Inhibition of arachidonic acid metabolism by the Andean crude drug *Parastrephia lucida* (Meyen) Cabrera

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ABSTRACT

Ethnopharmacological relevance: Parastrephia lucida (Meyen) Cabrera is used in the traditional medicine of Argentinean highlands as an antiseptic and anti-inflammatory medicinal plant. To give scientific support to the ethnopharmacological claim of *Parastrephia lucida* as an anti-inflammatory crude drug the effect of *Parastrephia lucida* extracts and fractions was assessed on key enzymes of the biosynthesis of pro-inflammatory eicosanoids mediators from arachidonic acid (AA).
Materials and methods: A bio-guided fractionation of the plant extract was carried out to find out the compounds or mixtures responsible for the anti-inflammatory effect. The extracts and fractions were tested *in vitro* for their ability to inhibit the enzymes cyclooxygenase (COX)-1 and COX-2, lipoxygenase (LOX) and phospholipase (sPLA2). Fractions were analyzed by HPLC–MS, HPLC–ESI-MS/MS and NMR to relate the effect with groups of secondary metabolites.
Results: Parastrephia lucida was more effective inhibiting COX and $sPLA_2$ than LOX. Assay-guided isolation led to the active fractions C and F which showed different effect on the selected enzymes. The fraction C was more effective inhibiting LOX while fraction F showed better activity against $sPLA_2$ and COX-2. Both fractions were further worked-up following the isolation of the anti-inflammatory agents with the selected enzyme assays. The main compounds identified in the most active fractions were 5,4′-dihydroxi-7-methoxyflavanone, apigenin, apigenin methyl ether and apigenin trimethyl ether, methyl and dimethyl ethers from quercetin, kaempferol and luteolin methyl ether, ferulic acid esters, cinnamic acid and vanillin.
Conclusions: Parastrephia lucida extract inhibit AA metabolism via several enzymes. The results give support to the traditional use of this plant for the treatment of inflammatory disorders.

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1. Introduction

The Asteraceae *Parastrephia lucida* (Meyen) Cabrera is a shrub common in the highlands of Antofagasta de la Sierra, Provincia de Catamarca, Argentina. In traditional medicine, *Parastrephia lucida* is used as an anti-inflammatory agent, to treat toothache by applications of leaves. The plant resin in poultices is used for rapid healing of wounds, bruises and to consolidate bone dislocation and fractures (Villagrán et al., 2003). *Parastrephia lucida* has been widely used by Amerindian cultures living in the South American highlands from pre-Columbian times. It was incorporated into the Argentinian traditional medicine, largely without untoward incident, and is considered generally safe. In spite of the widespread use of *Parastrephia lucida*, the reputed anti-inflammatory effect of the plant was not yet supported by scientific studies. The underlying mechanisms that account for their anti-inflammatory activity and active components remain largely to be disclosed. The plant was reported to have acaricide, antifungal, antibacterial and antioxidant activities (Ayma et al., 1995; Sayago et al., 2006; Zampini et al., 2008, 2009; Rojo et al., 2009; D'Almeida et al., 2012).

The use of plants for the treatment of human diseases is associated with cultures and traditional medicine from different parts of the world. Inflammation and inflammation-related ailments such as rheumatism, muscle swelling, cut wound, accidental bone fracture, insect bites, pains and burn by fire and hot water are frequently treated with medicinal plants (World Health Organization

(WHO), 2001). Amongst the *in vitro* anti-inflammatory assays, the ones examining the effects of an extract or compound on arachidonic acid (AA) metabolizing enzymes are most commonly used. AA is the substrate of different oxygenases such as cyclooxygenases (COXs) and lipoxygenases (LOXs), key enzymes in the synthesis of eicosonoids. The release of AA from cell membrane phospholipids is the result of activation of phospholipases A_2 (PLA$_2$) by cell stimulation, which is a rate limiting step of the production of pro-inflammatory lipid mediators such as prostaglandins (PGs), leukotrienes (LTs), lipoxins, and platelet activating factor (PAF) (Khanum et al., 2005). These mediators are responsible for the maintenance of the inflammatory process. Physiological or acute inflammation is a beneficial host response to tissue damage, but when timely resolution is delayed, the overproduction of the inflammatory mediators may lead to many diseases, such as rheumatoid arthritis, inflammatory bowel disease, cancer and atherosclerosis. Thus, inhibition of the production of these inflammatory mediators may prevent or suppress a variety of inflammatory diseases.

