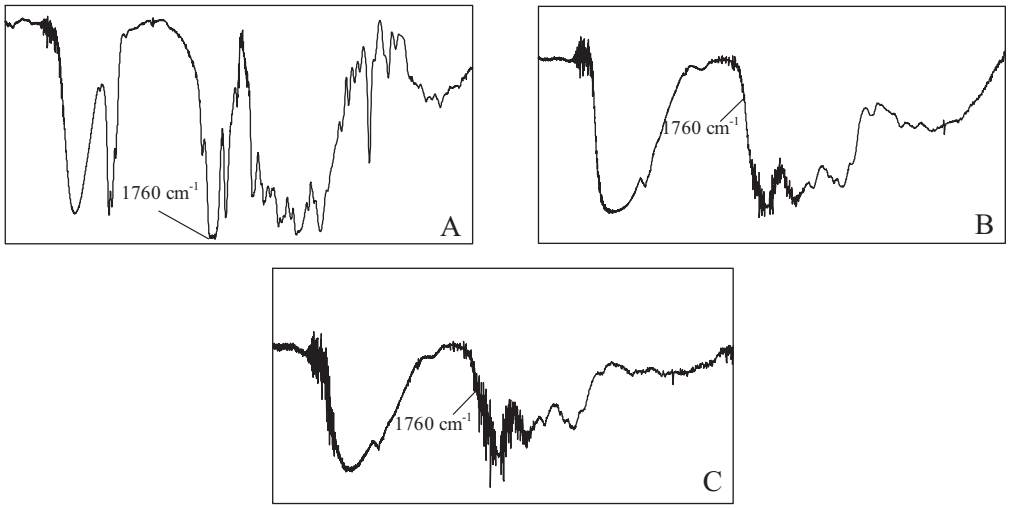

Fig. 1. Infrared spectra of LRE (A), PE (B) and infusion (C). Only LRE shows the typical strong band of carbonyl stretching of γ-lactone at 1760 cm^{-1}.

showed significant anti-oedematogenic activity (p.o.) at all tested doses (Fig. 4). The effect of the reference drug (indomethacin) and the LRE began in the third hour after treatment and remained throughout the experiment. Interestingly, the PE, at doses of 10 and 50 mg/kg (mean inhibition of 64.3 and 66.4%, respectively), showed a higher anti-oedematogenic effect than the reference drug and LRE (mean inhibition of 45.4% and 52.1%, respectively). Moreover, the activity of PE at doses of 50 and 100 mg/kg began in the first hour, before the indomethacin and LRE, which began showing effects only in the third hour. In contrast, the infusion, which is chemically similar to the PE (Fig. 2C and D), showed very different outcomes because all tested doses showed no significant anti-oedematogenic activity. A very low activity could be observed only at the largest doses (100 and 150 mg/kg; mean inhibition

