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Outstanding Anti-inflammatory Potential of Selected Asteraceae Species through the Potent Dual Inhibition of Cyclooxygenase-1 and 5-Lipoxygenase

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Abstract

Cyclooxygenase and 5-lipoxygenase are enzymes that catalyze important inflammatory pathways, suggesting that dual cyclooxygenase/lipoxygenase inhibitors should be more efficacious as anti-inflammatory medicines with lower side effects than the currently available nonsteroidal anti-inflammatory drugs. Many plants from the family Asteraceae have anti-inflammatory activities, which could be exerted by inhibiting the cyclooxygenase-1 and 5-lipoxygenase enzymes. Nevertheless, only a small number of compounds from this family have been directly evaluated for their ability to inhibit the enzymes in cell-free assays. Therefore, this study systematically evaluated 57 Asteraceae extracts *in vitro* in enzyme activity experiments to determine whether any of these extracts exhibit dual inhibition of cyclooxygenase-1 and 5-lipoxygenase. The chemical profiles of the extracts were obtained by the high-performance liquid chromatography-ultraviolet-diode array detector method, and their major constituents were dereplicated. Of the 57 tested extracts, 13 (26.6%, IC_{50} range from 0.03–36.2 µg/mL) of them displayed dual inhibition. Extracts from known anti-inflammatory herbs, food plants, and previously uninvestigated species are among the most active. Additionally, the extract action was found to be specific with IC_{50} values close to or below those of the standard inhibitors. Thus, the active extracts and active substances of these species are potent inhibitors acting through the mechanism of dual inhibition of cyclooxygenase-1 and 5-lipoxygenase. The extracts were prepared for this study using nontoxic extraction solvents (EtOH-H_2O), requiring only a small amount of plant material to carry out the bioassays and the phytochemical analyses. In summary, this study demonstrated the potential of the investigated species as dual inhibitors, revealing their potential as pharmaceuticals or nutraceuticals.

Abbreviations

COX:	cyclooxygenase
LOX:	lipoxygenase
NO:	nitric oxide
NSAIDs:	nonsteroidal anti-inflammatory drugs
NF-κB:	nuclear factor κB
TNF-α:	tumor necrosis factor-α

Introduction

COX-1, COX-2, and 5-LOX enzymes are among the major enzymes involved in inflammatory processes. Therefore, multitarget inhibitors of these enzymes should be more effective anti-inflammatory medicines with lower side effects than the currently available NSAIDs [1–6]. While NSAIDs are among the most used drugs in the world, some inflammatory diseases still lack suitable treatment options, such as rheumatoid arthritis, Alzheimer's disease, and atherosclerosis. Therefore, the search for novel anti-inflammatory compounds is of great interest [2, 7] (**Fig. 1**).

Currently available NSAIDs are able to inhibit COX-1 or COX-2, but not 5-LOX. The COX enzymes and 5-LOX metabolize the arachidonic acid produced in inflammatory processes. When the COX enzymes are inhibited, increased amounts of leukotrienes are produced through the LOX pathway, and this excess is correlated with the main side effects of the current NSAIDs [2, 5, 8, 9]. Clinical studies have revealed that selective COX-2 inhibitors have more severe side effects than COX-1 inhibitors [5, 10]. The inhibition of other pathways involved in the genesis or maintenance of inflammatory processes, such as NF-κB and phospholi-

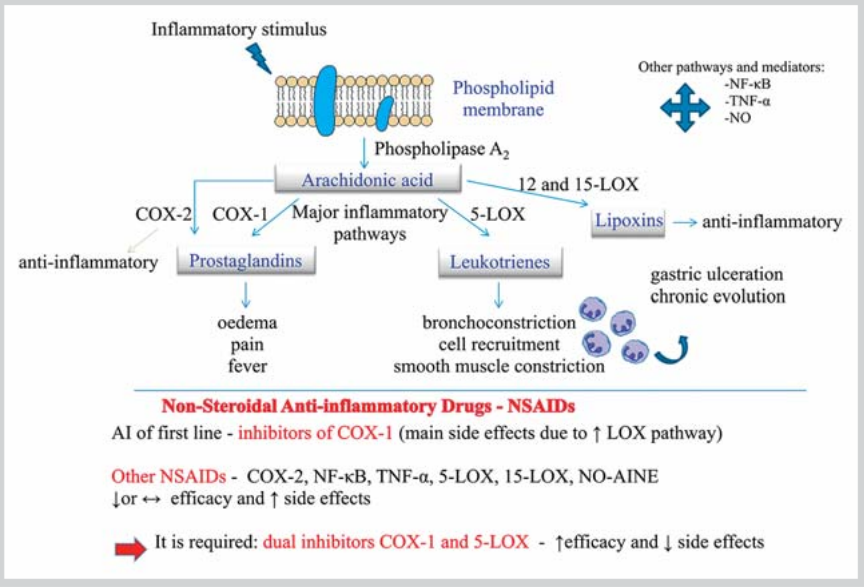

Fig. 1 Main proinflammatory pathways and the requirements for dual inhibitors that would be superior to NSAIDs. (Color figure available online only.)

pase A_2, would also lead to severe side effects [11–14] (**Fig. 1**). However, dual inhibitors of COX-1 and 5-LOX would have better efficacy and fewer side effects compared with current drugs for the following reasons: i) the inhibition of COX-1 prevents the production of proinflammatory mediators (prostaglandins and thromboxanes) that cause pain, edema, fever, and proinflammatory mediation [2,5,8]; and ii) the inhibition of 5-LOX prevents the production of the proinflammatory leukotrienes that are responsible for the recruitment and activation of inflammatory cells, smooth muscle constriction, and gastric ulceration [2,5,8]. Moreover, because 15- and 12-LOX would not be inhibited by such dual inhibitors, these enzymes would metabolize the arachidonic acid into anti-inflammatory mediators (lipoxins), increasing the anti-inflammatory properties of the dual inhibitors of COX-1 and 5-LOX [3,8] (**Fig. 1**).

A multitarget inhibitor that is able to inhibit COX-1, COX-2, and 5-LOX could be a better anti-inflammatory drug than any current NSAID [5,8,15]. The main side effects of selective COX-2 inhibitors, such as the prothrombotic side effects, could be reduced by the concomitant inhibition of platelet aggregation by COX-1; the gastric effects due to COX-1 inhibition could be inhibited through 5-LOX inhibition [8,15]. In this way, simultaneous inhibition of COX-1, COX-2, and 5-LOX would control the main inflammatory pathways, resulting in a far more potent and efficient anti-inflammatory effect compared with the effects of current NSAIDs.

However, for some physiological functions, the inhibition of only one COX isoform can be compensated by the other non-inhibited isoform [8]. Considering the compensatory effect between COX isoforms, the concomitant inhibition of COX-1 and COX-2 may have relevant side effects. COX-2 has been proven to be essential to the stage of inflammation resolution, while COX-1 is not [8, 16–18].

Thus, based on current knowledge about inflammatory pathways, the ideal anti-inflammatory mechanism of action of a drug seems to be COX-1 and 5-LOX inhibition.