Inhibitors of COXs are the main strays of current therapy aimed to modulate inflammation, pain, and to control fever. The constitutive form COX-1 is responsible for the maintenance of physiological prostanoid biosynthesis. In contrast, COX-2 is an inducible isoform linked to inflammatory cell types and tissues (Vane, 1994). Prolonged use of non-steroidal anti-inflammatory drugs (NSAIDs) is also associated with severe side effects such as gastrointestinal hemorrhage due to COX-1 inhibition (Lee et al., 2003). Many COX-2 inhibitors have been developed as drugs to treat inflammation; however, some have been withdrawn from the market, indicating a need for inhibitors free of side effects (Viji and Helen, 2008). On the other hand, NSAIDs induced gastric inflammation has been associated with shunting of the AA to generate LTs from 5-LOX (Celotti and Durand, 2003).

Many compounds proved to be potent and selective inhibitors of LOX, but could not enter the pharmaceutical market due to severe side effects or inefficacy in clinical trials (Viji and Helen, 2008). Reports have appeared regarding so-called "dual inhibitors", agents that inhibit not only COX-1 and COX-2, but also 5-LOX-mediated AA metabolism (Celotti and Durand, 2003; Altavilla et al., 2009). These agents may be particularly effective for minimizing both gastric and cardiovascular side effects by balancing AA metabolism in the body (Altavilla et al., 2009).

Due to the role of reactive species in the inflammatory process (Halliwell et al., 1988), the hydrogen donating ability and the scavenging effects on reactive oxygen species (ROS) of *Parastrephia lucida* extracts (Zampini et al., 2008) could be considered relevant for the plant anti-inflammatory effect. Besides the importance of the scavenging activity against ROS, a putative direct anti-inflammatory activity may be relevant in the treatment of inflammatory affections by *Parastrephia lucida*. Thus, the aim of the present study was to investigate if *Parastrephia lucida* extracts can decrease the activity of COX, LOX and sPLA$_2$, key enzymes in the formation of pro-inflammatory eicosanoids mediators from AA, in order to support the traditional use of this plant as an anti-inflammatory crude drug. Furthermore, bio-guided fractionation of the extracts and active fractions was undertaken to get an insight into the chemical identity of the active anti-inflammatory agents.

2. Materials and methods

2.1. Chemicals

Soybean lipoxygenase-1, diphenylboric acid-β-ethylamino ester (NP), tannic acid, caffeic acid and ethylenediaminetetraacetic acid (EDTA) were purchased from Sigma-Aldrich (MO, USA). Linoleic acid and dimethyl sulfoxide (DMSO) were obtained from Merck

(Germany). Triton X-100 was supplied from Fluka Chemical Corp. (USA). 1,2-diheptanoilthio-glycerophosphocholine (1,2dHGPC), secretory phospholipase A_2 (sPLA$_2$) from bee venom and 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) were obtained from Cayman Chemical Co. (MI, USA). Dichloromethane (DCM) and methanol (MeOH) were purchased from Cicarelli (Buenos Aires, Argentina). Analytical TLC was carried out using pre-coated plates (Kieselgel 60 F254, 0.2 mm, Merck, Germany). Other chemicals were purchased from local commercial sources and they were of analytical grade quality.

2.2. Plant material

The aerial parts of *Parastrephia lucida* (Meyen) Cabrera were collected from January to February 2006 at 3600 m over sea level (m.o.s.l) in Antofagasta de la Sierra, Provincia de Catamarca, Puna de Atacama. A voucher Specimen no. 607923/LIL, was deposited in the Herbarium of "Fundación Miguel Lillo", Tucumán, Argentina. The plant was authenticated by Dra. Soledad Cuello. The samples were dried at 40 °C. The parts used were leaves and stems (aerial parts), according to the traditional use.