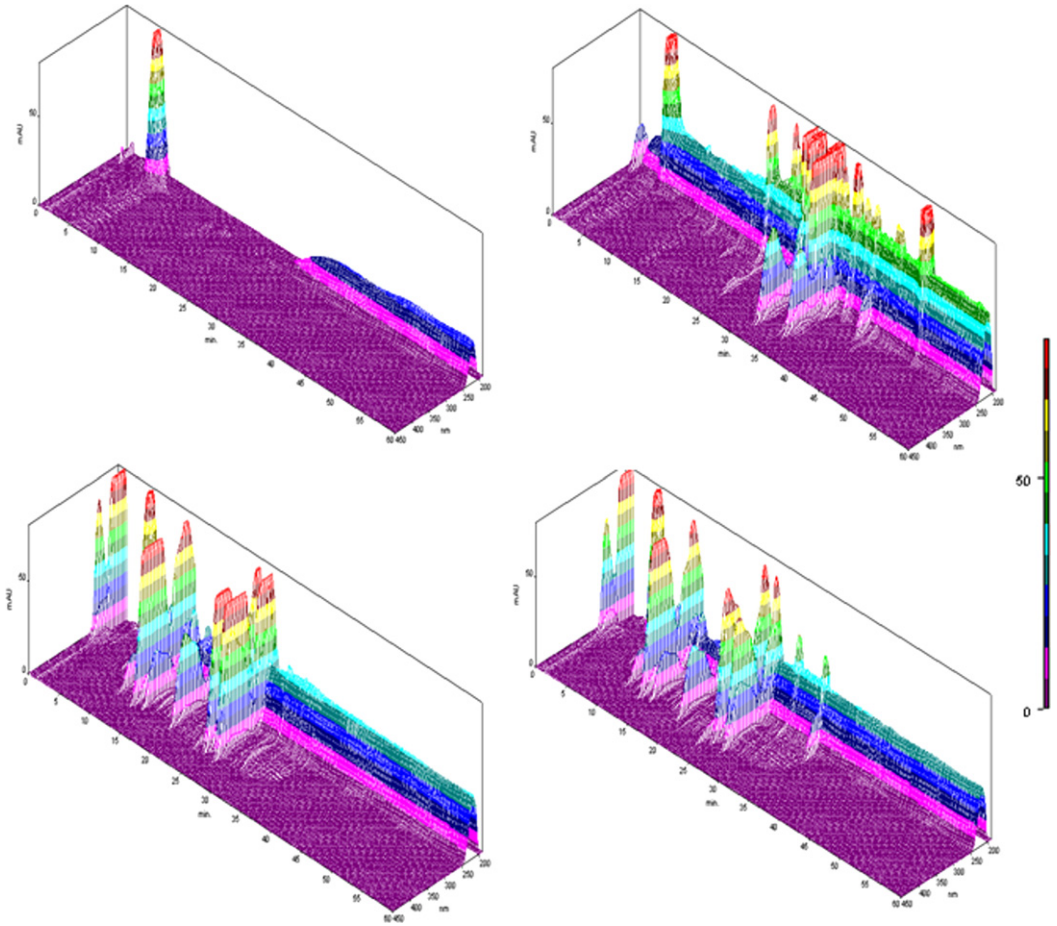

Fig. 2. 3D chromatograms of the mobile phase—MeCN:H_2O (A), LRE (B), PE (C), and infusion (D) obtained by HPLC-UV-DAD using two coupled C18 monolithic columns in series (3 mm × 100 mm); 1.2 ml/min; 0–25% MeCN in 30 min, 25% MeCN isocratic until 60 min and 100% MeCN for 5 min; 0.2% AcOH.