Therefore, the first step to discovering an anti-inflammatory treatment option superior to current options is to find compounds with a distinct mechanism of action. After this step, further studies will be required to prove whether these compounds are truly superior than the current drugs. One interesting approach to selecting biologically active compounds is to screen the natural world, particularly plants.

Arnica (*Arnica montana* L.) [19], feverfew (*Tanacetum parthenium* L. Sch. Bip.) [20,21], pot marigold (*Calendula officinalis* L.) [22], and chicory (*Cichorium intybus* L.) [23] are among the well-known anti-inflammatory species of the family Asteraceae [6,7]. Many plants from this family have compounds with the potential to inhibit COX and LOX [6,7]. Nevertheless, so far, only a few of these species have been evaluated in cell-free assays, i.e., in direct interaction with the enzymes of interest. In most studies, the effects of extracts and pure compounds on these two enzymes are demonstrated indirectly by measuring the inhibition of the production of mediators (prostaglandins and leukotrienes). Thus, the biological activity of these species or their compounds could be due to their direct effects on the COX-1, COX-2, or 5-LOX enzymes or indirectly through the inhibition of other pathways. Consequently, the results of these studies cannot be compared because they followed different experimental protocols [6,7,24]. Additionally, the literature indicates that natural products usually exert a more inhibitory effect on COX-1 than on COX-2 [25].

Thus, in this work, several extracts from Asteraceae species, which are rich in potential anti-inflammatory compounds, were systematically evaluated for their dual inhibition of COX-1 and 5-LOX, and this evaluation was performed directly on these enzymes. Moreover, the chemical profiles of the extracts were assessed by the high-performance liquid chromatography-ultraviolet-diode array detector (HPLC-UV-DAD) method with the aim of characterizing their main constituents by dereplication. The selected Asteraceae species investigated here belong to three groups: i) known anti-inflammatory herbs (with ethnobotanical information); ii) food plants; and iii) species that have not yet been investigated from the phytochemical or anti-inflammatory point of view.

Results and Discussion

Preliminary tests using different extraction methodologies were carried out with the dried and powdered aerial parts of two plant species that belong to different Asteraceae tribes and consequently contain different sets of secondary metabolites. The aim of these tests was to obtain extracts that contain compounds of intermediate to high polarities, which could display anti-inflammatory activity [6,7,20]. Detailed information about the plant material and yield of each extract is listed in **Table 1**.

Solvent mixtures consisting of EtOH-H_2O and MeOH-H_2O were evaluated in three ratios (7 : 3, 1 : 1, and 3 : 7), with extracts prepared by maceration in an orbital shaker (24 and 48 h, with fresh solvent added every 24 h) or ultrasonication (10 and 20 min, with fresh solvent added every 10 min).

The chemical profiles determined by HPLC-UV-DAD for the material extracted with EtOH-H_2O were very similar to those extracted with MeOH-H_2O (**Fig. 2**), independent of the extraction method and solvent ratio. Thus, EtOH-H_2O was chosen as the extraction solvent because EtOH is cheaper, less toxic, and less pollutant than MeOH. Different proportions of EtOH in H_2O led to differences in the chemical profiles of the extracts, and EtOH : H_2O 7 : 3 (v/v) was chosen because it provided higher yields and a larger variety of compounds.

The extractions by maceration in an orbital shaker and ultrasonication revealed differences in the yields of the extracts, with higher yields observed for the extracts prepared by ultrasonication (**Table 2**). However, the chromatograms of the extracts prepared by ultrasonication exhibited lower peak intensities (**Fig. 3**). As all of the extracts were injected at the same concentration, the best preparation method was found to be maceration in a shaker for 24 h. In summary, the best extraction method was maceration in an orbital shaker for 24 h using EtOH-H_2O 7 : 3 (v/v) as the extraction solvent. This method was then used to prepare all 57 extracts studied here.

A second maceration of the plant material was also carried out, revealing that only the major compounds were extracted in the second extraction (**Fig. 4**). Because the major compounds could dilute the minor compounds, performing a second extraction would decrease the diversity of the composition of the extracts, and therefore only a single extraction was performed.

It is also important to highlight that the extracts prepared by the best method provided potent dual inhibitors using only a small amount of plant material (**Tables 1** and **3**).

The chemical profiles of the extracts determined by HPLC-UV-DAD and comparison of these profiles with data on compounds from Asteraceae in our in house database AsterDB showed that the prepared extracts are basically composed of chlorogenic acids (*trans*-cinnamic acid derivatives), flavonoids, and sesquiterpene lactones (**Table 4**). The dereplication results were confirmed by data from the literature. Some compounds were found to be more widespread than others, occurring in different either active or non-active extracts, while other compounds are exclusive of a certain species (**Table 4**). Although the dereplication procedure described here was successful for our purposes, further studies should be conducted using a more sensitive and comprehensive analytical technique such as UHPLC-UV-MS. Moreover, the chemical data combined with the biological data followed by proper multivariate statistical analysis could determine whether the active compounds are the same across different extracts. This approach, which is currently in progress, could also guide the determination of which active compounds in the active extracts are responsible for the dual inhibition of the COX-1 and 5-LOX enzymes.

Of the 57 extracts tested against the COX-1 and 5-LOX enzymes, 13 of them (26.6%, IC_{50} range from 0.03–36.2 µg/mL) displayed dual inhibition (**Table 3**, **Fig. 1**). Interestingly, species belonging to the following three previously described plant groups were found to be active (**Table 3**): (i) known anti-inflammatory herbs [*Solidago microglossa* DC., *Tithonia diversifolia* (Hemsl.) A. Gray, *Vernonia polyanthes* Less. and *Viguiera robusta* Gardner]; (ii) food plants (*C. intybus*); and (iii) previously uninvestigated species [*Minasia scapigera* H. Rob., *Piptolepis monticola* Loeuille, *Prestelia eriopus* Sch. Bip., *Sphagneticola trilobata* (L.) Pruskei, *Vernonia herbacea* (Vell.) Rusby, *Vernonia platensis* (Spreng.) Less., *Vernonia rubriramea* Mart. ex DC. and *Viguiera trichophylla* Dusén].

The relatively high percentage of biologically active extracts corroborates the well-known potential of the family Asteraceae to display anti-inflammatory properties. Extracts of 39 species, or 68.4%, were biologically active, which was defined as the ability to inhibit COX-1 only, 5-LOX only, or both enzymes (**Table 3**). However, of the group of species known as anti-inflammatory herbs, three species were able to inhibit both enzymes (**Table 3**), thus displaying the required mechanism of action (**Fig. 1**). Most of the extracts exhibiting dual inhibition belong to the group of previously uninvestigated species, especially those from the tribe Vernonieae (**Tables 1** and **3**), and all of them are from the Brazilian Cerrado biome. The Cerrado, also known as the Brazilian savannah, is a biome recognized as a biodiversity hotspot for conservation priorities because it is home to many species facing an extremely high risk of extinction [26]. These results demonstrate the importance of investigating species from this biome, especially by the approach established here, which requires only a small amount of plant material for phytochemical studies and bioassays.

The inability of some of the tested extracts to inhibit any enzymes does not imply that these extracts are not anti-inflammatory at all, as they could simply display other mechanisms of action (**Fig. 1**). This explains why some of the well-known anti-inflammatory plants were not able to inhibit the COX-1 and/or 5-LOX enzymes.