2.3. Extraction and fractionation of bioactive compounds

The extraction and fractionation of bioactive compounds was performed according to D'Almeida et al. (2012) (Fig. 1). Briefly, the dried plant material was finely powdered and macerated with MeOH (250 g of dry plant material per liter) for 7 days at room temperature with gentle shaking or stirring (40 cycles/min). A liquid–liquid partition of the crude MeOH extract was performed with dichloromethane (DCM) and water (AQ). The DCM phase was more active as anti-inflammatory than the AQ phase and was further worked-up to disclose the active fractions. The DCM extract was permeated on Sephadex LH-20 and eluted with methanol. Seven fraction pools were obtained (A–G) based on TLC profiles revealed with NP reagent. Fractions C and F showed the highest enzyme inhibitory activities and were fractionated by silica gel column chromatography using a gradient of increasing polarity as follows.
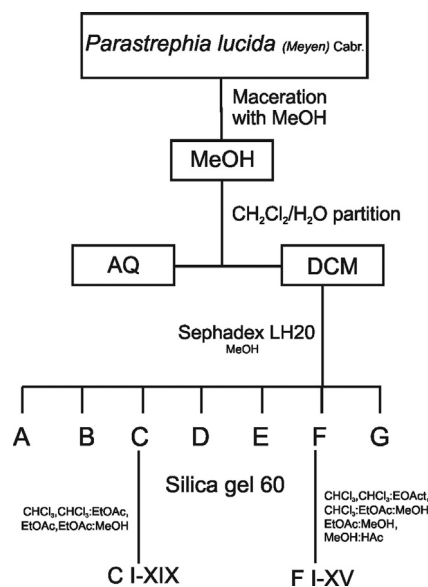

Fig. 1. Bio-guided fractionation of anti-inflammatory compounds from *Parastrephia lucida* aerial parts. Methanol (MeOH), aqueous (AQ) and dichloromethane (DCM) extracts.

Fraction C: The column was eluted with $CHCl_3$ followed by $CHCl_3$/EtOAc mixtures to reach 100% EtOAc and then EtOAc:MeOH (90:10)

Fraction F: The column was eluted with $CHCl_3$ followed by $CHCl_3$/EtOAc mixtures to reach 50% EtOAc. Then, $CHCl_3$:EtOAc: MeOH (45:45:10), EtOAc:MeOH (70:30), and MeOH: HAc (50:50). Fractions of 10 mL each were collected and combined into pools based on TLC analysis affording the sub-fractions C_I–C_{XIX} and F_I–F_{XV} (Fig. 1). All extracts, fractions and sub-fractions were suspended in DMSO to obtain stock solutions of 20 mg/mL. The solutions were kept at 4 °C for further experimental use.

2.4. Enzyme assays

2.4.1. Lipoxygenase

LOX activity was determined according to Taraporewala and Kauffman (1990), based on the enzymatic oxidation of linoleic acid to the corresponding hydroperoxide. The assay mixture containing soybean lipoxygenase (948 U), linoleic acid (10–75 μM), sodium borate buffer (200 mM, pH 9.0) and different concentrations of natural products (up to 400 μg/mL for MeOH and DCM extracts and up to 1200 μg/mL for AQ extract) were incubated at 25 °C during 4 min. The positive and negative controls were also done. The absorption at 234 nm was recorded as a function of time during 4 min every 30 s on a Spectronic Unicam (Genesys) spectrophotometer. Caffeic acid (up to 160 μg/mL) and naproxen sodium (up to 50 μg/mL) were used as reference compounds.

The anti-inflammatory effect was expressed as inhibition percent of hydroperoxide production and the IC_{50} value was defined as test compound concentration necessary to produce 50% inhibition of hydroperoxide-release. The double-reciprocal Lineweaver-Burk plot of $1/V$ $vs.$ $1/S$ was plotted to determine inhibition type. Michaelis Menten constant (K_m) and inhibition constant (K_i) were determined.

2.4.2. Cyclooxygenase

The ability of the extracts and/or fractions to inhibit the conversion of arachidonic acid to PGH_2 by ovine COX-1 and human recombinant COX-2 was determined by enzyme immunoassay, EIA (Kit no. 560131; Cayman Chemical, MI, USA). Cyclooxygenase catalyzes the first step in the biosynthesis of arachidonic acid to PGH_2. $PGF_{2\alpha}$ produced from PGH_2 by reduction with stannous chloride was measured by EIA in a microplate reader (Biotek ELx 808). The assay to obtain the 100% of COX activity was performed with and without DMSO as solvent control. The inhibitory assays were performed in presence of 50 μg/mL of natural products or commercial anti-inflammatory drugs (indomethacin and nimesulide selective for COX-1 and COX-2, respectively). Enzyme control was performed with inactivated enzymes by boiling during 3 min. Detection limit was 29 pg of PG/mL.

