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

Daniela Aparecida Chagas-Paula[a], Rejane Barbosa de Oliveira[a], Vanessa Cristina da Silva[a], Leonardo Gobbo-Neto[b], Thaís Helena Gasparoto[c], Ana Paula Campanelli[c], Lúcia Helena Faccioli[d], Fernando Batista Da Costa[a,*]

[a] *Faculdade de Ciências Farmacêuticas de Ribeirão Preto (FCFRP), Universidade de São Paulo (USP), Departamento de Ciências Farmacêuticas. Av. Café s/no., Monte Alegre, 14040-903 Ribeirão Preto, SP, Brazil*
[b] *FCFRP, USP, Departamento de Física e Química, Brazil*
[c] *Faculdade de Odontologia de Bauru, USP, Departamento de Ciências Biológicas, Al. Dr. Octávio Pinheiro Brisolla 9-75, Vila Universitária, 17043-101 Bauru, SP, Brazil*
[d] *FCFRP, USP, Departamento de Análises Clínicas, Toxicológicas e Bromatológicas, Brazil*

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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

* Corresponding author. Tel.: +55 16 3602 0661; fax: +55 16 3602 4879.
E-mail address: febcosta@fcfrp.usp.br (F.B. Da Costa).

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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