The results summarized in **Table 3** also reveal that the enzymatic inhibition displayed by the active extracts is not nonspecific, meaning that these extracts have specificity (inhibit only COX-1 or 5-LOX) or some selectivity in their inhibition (inhibit one enzyme more than other). All of the samples were tested experimentally for protein precipitation, with no samples exhibiting protein precipitation except for the positive control (tannic acid). The literature information and dereplication data (**Table 4**) also corroborate this information, i.e., compounds that are able to precipitate proteins (nonspecific inhibition) have not been previously reported in Asteraceae plants [27,28]. Thus, the enzymatic effects of the extracts investigated here are not nonspecific. Moreover, note that the IC_{50} values of the active extracts are near to or lower than those of the standard inhibitors (**Table 3**). Considering that an extract contains a highly complex mixture of compounds, with active constituents present in lower concentrations than the standard compounds used in our experiments, the extracts with low IC_{50} values might contain active compounds with highly potent inhibitory activity.

Additionally, the results of the inhibition of the COX-1 and 5-LOX enzymes (**Table 3**, **Fig. 1**) by *T. diversifolia* corroborate a previous *in vivo* study [29] in which this extract demonstrated the

Table 1 Information about the plant material: sample codes, species groups, species names, their respective tribes, sources, and the yield (%) of their extracts. All species belong to the family Asteraceae. (1) Species from the Cerrado biome; (2) species known as anti-inflammatory; (3) food plants. *Inflorescences instead of leaves; **the genus *Viguiera* was recently reclassified to *Aldama* [77].

Sample codes	Group	Species	Tribe	Location/source	Yield (%)
1	(2) [6, 36]	*Achillea millefolium* L. [yarrow]	Anthemideae Cass.	Sítio Irmãs Maries	3.6
2	(2) [37, 38]*	*Achyrocline satureioides* (Lam.) DC.	Gnaphalieae (Cass.) Lecoq & Juill.	Sítio da Mata	1.7
3	(3) [39]	*Acmella oleracea* (L.) R. K. Jansen [toothache plant]	Heliantheae Cass.	Supermarket	3.9
4	(2) [40]	*Ageratum conyzoides* L. [billygoat-weed]	Eupatorieae Cass.	Sítio Irmãs Maries	3.6
5	(1) [41]	*Anteremanthus hatschbachii* H. Rob.	Vernonieae Cass.	Cristália-MG 16°35′37.6′′ S and 42°54′07.7′′ W	3.3
6	(2)/(3) [42, 43]	*Arctium lappa* L. [burdock]	Cynareae Less.	Sítio Irmãs Maries	2.5
8	(2) [19]*	*Arnica montana* L.	Heliantheae Cass.	Santos Flora	3.8
9	(2) [44]	*Artemisia absinthium* L. [absinth wormwood]	Anthemideae Cass.	Sítio Irmãs Maries	3.0
10	(2) [45, 46]	*Artemisia annua* L. [sweet wormwood]	Anthemideae Cass.	Sítio Irmãs Maries	16.9
12	(2) [47]	*Baccharis dracunculifolia* DC. [alecrim do campo]	Astereae Cass.	Sítio Irmãs Maries	22.8
13	(2) [48, 49]	*Baccharis trimera* (Less.) DC.	Astereae Cass.	Sítio Irmãs Maries	4.7
14	(2) [50–52]	*Bidens pilosa* L.	Coreopsideae Lindl.	Sítio Irmãs Maries	3.2
15	(1) [41]	*Calea cuneifolia* DC.	Neurolaeneae Rydb.	Altinópolis-SP Morro do Forno	2.9
16	(2) [53, 54]	*Calendula officinalis* L. [marigold]	Calenduleae Cass.	Sítio Irmãs Maries	7.0
18	(1) [41, 55]	*Chronopappus bifrons* (DC. ex Pers.) DC.	Vernonieae Cass.	Santo Antônio do Itambé-MG	3.8
19	(3) [56]	*Cichorium intybus* L. [chicory]	Cichorieae Lam. & DC.	Supermarket	7.3
20	(3) [56]	*Cynara scolymus* L. [artichoke]	Cardueae Cass.	Sítio Irmãs Maries	2.9
21	(1) [41, 57]	*Dasyphyllum brasiliense var. latifolium* (D.Don) Cabrera	Barnadesieae D. Don	Furnas-MG 20°39′50′′ S and 46°21′79.3′′W	6.8
22	(2) [58]	*Echinacea purpurea* (L.) Moench [cone flower]	Heliantheae Cass.	Sítio Irmãs Maries	3.5
23	(2) [59]	*Emilia sonchifolia* L. DC. [lilac tassel flower]	Senecioneae Cass.	Sítio Irmãs Maries	8.6
24	(1) [41]	*Eremanthus polycephalus* (DC.) MacLeish	Vernonieae Cass.	Diamantina-MG 18°11′ 52.5′′ S and 43°37′33.1′′ W	1.5
26	(3) [56]	*Helianthus annuus* L. [sunflower]	Heliantheae Cass.	MG runway M-050; 20°39′ 09.2′′ S and 46°13′54.4′′W	3.7
27	(1) [41, 55]	*Heterocoma gracilis* Loeuille, J. N. Nakaj. & Semir	Vernonieae Cass.	São Gonçalo do Rio Preto-MG	4.5
28	(3) [56]	*Lactuca sativa* L. [common lettuce]	Cichorieae Lam. & DC.	Supermarket	3.8
29	(1) [41]	*Lychnophora diamantinana* Coile & S. B. Jones	Vernonieae Cass.	Diamantina-MG 18°11′ 52.5′′ S and 43°37′33.1′′ W	1.6
33	(1) [41, 60, 61]	*Lychnophora ericoides* Mart.	Vernonieae Cass.	Sítio Irmãs Maries	4.8
34	(1) [41, 55]	*Lychnophora tomentosa* (Mart. ex DC.) Sch. Bip.	Vernonieae Cass.	Diamantina-MG 18°12′ 52.9′′ S and 43°35′44.1′′ W	2.0
35	(2) [53]	*Matricaria chamomilla* L. [chamomile]	Anthemideae Cass.	Sítio Irmãs Maries	6.2
37	(2) [30]	*Mikania glomerata* Spreng. [guaco]	Eupatorieae Cass.	Sítio Irmãs Maries	1.8
38	(1)/(2) [41]	*Mikania hirsutissima* DC.	Eupatorieae Cass.	Sítio Irmãs Maries	1.9
39	(2) [30]	*Mikania laevigata* Schultz Bip. ex Baker [guaco]	Eupatorieae Cass.	Sítio Irmãs Maries	3.0
40	(1) [41]	*Minasia scapigera* H. Rob.	Vernonieae Cass.	Diamantina-MG 18°12′ 52.9′′ S and 43°35′44.1′′ W	2.1
41	(1) [41, 55]	*Piptolepis monticola* Loeuille	Vernonieae Cass.	Santo Antônio do Itambé-MG	12.5
42	(1) [41, 55]	*Prestelia eriopus* Sch. Bip.	Vernonieae Cass.	Santana do Riacho-MG 19°17′28.1′′ S and 43°36′ 01.5′′ W	2.8
43	(2) [62]	*Pluchea quitoc* DC.	Inuleae Cass.	Sítio Irmãs Maries	4.2
45	(2)/(3) [56, 63, 64]	*Smallanthus sonchifolius* (Poepp. & Endl.) H. Robinson [yacon]	Heliantheae Cass.	Sítio Irmãs Maries	2.3
46	(2) [65]	*Solidago microglossa* DC.	Astereae Cass.	Sítio Irmãs Maries	4.0
48	(3) [66]	*Sonchus oleraceus* L. [sowthistle]	Cichorieae Lam. & DC.	Sítio Irmãs Maries	3.1
49	(1) [41]	*Sphagneticola trilobata* (L.) Pruskei	Heliantheae Cass.	Campus USP Ribeirão Preto	1.9
50	(3) [67]	*Stevia rebaudiana* (Bertoni) Bertoni [sweetleaf]	Eupatorieae Cass.	Sítio da Mata	5.9
51	(1) [41]	*Tridax procumbens* L.	Heliantheae Cass.	Campus USP Ribeirão Preto	2.8
53	(2) [6, 20, 21, 68]	*Tanacetum parthenium* L. (feverfew)	Anthemideae Cass.	Sítio Irmãs Maries	24.0
54	(2) [69]	*Tanacetum vulgare* L. [tansy]	Anthemideae Cass.	Sítio Irmãs Maries	3.9
55	(2)/(3) [70, 71]	*Taraxacum officinale* Weber ex FH Wigg. [dandelion]	Cichorieae Lam. & DC.	Sítio Irmãs Maries	3.1
56	(2) [29, 72, 73]	*Tithonia diversifolia* (Hemsl.) A. Gray [tree marigold]	Heliantheae Cass.	Sítio Irmãs Maries	3.6
25	(1)/(2) [41, 74]	*Vernonia condensate* Baker [boldo baiano]	Vernonieae Cass.	Sítio Irmãs Maries	3.1