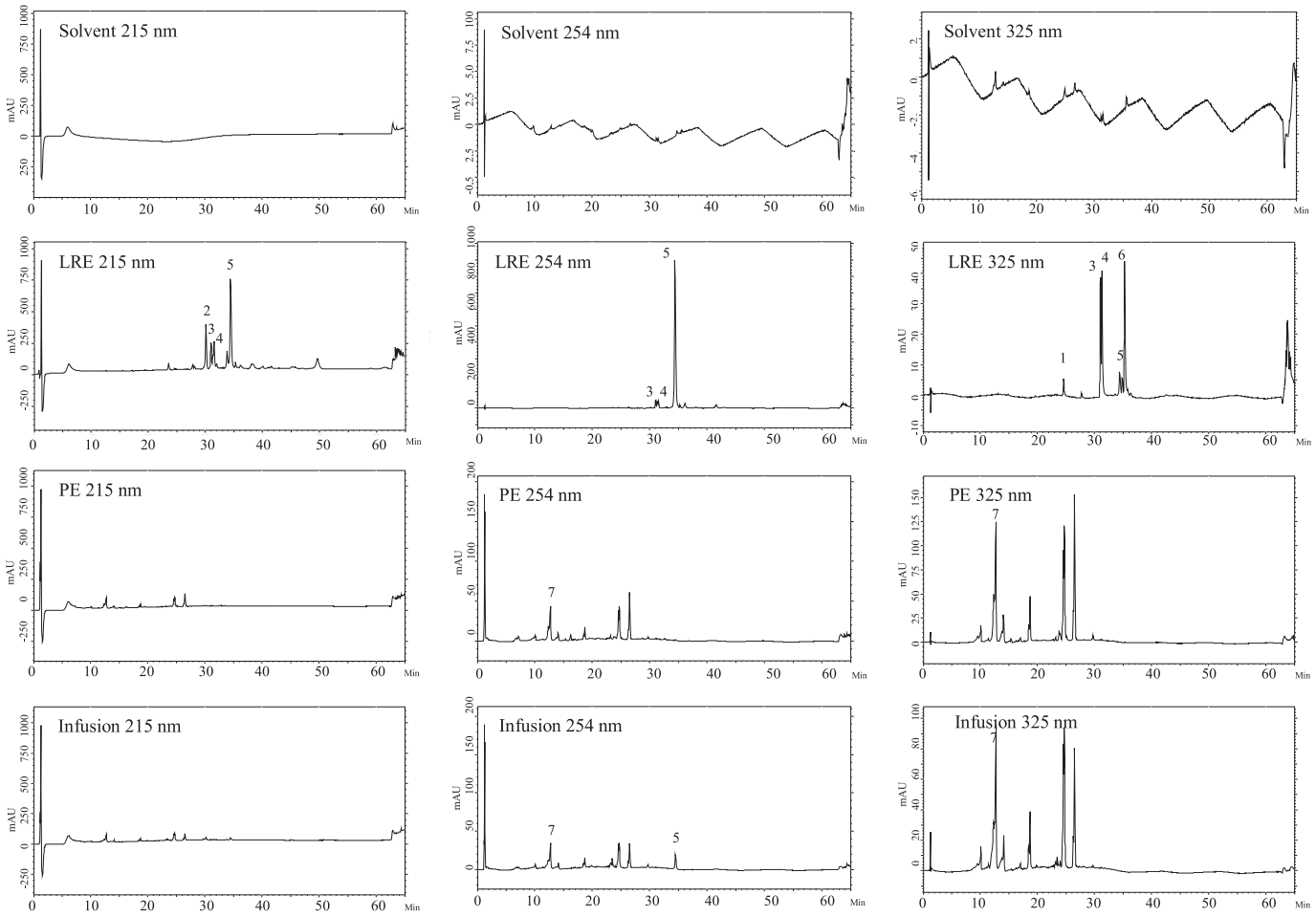

Fig. 3. Chromatograms recorded at 215, 254 and 325 nm of the mobile phase and three extracts (LRE, PE and infusion). The profiles were obtained by HPLC-UV-DAD using two coupled C18 monolithic columns in series (3 mm × 100 mm); 1.2 ml/min; 0–25% MeCN over 30 min, 25% MeCN isocratic until 60 min and 100% MeCN for 5 min; 0.2% AcOH. Intense peaks 2 and 5 are sesquiterpene lactones; weak peaks 1, 3, 4 and 6 are flavonoids; peak 7 is chlorogenic acid.

of 19.6 and 35.5%, respectively) in the sixth hour after treatment (Fig. 4).

3.2.2. *Croton oil ear oedema*

The PE showed significant anti-oedematogenic activity at all tested doses, when applied topically (Fig. 5, mean inhibition of 26.6%). The infusion did not show significant activity in diminishing croton oil-induced oedema at any tested dose, even though its activity was intermediary (mean inhibition of 16.0%) between the negative (vehicle) and positive (indomethacin; mean inhibition of 29.9%) control groups. The LRE, rich in STLs, demonstrated significant effect only at the largest doses (1, 2 and 3 mg/ear; mean inhibition of 28.2%); doses of 0.05 and 0.5 mg/ear showed only intermediary effect (mean inhibition of 16.2%).

3.2.3. *Measurement of neutrophil migration*

Reference drug and negative control groups were similarly inactive at inhibiting neutrophil migration (Fig. 6). Interestingly, for all extracts, including the infusion, the lowest doses (0.05 and 0.5 mg/ear) were significantly more active for inhibiting neutrophil migration (mean inhibition of 86.5, 83.1 and 79.7% for LRE, PE and infusion, respectively), while the highest doses (1, 2 and 3 mg/ear) did not significantly affect neutrophil recruitment to the site of inflammation (Fig. 6). The LRE at 3 mg/ear showed some, but not significant, inhibition of MPO activity (mean inhibition of 41.7%; Fig. 6).