cont.

Table 1 *Continued*

Sample codes	Group	Species	Tribe	Location/source	Yield (%)
57	(1) [41]	*Vernonia herbacea* (Vell.) Rusby	Vernonieae Cass.	Mogi Guaçu-SP 22°14′ 59.0″ S and 47°09′23.3′′ W	10.4
58	(1) [41]	*Vernonia platensis* (Spreng.) Less.	Vernonieae Cass.	Botucatu-SP 22°53′25.9′′ S and 48°30′05.2′′ W	8.5
59	(2) [75]	*Vernonia polyanthes* Less. [assa peixe]	Vernonieae Cass.	Highway Sacramento to Araxá-MG 19°52′47.8′′ S and 47°21′18.1′′ W	23.0
60	(1) [41]	*Vernonia rubriramea* Mart. ex DC.	Vernonieae Cass.	Sítio Irmãs Maries	23.4
61	(1) [41]	*Viguiera arenaria* Baker**	Heliantheae Cass.	Itirapina-SP 22°13′ S and 47°54′ W	3.6
62	(1) [41]	*Viguiera bracteata* Gardner**	Heliantheae Cass.	Road to Curvelo-MG	3.1
63	(1) [41]	*Viguiera discolor* Baker**	Heliantheae Cass.	Road to Altinópolis-MG	2.6
64	(1) [41]	*Viguiera filifolia* Sch. Bip. ex Baker**	Heliantheae Cass.	Alto Paraíso de Goiás-GO 25°22′53.4′′ S and 49°48′ 24.0′′ W	2.9
65	(1) [41]	*Viguiera linearifolia* Chodat & Hassl**	Heliantheae Cass.	Ponta Porã-MS 22°16′38.5″ S and 55°40′28.8′′ W	2.0
66	(1)/(2) [41, 76]	*Viguiera robusta* Gardner**	Heliantheae Cass.	Mogi Guaçu-SP 22°16′ S and 47°11′ W	3.6
67	(1) [41]	*Viguiera trichophylla* Dusén**	Heliantheae Cass.	Palmeira-PR 25°22′53.4′′ S and 49°48′21.0′′ W	3.5

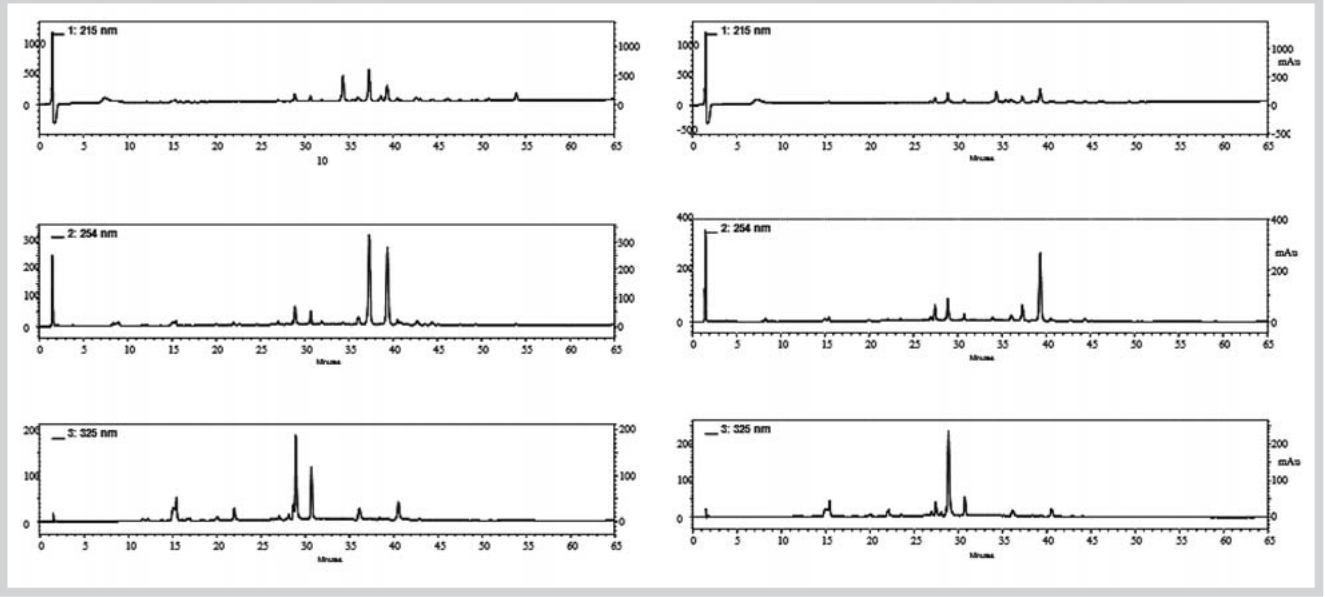

Fig. 2 HPLC-UV-DAD chromatograms (0–45 % MeCN in 60 min; 0.5 % HOAc; C-18 monolithic column) of the extracts of *T. diversifolia* prepared in an orbital shaker (left) and by ultrasonication (right) using EtOH-H_2O 7 : 3 (v/v) as the extraction solvent. Chromatograms were obtained at 215, 254, and 325 nm (top, middle, and bottom chromatograms, respectively).

inhibition of edema and the recruitment of inflammatory cells (neutrophils). Another example is the species *Mikania glomerata* Sprengl. and *Mikania laevigata* Schultz Bip. ex Baker, which are used in popular medicine in Latin America to treat inflammatory diseases, where the LOX pathway and the recruitment of inflammatory cells are involved (bronchitis, pleurisy, and asthma; ◉ **Fig. 1**) [30–32]. In this work, the extracts of these two species in fact inhibited this pathway (◉ **Table 3**), and it should be emphasized that this is the first report of the direct inhibition of 5-LOX. Some previous experiments with other species using inflammatory cells show the inhibition of the production of leukotrienes [33], and this inhibition probably occurs due to the direct inhibition of 5-LOX, as shown by our results with the extracts of *Echinacea purpurea* (L.) Moench and *T. parthenium* (◉ **Table 3**). Thus, following this same argument and based on our results, the extracts with positive *in vitro* results that have never been studied *in vivo* have high chances of displaying the same properties *in vivo*.

The focus of our work was to identify COX-1 and 5-LOX inhibitors, as these activities represent a probable mechanism of action that

Table 2 Yield (mg) of the extracts of *T. diversifolia* (Td) and *V. gardneri* (Vg) obtained by maceration in a shaker or an ultrasonic bath.

	Td/shaker		Vg/shaker		Td/ultrasonic bath		Vg/ultrasonic bath	
	maceration	re-maceration	maceration	re-maceration	maceration	re-maceration	maceration	re-maceration
EtOH-H_2O	2.8	1.8	2.6	1.4	3.9	1.6	3.6	2.5
	3.0	1.5	2.9	2.2	4.7	2.0	4.4	2.0
	3.7	1.4	2.8	1.4	4.0	1.6	3.9	2.5
Mean	3.2	1.6	2.8	1.7	4.2	1.7	4.0	2.3
Yield percentage	32%	16%	28%	17%	42%	17%	40%	23%
MeOH-H_2O	2.5	1.8	2.9	1.4	4.1	1.7	3.5	1.9
	2.7	1.6	2.6	1.5	3.8	1.7	3.9	2.9
	2.7	1.3	2.6	1.7	3.8	1.1	4.1	1.9
Mean	2.6	1.6	2.7	1.5	3.9	1.5	3.8	2.2
Yield percentage	26%	16%	27%	15%	39%	15%	38%	22%

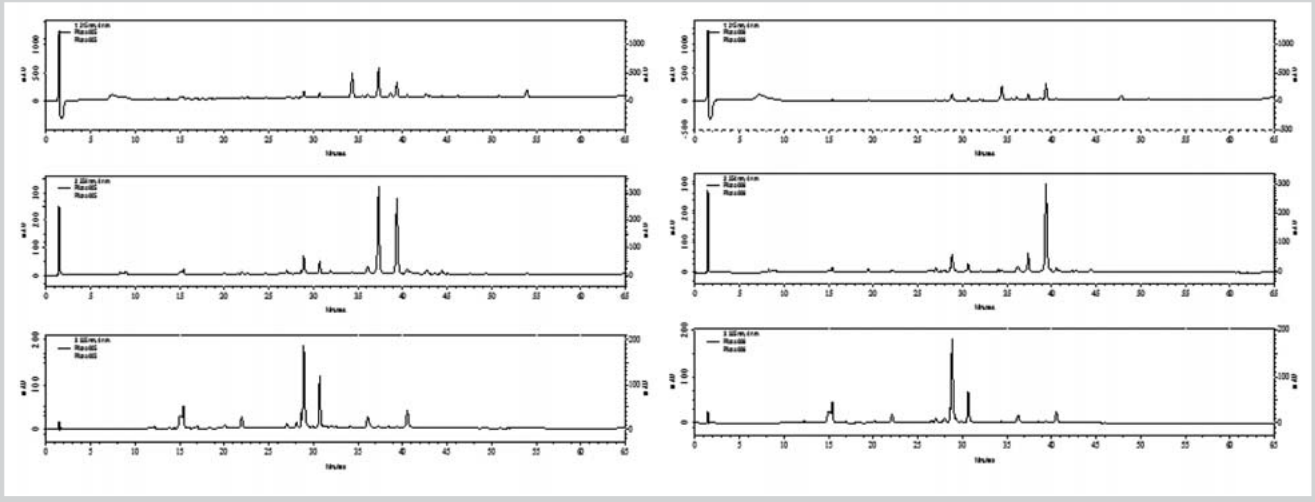

Fig. 3 HPLC-UV-DAD chromatograms (0–45% MeCN in 60 min; 0.5% HOAc; C-18 monolithic column) of the extracts of *T. diversifolia* were prepared with EtOH-H_2O 7:3 (v/v) (left) and MeOH-H_2O 7:3 (v/v) (right) as the extraction solvents. Chromatograms were obtained at 215, 254, and 325 nm (top, middle, and bottom, respectively).

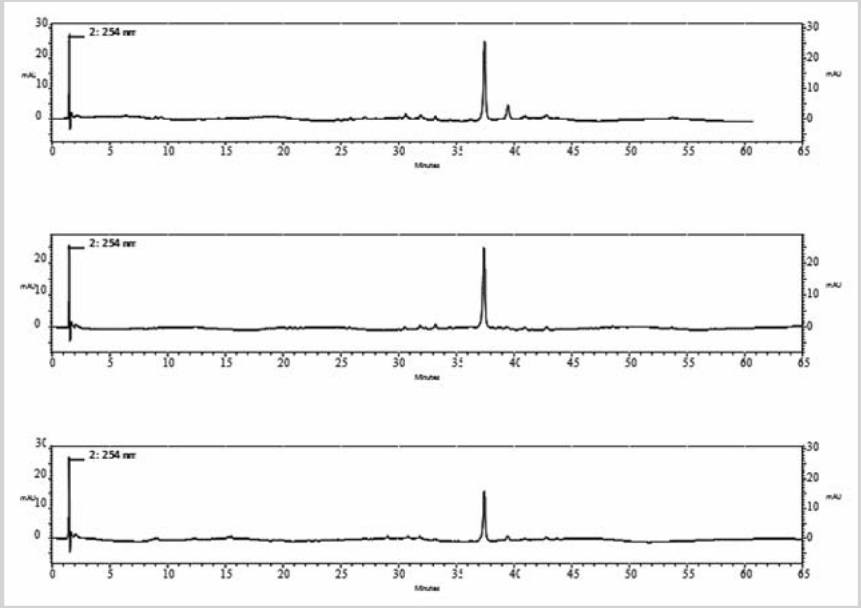

Fig. 4 HPLC-UV-DAD chromatograms (0–45% MeCN in 60 min; 0.5% HOAc; C-18 monolithic column) of the extracts of *T. diversifolia* prepared by a second maceration with EtOH-H_2O 3:7, 1:1 and 7:3 (v/v), shown in the top, middle, and bottom chromatograms, respectively. These chromatograms were obtained at 254 nm.

Table 3 The 50% inhibitory concentration (IC_{50}) of 5-LOX and COX-1 of the extracts. Species printed in **bold** denote dual inhibitor extracts. (1) Species from the Brazilian Cerrado biome; (2) species known as anti-inflammatory; (3) food plants. *Standard inhibitor (IC_{50} values): nordihydroguaiaretic acid for 5-LOX (15.0 µg/ml) and indomethacin for COX-1 (0.1 µg/ml). **Previous evidence of inhibition of the respective pathway (COX-1 or 5-LOX) through *in vivo* or *ex vivo* experiments. The symbol (–) denotes $IC_{50} > 50$ µg/mL.