4. Discussion

Obtaining three chemically different extracts from the leaves of *T. diversifolia* was successful (Figs. 1–3) and allowed us to evaluate the contribution of different classes of secondary metabolites to the observed anti-inflammatory activity and gain insights into their mechanisms of action. The pharmacological effect of STLs and flavonoids could be evaluated by studying the LRE, while the effect of CAs was discovered by analysing the PE. Moreover, the effect of the infusion could also be evaluated.

In this study, it was observed that the PE and LRE, when topically applied, inhibited oedema (Fig. 5) and neutrophil migration (Fig. 6). These extracts possibly act by similar way to the inhibition of COX by NSAIDs. Additionally, they inhibit neutrophil migration, mechanisms that NSAIDs do not display (Ferreira and Vane, 1974; Parente, 2001). Therefore, the PE and LRE have better mechanisms of action than NSAIDs.

When applied topically, PE at the lowest doses (0.05 and 0.5 mg/ear) showed both mechanisms of action simultaneously (inhibition of oedema and neutrophil migration; Figs. 5 and 6). The anti-inflammatory effect of LRE and indomethacin was outperformed by the PE due to another reason: the PE p.o. at the lowest doses appeared to act by an additional mechanism of action. The oral anti-oedematogenic effect was observed in the first hour, i.e., before the effect of LRE and indomethacin began in the third hour, and remained constant during the entire experi-

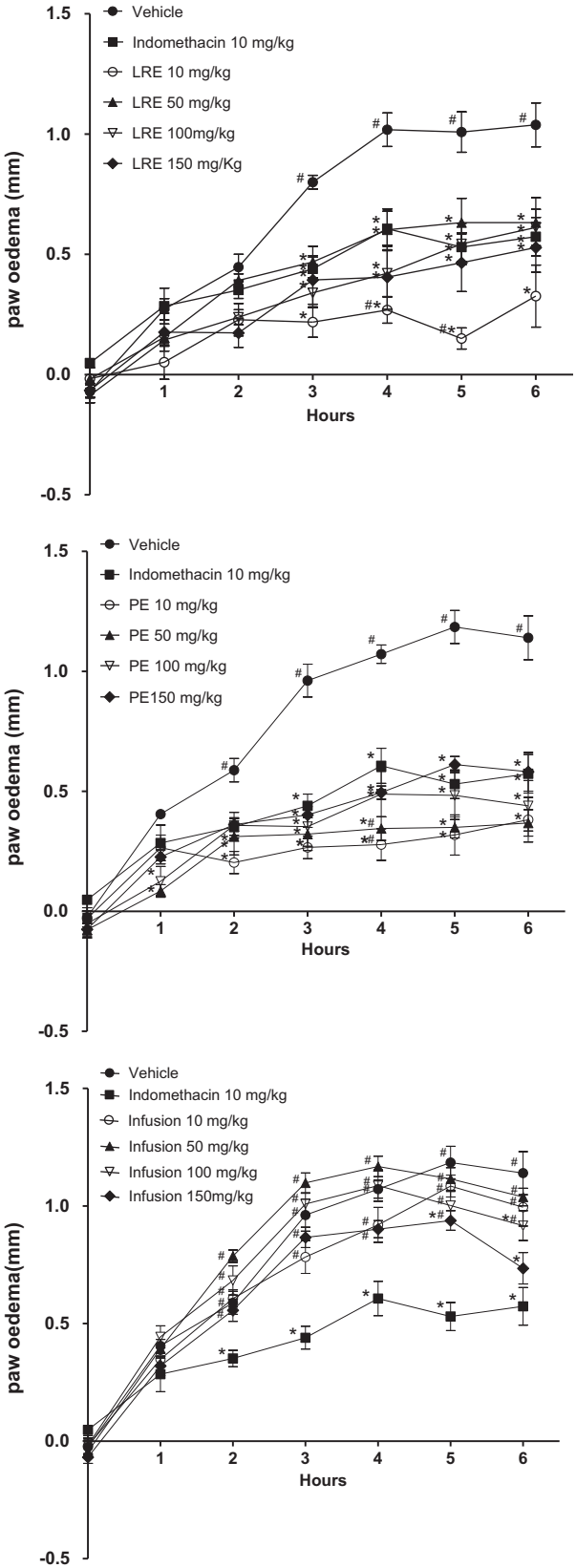

Fig. 4. Oral effect of different extracts (LRE, PE and infusion) in the carrageenan-induced mice paw oedema assay. The values represent the mean ± SEM ($n = 6$). *$P < 0.05$ when compared to the respective negative control group and #$P < 0.05$ when compared to the indomethacin group (two-way ANOVA following the Bonferroni's test).