Groups	Species and standard compounds[1]	Sample codes	5-LOX (µg/mL)*	COX-1 (µg/mL)*
(2)	*Achillea millefolium* Ledeb.	1	–	–
(2)	*Achyrocline satureioides* (Lam.) DC.	2	–	–
(3)	*Acmella oleracea* (L.) R. K. Jansen	3	–	–
(2)	*Ageratum conyzoides* L.	4	–	–
(1)	*Anteremanthus hatschbachii* H. Rob.	5	–	–
(2)/(3)	*Arctium lappa* L.	6	17.6	–
(2)	Arnica montana L.	8	0.009** [78]	–
(2)	*Artemisia absinthium* L.	9	13.2	–
(2)	*Artemisia annua* L.	10	–	–
(2)	*Baccharis dracunculifolia* D. C.	12	–	–
(2)	*Baccharis trimera* (Less.) DC.	13	–	–
(2)	*Bidens pilosa* L.	14	–	–
(1)	*Calea cuneifolia* DC.	15	–	–
(2)	*Calendula officinalis* L.	16	–	0.1** [53]
(1)	*Chronopappus bifrons* (DC. ex Pers.) DC.	18	–	4.6
(3)	***Cichorium intybus* L.**	19	**0.3**	**3.6**
(3)	*Cynara scolymus* L.	20	–	0.4** [79]
(1)	*Dasyphyllum brasiliense* var. *latifolium* (D. Don) Cabrera	21	–	0.02
(2)	*Echinacea purpurea* (L.) Moench	22	12.29** [6]	–
(2)	*Emilia sonchifolia* L. DC.	23	–	–
(1)	*Eremanthus polycephalus* (DC.) MacLeish	24	–	0.006
(3)	*Helianthus annuus* L.	26	–	–
(1)	*Heterocoma gracilis* Loeuille, J. N. Nakaj. & Semir	27	–	50.0
(3)	*Lactuca sativa* L.	28	13.7	–
(1)	*Lychnophora diamantinana* Coile & S. B. Jones	29	17.5	–
(1)	*Lychnophora ericoides* Mart.	33	0.6** [80]	–
(1)	*Lychnophora tomentosa* (Mart. ex DC.) Sch. Bip.	34	16.6	–
(2)	*Matricaria chamomilla* L.	35	0.7	–
(2)	*Mikania glomerata* Spreng.	37	2.6	–
(1)/(2)	*Mikania hirsutissima* DC.	38	1.9** [31]	–
(2)	*Mikania laevigata* Schultz Bip. ex Baker	39	8.1** [31]	–
(1)	***Minasia scapigera* H. Rob.**	40	**4.6**	**5.6**
(1)	***Piptolepis monticola* Loeuille**	41	**4.8**	**6.7**
(1)	***Prestelia eriopus* Sch. Bip.**	42	**20.6**	**6.7**
(2)	*Pluchea quitoc* DC.	43	–	–
(2)/(3)	*Smallanthus sonchifolius* (Poepp. & Endl.) H. Robinson	45	–	0.001** [63]
(2)	***Solidago microglossa* DC.**	46	**0.08**	**5.5**
(3)	*Sonchus oleraceus* L.	48	0.92	–
(1)	***Sphagneticola trilobata* (L.) Pruskei**	49	**12.2**	**11.9**
(3)	*Stevia rebaudiana* (Bertoni) Bertoni	50	0.01	–
(1)	*Tridax procumbens* L.	51	0.05	–
(2)	*Tanacetum parthenium* L.	53	0.01** [6]	–
(2)	*Tanacetum vulgare* L.	54	–	–
(2)	*Taraxacum officinale* Weber ex FH Wigg.	55	–	0.002
(2)	***Tithonia diversifolia* (Hemsl.) A. Gray**	56	**11.6**** [29]	**28.7**** [29]
(1)	*Vernonia condensata* Baker	25	–	–
(1)	***Vernonia herbacea* (Vell.) Rusby**	57	**24.6**	**0.2**
(1)	***Vernonia platensis* (Spreng.) Less.**	58	**15.8**	**0.1**
(2)	***Vernonia polyanthes* Less.**	59	**3.00**	**0.06**** [81]
(1)	***Vernonia rubriramea* Mart. ex DC.**	60	**1.79**	**0.03**
(1)	*Viguiera arenaria* Baker	61	16.5	–
(1)	*Viguiera bracteata* Gardner	62	3.6	–
(1)	*Viguiera discolor* Baker	63	8.4	–
(1)	*Viguiera filifolia* Sch. Bip. ex Baker	64	5.6	–
(1)	*Viguiera linearifolia* Chodat & Hassl.	65	–	–
(1)/(2)	***Viguiera robusta* Gardner**	66	**36.0**** [76]	**0.1**** [76]
(1)	***Viguiera trichophylla* Dusén**	67	**4.0**	**2.6**
	Nordihydroguaiaretic acid (Sigma-Aldrich®)		15.0	–
	Indomethacin (Sigma-Aldrich®)		–	0.1

Table 4 Compounds identified by HPLC-UV-DAD data [retention time (t_R) and UV absorption] and comparison with data from our in-house database. #For all substances with references in this column, previous evidence indicates that the LOX pathway is inhibited through *in vitro, in vivo*, or ex *vivo* experiments, except for substances marked with (−) for which previous evidence of a lack of inhibition of the LOX pathway is available through *in vitro, in vivo*, or *ex vivo* experiments. ##For all substances with references in this column, previous evidence is available indicating the inhibition of the COX (1 or 2, not necessarily specified) pathway through *in vitro, in vivo*, or *ex vivo* experiments, except for substances marked with (−) for which previous evidence of a lack of inhibition of the COX pathway is available through i*n vitro, in vivo*, or *ex vivo* experiments (sh = shoulder). ###Extract codes – **Table 1**. Dual inhibitor samples are printed in **bold**. *The previous evidence was from a direct evaluation with 5-LOX enzymes; **the previous evidence was from a direct evaluation with COX-1 enzymes.