ment (Fig. 4). During carrageenan-induced oedema, up until 2.5 h, the predominant inflammatory factors involved are histamine, 5-hydroxytryptamine, complement, nitric oxide and bradykinin (Di Rosa, 1972; Salvemini et al., 1996; Posadas et al., 2004). In addition, the doses of 10 and 50 mg/kg were more anti-oedematogenic than indomethacin at 10 mg/kg, which is considered one of the most potent NSAIDs in clinical use (Ferreira and Vane, 1974).

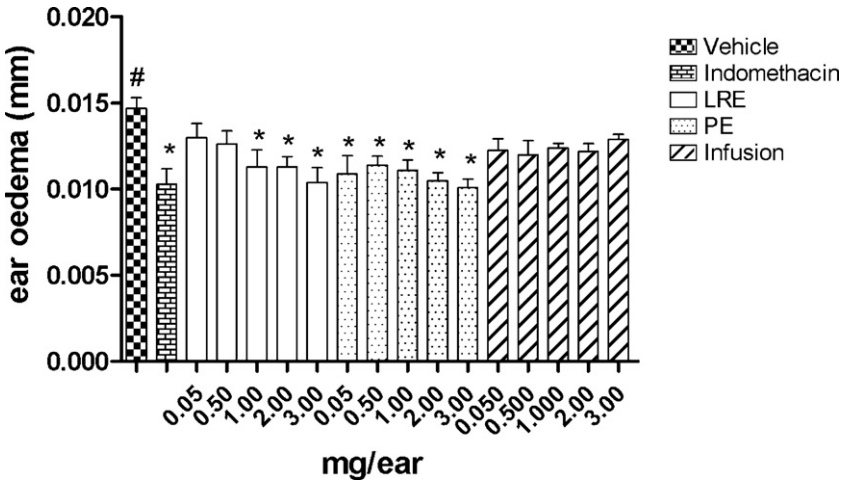

Fig. 5. Topical effect of different extracts (LRE, PE and infusion) on croton oil ear oedema assay. The values represent the mean ± SEM ($n = 8$). *$P < 0.05$ when compared to matched negative control group and #$P < 0.05$ when compared to the indomethacin group (one-way ANOVA following the Dunnett's multiple comparison test).

The previously reported anti-inflammatory effects of some pure CAs (Huang et al., 1991; Santos et al., 2006) are modest when compared with our present results. Thus, the PE from the leaves of *T. diversifolia* seems to be an efficient pool of anti-inflammatory CAs that may act synergistically. Another important issue is that the only apparent difference between the chemical compositions of the PE and the infusion is the presence of STLs at low concentrations in the infusion (Figs. 1–3). We do not believe that this difference is enough to justify the absence of anti-oedematogenic activity of the infusion compared with that of PE (Figs. 4–6). Owoyele et al. (2004) showed a significant anti-odematogenic activity for the methanolic extract of *T. diversifolia*, which theoretically would have similar composition to the infusion evaluated in this study (CAs and some STLs). Thus, we suggest the occurrence of antagonist compounds only in the infusion, which are probably produced or extracted during the preparation process (hot water). However, infuse inhibit neutrophil recruitment, so it can be useful for treatment of inflammatory process that present high cell infiltration and oedema is not involved (Witko-Sarsat et al., 2000; Castelucci et al., 2007; Venkatesha et al., 2011). In fact, the results justify the use of *T. diversifolia* in traditional medicine.