t_R (min)	Compound	Previous evidence of LOX inhibition#	Previous evidence of COX inhibition##	Peak of maximum absorption on the UV (nm)	Sample###
2.5	gallic acid	[82] Weakly* [83]	Weakly** [83]	276	21, 61
3.4	mono-O-E-caffeoylaltraricacid			302, 327	45
4.6	protocatechuic acid			257, 294 sh	1, 5, 6, 21, 25, 29, 34, **56, 58**, 61, 64, 65
5.5	mono-O-E-caffeoylaltraricacid			302, 327	45, **58**
7.0	mono-O-E-caffeoylaltraricacid			302, 327	45, **58**
8.2	3-O-E-caffeoylquinic acid			297, 324	1, 5, 6, 9, 13, 14, 16, 21, 24, 34, 37,38, 39, **40**, 43, **56, 59**, 61, 62
8.8	mono-O-E-caffeoylaltraricacid			302, 327	45, **58**
10.5	aesculin			280, 324 sh	45, 55, **58**, 62
11.6	5-O-E-caffeoylquinic acid	Weakly [84]	** [85]	295, 325	1, 2, 3, 4, 5, 6, 8, 9, 10, 12, 13, 14, 15, 16, 18, **19**, 20, 21, 22, 23, 24, 26, 27, 28, 33, 34, 35, 37, 38, **40, 41, 42**, 43, 45, **46**, 48, **49**, 50, 51, 53, 54, 55, **56, 57, 58, 60**, 61, 63, 64, 65, **66, 67**
13.0	4-O-E-caffeoylquinic acid			295, 325	1, 2, 3, 4, 5, 6, 8, 9, 10, 12, 13, 14, 15, 16, 18, **19**, 20, 21, 22, 23, 24,26, 27, 28, 33, 34, 35, 37, 38, **40, 41, 42**, 43, 45, **46**, 48, **49**, 50, 51, 53, 54, 55, **56, 57, 58, 59, 60**, 61, 63, 64, 65, **66, 67**
14.3	3-O-E-cumaroylquinic acid			295, 323	24, 37, **58**
15.1	mono-O-E-cafeoylchiquimicacid			300, 324	15, 21, 65
15.3	cumaric acid			299, 324	29, 37, 61
15.4	caffeoylchiquimic acid			280, 316	21
16.0	caffeoylchiquimic acid			280, 316	21
17.8	ferulic acid	(−) [84]	(−) [84]	300, 325	9, 22, **57**, 61
18.5	mono-O-caffeoylpentose			300, 327	22, **56, 57**, 65
22.1	hyperoside			286	1, 9, 13, 14, 21, 62, **66**
22.3	quercetrin			251, 262, 326	1, 5, 6, 9, 13, 14, 15, 21, 24, 34, 38, **57, 58, 59**, 61, 64, **66**
22.6	isoquercetrin			251, 265, 344	1, 9, 13, 15, 21, 22, 24, 25, 34, 39, 45, 55, **57, 58, 59**, 62, 64, 65, **66**
24.9	di-O-E-caffeoylaltraricacid			300, 327	45
25.2	3,4-di-O-E-caffeoylquinic acid	[86]	(−) [86]	298, 326	1, 5, 6, 9, 13, 14, 15, 16, 21, 22, 24, 29, 34, 37, 38, 39, **42**, 43, **56, 59**, 61, 64, **66**
25.9	3,5-di-O-E-cafeoilquinic acid	[86]	(−) [86]	298, 326	1, 13, 21, 22, 29, **56, 58, 66**
27.5	4,5-di-O-E-caffeoylquinic acid	[86]	(−) [86]	298, 326	1, 2, 3, 4, 5, 6, 8, 9, 10, 12, 13, 14, 15, 16, 18, **19**, 20, 21, 22, 23, 24, 26, 27, 28, 29, 33, 34, 35, 37, 39, **40, 41, 42**, 43, 45, **46**, 48, **49**, 50, 51, 53, 54, 55, **56, 57, 58, 59, 60**, 61, 63, 64, 65, **66, 67**
28.1	tri-O-E-caffeoylaltraricacid			293, 329	45
28.8	tri-O-E-caffeoylaltraricacid			293, 329	45
29.3	tri-O-E-caffeoylaltraricacid			293, 329	45, **66**
31.0	tri-O-E-caffeoylaltraricacid			293, 329	45
31.8	quercetin	[24, 87, 88]* [89]	** [88] (−) [89]	253, 265, 349	1, 5, 13, 21, 24, 38, 55
32.1	nepetin			252, 339	15, **56**
32.2	luteolin	[24]		274, 334	13, 21
32.5	goyazensolide			268	24, 29, 33, 34, **40, 41, 42**
32.6	isorhamnetin			252, 268, 331	24, 29, 34, 39, **40**
32.6	3-O-methylquercetin			266, 327	1
32.9	arctiin			237	5
34.0	hispidulin	[90]		270, 334	13, 37, **59**
35.0	tagitinin C			249	56

cont.

Table 4 *Continued*

t_R (min)	Compound	Previous evidence of LOX inhibition[#]	Previous evidence of COX inhibition[##]	Peak of maximum absorption on the UV (nm)	Sample[###]
35.2	3,4,5-tri-O-caffeoylquinic acid			300, 325	1, 5, 13, 24, 34, 43
35.8	apigenin	* (−) [88]	** (−) [88]	270, 334	1, 24, 37, **58**
36.0	hesperetin	(−) [91]	[92]	285, 331 sh	15
36.6	chrysoeriol			250, 265, 345	5, 24, **56**
37, 49	2-one-8β-metacriloiloxy-guai-3,10 (14),11(13)-trien-6α,12-olide-16α-(1′,2′-epoxy-1′-methylprop)-eremantholide			265	42
40.8	3′,4′,5-trihydroxy-7-methoxyflavanone			288	45, **59**
40.9	4,5-dihydro-15-desoxigoyazensolide			264	40
42.7	enhydrin			232	45
44.1	eremantholide C			267	40
44.2	3′,5,7-trihydroxi-3,4′-dimethoxiflavone			253, 266, 352	45
48.1	acacetin	* (−) [93]	** [94]	267, 332	59
48.9	uvedalin			236	45
52.2	8β-angeloxy-germacra-1(10)Z,4E,11 (13)-trien-6α,12-olide-14-oic acid			234	45
60.3	sonchifolin			242	45

should provide a treatment with greater efficacy and lower side effects. Nevertheless, we did not evaluate other pathways such as COX-2 and NF-*k*B inhibition or cytokine and NO release (**Fig. 1**). Therefore, the dual inhibitors (**Table 3**) can be either selective COX-1 and 5-LOX inhibitors or multitarget inhibitors that also affect other inflammation pathways (**Fig. 1**, **Table 3**). Further studies are necessary to determine the effect of the extracts on other targets involved in inflammation.

Regarding whether a certain anti-inflammatory effect observed *in vitro* also occurs *in vivo*, we observed that our *in vitro* results could be coherently correlated with the results from previous *in vivo* and/or *ex vivo* studies (**Table 3**). This result is because this study used species from a plant family with a long historic tradition of use for its anti-inflammatory activity. However, it is important to note that the coherent correlation observed in our study was only possible for some selected plants (**Table 3**) that have been previously evaluated through *in vivo* and/or *ex vivo* experiments.

Unfortunately, the results of *in vitro* enzyme inhibition tests are sometimes not reproducible *in vivo*. However, a great advantage of the *in vitro* tests is that the direct evaluation of the inhibition is a straightforward way to determine the exact mechanism of action. Additionally, screening studies based on enzymes are important ways to detect potential candidates for further studies such as drug design or virtual screening. For example, if a certain highly potent compound loses its activity when tested *in vivo*, it can be further submitted to derivatization using the tools of modern medicinal chemistry.

The *ex vivo* (experiments performed in cells) and *in vivo* (experiments performed in animals) assays involve the inflammatory cascade, and altering one step of this cascade interferes with the results of the next steps [6,24]. Thus, differences from the normal levels of products of inflammatory pathways suggest that a pathway is inhibited. However, further direct enzymatic studies are needed to determine the exact mechanism of action [6,24]. The main disadvantage of *in vivo* or *ex vivo* assays is that at the end of the experiment, the active substances may have standard mechanisms of action [6,24]. In contrast, the main advantage of our protocol is that we can focus on the discovery of active products with an unusual mechanism of action.

In summary, based on our results, the active extracts isolated here acted in a specific way and had IC_{50} values that were lower than or near those of the standard inhibitors. Thus, these extracts are potent inhibitors that could be interesting subjects for structural isolation followed by drug discovery studies, and these results corroborate the potential of the Asteraceae family as a rich source of anti-inflammatory compounds. This is the first report of the direct inhibition of both enzymes as a mechanism of action for the species investigated here. Moreover, one of the species is a food plant (chicory) that could be useful as a nutraceutical for consumption by people suffering from inflammatory diseases, pending further investigation. The chemical profiles of the extracts enabled the dereplication of most of the major compounds. Additionally, this work showed that our proposed HPLC-UV-DAD-based analysis combined with *in vitro* enzymatic evaluations can be carried out using only a small amount of plant material, and nontoxic and non-pollutant extraction solvents were used to obtain a highly diverse pool of biologically active compounds from plant material.

Materials and Methods

Plant material

Leaves from all of the Asteraceae species were purchased (authenticity documented) or donated. All of the information about the species is summarized in **Table 1**. The donated species have vouchers deposited in the SPF herbarium at the Department of Botany, Institute of Biosciences, University of São Paulo, São Paulo, SP, Brazil, and their respective collection numbers are as follows: species code #1 – A.M.S. Pereira 1428; #5 – Loeuille et al. 537; #13 – A.M.S. Pereira 1426; #14 – A.M.S. Pereira 1424; #15 – M. Nogueira & L.E. Gregório 35; #16 – A.M.S. Pereira 1430; #18 – Lusa et al. 63; #21 – D.A. Chagas-Paula 10; #22 – A.M.S. Pereira

1425; #23 – D.A. Chagas-Paula 06; #24 – Loeuille et al. 531; #25 – A.M.S. Pereira 1418; #26 – D.A. Chagas-Paula 09; #27 – Lusa et al. 61; #28 – D.A. Chagas-Paula 08; #29 – Loeuille et al. 530; #34-Loeuille et al. 528; #38 – A.M.S. Pereira 1400; #39 – A.M.S. Pereira 1419; #40 – Loeuille et al. 529; # 41 – Lusa et al. 62; #42 – Loeuille et al. 524; #43 – A.M.S. Pereira 1427; #45 – A.M.S. Pereira 1422; #46 – A.M.S. Pereira 1421; #48 – A.M.S. Pereira 1429; #49 – D.A. Chagas-Paula 05; #51 – D.A. Chagas-Paula 07; #54 – A.M.S. Pereira 1420; #56 – A.M.S. Pereira 1423; #57 – E.S.A. 94148; #58 – E.S.A. 94146; #61 – M. Magenta et al. 275; #62 – M. Magenta et al. 440; #63 – M. Magenta et al. 307; #64 – A.B. Bombo et al. 56; #65 – A.B. Bombo et al. 62; #66 – M. Magenta et al. 454; #67 – A.B. Bombo et al. 51. The other species were purchased, following their code number is the name of their origin: #2 – Sítio da Mata, Cajuru-SP; #3 – Market, Belém-PA; #4,#6, #9, #10, #12, #20, #33, #35, #37, #53, #55, #59, #60 – Sítio Irmas Maries, Jardinópolis-SP; #8 – Santos Flora, São Paulo-SP; #19 – Market, Ribeirão Preto-SP; #50 – Sítio da Mata, Cajuru-SP. Documents attesting to the identity of the samples are available for all species.

The species included in this study were separated into the following three groups as previously explained: food plants, plants known as anti-inflammatory herbs, and previously uninvestigated species.

For species #2, #8, and #53, as the inflorescences are correlated with the anti-inflammatory activity rather than the leaves, the inflorescences of these species were investigated instead their leaves.

This project was authorized by the Genetic Heritage Management Council (CNPq, process #010055/2012–6).

Extraction

Dried plant materials were ground in an analytical mill (A 11 basic, IKA®). The particle size was under 0.42 mm. After that, 20 mg of each plant powder was extracted with 2 mL of EtOH-H_2O (7 : 3, v/v) in an orbital shaker (110 rpm and 30 °C) for 24 h.

As already explained, preliminary tests using different extraction methodologies were carried out to choose the best extraction method. These tests were carried out in triplicate with two plant species from different subtribes. The extracts were evaluated by HPLC-UV-DAD, and the best method was used for all of the plant material.

The extracts were partitioned with *n*-hexane to eliminate the fatty acids or waxes, filtered through a 0.2-µm PTFE membrane (Millipore©), and then the solvent was eliminated by reducing the pressure. The final extracts were split into two aliquots that were used for both HPLC-UV-DAD analysis and anti-inflammatory assays.

High-performance liquid chromatography-ultraviolet-diode array detector method and dereplication

The HPLC-UV-DAD profiling of the extracts was performed on a Shimadzu liquid chromatograph (LC-10 Avp pumps, SCL-10 Avp controller, SPD-10 Avp diode array detector, and software Class VP, version 5.02) using two C-18 Onix monolithic columns (3 × 100 mm; Phenomenex) coupled in series, with a flow rate of 1.4 ml/min and the following gradient elution: MeCN 1% AcOH (B)/H_2O(A) 1% AcOH; 0–45% B in 60 min (linear gradient), 45–100% B in 5 min (linear gradient), and 100% B in 5 min (isocratic). The samples were solubilized in 1 : 1 A/B to obtain a final concentration of 1 mg/mL and the injected volume 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 dereplication of the main compounds was carried out by comparing the retention times and UV data from several pure compounds previously run under the same conditions (in-house database AsterDB, www.asterbiochem.org/asterdb). All of the compounds were isolated from species of the family Asteraceae.

Anti-inflammatory assays – cyclooxygenase-1 and 5-lipoxygenase

The anti-inflammatory assays were performed using COX-1 (catalog #560101) and 5-LOX (catalog #760700 and #60401) screening kits from Cayman Chemical's ACE™ according to the manufacturer's instructions and a previously reported method [34,35] using duplicates of five concentrations of the extracts and standards (100, 10, 1, 0.1, and 0.01 µg/mL). The extracts were dissolved in the buffer from the kit, and the standards in DMSO were diluted in the appropriate buffers. The IC_{50} values were determined by the sigmoidal dose-response curves (GraphPadPrism 5.0®). Standard inhibitors were nordihydroguaiaretic acid (purity ≥ 97.0%, HPLC; Sigma®) for 5-LOX and indomethacin (purity ≥ 99%, TLC; Sigma®) for COX-1.

Protein precipitation

This test was carried out to eliminate the possibility of nonspecific enzyme inhibition due to protein precipitation. Hydrochloric acid (10%, one drop) was added to the extract solution (1 mg/100 µL of H_2O), and then a gelatin solution (2.5%) was added until precipitation occurred, which was followed by centrifugation. Tannic acid (purity ≤ 100%; Sigma-Aldrich®) was used as the standard for the precipitation assay.

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Conflict of Interest

The authors declare no conflict of interest.

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