Studying the anti-inflammatory effect of CAs, the main constituents of PE, is justified because CAs are ubiquitous in the plant kingdom, and there is evidence suggesting that the consumption of food rich in these compounds reduces the incidence of cancer, coronary heart disease and inflammatory diseases (Zhao and Moghadasian, 2010). As anti-inflammatory compounds like CAs are found in several plants, Calixto et al. (2004) suggested that even plants that are not used in folk medicine could present anti-inflammatory properties. However, the difference between results of infusion and PE (rich in such ubiquitous compounds), indicate that although the majority of plants could present anti-inflammatory activities, to have or not such activity or the category of anti-inflammatory effect depends on a lack of antagonist compounds that can be correlated with the extraction method.

Finally, it is important to mention that some side effects of NSAIDs, as gastric damage, are associated the high neutrophil infiltration (Ferreira and Vane, 1974; Parente, 2001); thus, PE and LRE can inhibit oedema and neutrophil recruitment concomitantly and possible does not display this kind of side effect. Additionally, some studies attributed a protective effect on gastric mucosa to STLs (Giordano et al., 1992; Heinrich et al., 1998), which is very important given the concern about the gastric damage displayed by NSAIDs is taken into account. One piece of evidence corroborating this statement is the fact that budlein A, a promising anti-inflammatory LST, did not show gastric damage and therefore

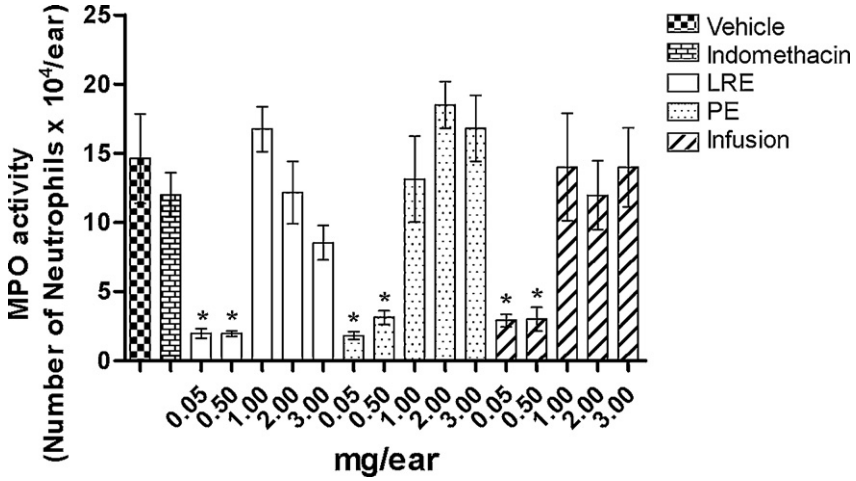

Fig. 6. Topical effect of LRE, PE and infusion on neutrophil recruitment to the inflammatory site. The measurements were on ear plugs obtained from the croton oil ear oedema assay. Reference drug and negative control groups were not significantly different, and neither inhibited neutrophil migration. The values represent the mean ± SEM ($n = 5$). *$P < 0.05$ when compared to controls groups (one-way ANOVA following the Dunnett's multiple comparison test).

may be useful for at least short-term therapies without the side effects displayed by known drugs (Valério et al., 2007). In summary, based on our results, the potential of CAs or STLs as oral and topic anti-inflammatory drugs is evident.

5. Conclusions

The STLs are not the only anti-inflammatory class of compounds from *T. diversifolia*. CAs also showed strong oral and topical activity in our models. The PE, rich in CAs, showed better results than indomethacin, one of the most potent NSAIDs, the STL-rich LRE and other CAs reported in the literature. The three extracts of *T. diversifolia*, including the infusion, seem to act by dose-dependent mechanisms that are different from the mechanisms of action of NSAIDs and can therefore be useful as alternative treatments for inflammatory diseases.

We also conclude that the mode of extract preparation also exerts influence on the activity; the infusion, which is chemically similar to the PE, showed different anti-inflammatory effect. This observation suggests that even though the majority of plant species have CAs, their anti-inflammatory effects will depend upon the absence, or at least on a low amount, of antagonist compounds.

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Glossary

CA: chlorogenic acids
COX: cyclooxygenase
IR: infrared
LOX: lipoxygenase
LRE: leaf rinse extract
MPO: myeloperoxidase
NSAID(s): non-steroidal anti-inflammatory drug(s)
PE: polar extracts from the rinsed leaves
STL(s): sesquiterpene lactone(s)