2.4.3. Phospholipase A_2

sPLA$_2$ activity was determined using 1,2-diheptanoylthio-gly-cerophosphocholine (1,2dHGPC) and Triton X-100 as substrates (Reynolds et al., 1992). The buffer Tris–HCl (10 mM pH 8) with $CaCl_2$ (10 mM), KCl (100 mM) and Triton X-100 (0.3 mM) was used for reconstitution of substrate to achieve a final concentration of 1.25 mM. The mixture contained 50 μL buffer Tris–HCl (10 mM pH 8), 10 μL DTNB (10 mM), 10 μL enzyme sPLA$_2$ (1 μg/mL) and 50 μg/mL of assayed natural products dissolved in DMSO or commercial anti-inflammatory drugs (naproxen, acetylsalicylic acid and indomethacin). The reaction was initiated by the addition of 150 μL of 1,2dHGPC (1.66 mM) and maintained during 20 min at 25 °C. The absorbance was read at 414 nm during 20 min every 2 min in a microplate reader (Biotek ELx 808).

2.5. Antioxidant ativity

2.5.1. Iron (III) to iron (II) reducing activity

The ability of the extracts to reduce iron (III) was assessed by the method of Oyaizu (1986). The natural product solution (10–50 μg/mL) was mixed with 3.5 mL of sodium phosphate buffer (0.2 M, pH 6.6) and 2.5 mL of 1% aqueous potassium hexacyano-ferrate [$K_3Fe(CN_6)$] solution. After 30 min incubation at 50 °C, 2.5 mL of 10% trichloroacetic acid was added, and the mixture was centrifuged at 4000g for 10 min. Finally, 2.5 mL of the upper layer was mixed with 2.5 mL of water and 0.5 mL of 0.1% aqueous $FeCl_3$, and the absorbance was recorded at 700 nm. The mean absorbance values were plotted against concentration, and a linear regression analysis was carried out. RC_{50} (micrograms per milliliter) is the concentration at which the absorbance was 0.5 for reducing power and was obtained by interpolation from linear regression analysis. Ascorbic acid was used as control.

2.5.2. Metal chelating activity

The chelation of ferrous ions by natural products was estimated by the method of Dinis et al. (1994). Briefly, 50 μL of 2 mM $FeCl_2$ was added to 1 mL of different concentrations of the samples (10–300 μg/mL). Na$_2$EDTA was used as positive control. The reaction was initiated by the addition of 0.2 mL of 5 mM ferrozine solution. The mixture was vigorously shaken and left to stand at room temperature for 10 min and the absorbance was measured at 562 nm. The percentage inhibition of ferrozine–Fe^{2+} complex formation was calculated and the concentration values of natural product required to complex 50% of Fe^{2+} (QC_{50}) were expressed as milligrams per milliliter. EDTA was used as control.

2.5.3. ABTS free radical scavenging activity

The antioxidant capacity assay was carried out by the improved $ABTS^{\bullet+}$ method as described by Re et al. (1999). $ABTS^{\bullet+}$ solution (1 mL) was added to different concentrations of natural products (5–100 μg/mL) and mixed thoroughly. Absorbance was recorded at 734 nm after 1 and 6 min. The percentage of inhibition was measured after 1 min of reaction, by the following formula: % inhibition $= [(A_0-A_s)/A_0] \times 100$; where A_0 is the absorbance of the control (blank, without extract) and As is the absorbance in presence of the extract. The SC_{50} values are the micrograms per milliliter required to scavenge 50% ABTS free radicals. Quercetin was used as control.

2.6. Compound identification

2.6.1. HPLC–ESI-MS/MS

An Agilent Series 1200 LC System (Agilent, USA) coupled to a MicrOTOF Q II (Bruker Daltonics, USA) was used for HPLC–ESI-MS/MS analysis. The HPLC system consisted in a micro vacuum degasser, binary pumps, an autosampler (40 μL sample loop), a thermostated column compartment, and a diode array detector. The mass spectrometer equipped with electrospray ion source and qTOF analyzer, was used in MS and MS/MS mode for the structural analysis of phenolics. HPLC analyses were performed on a thermostated (40 °C) Phenomenex Luna C18 250 × 4.6 mm (5 μm) column at a 0.4 mL/min flow rate using 0.5% (v/v) formic acid (solvent A) and methanol (solvent B) with the following gradient of composition: starting with 20% and changing to 50% B during 3 min, kept for 5 min, followed by a second ramp to 80% B in 5 min, maintained for 17 min, a third ramp to 20% B in 1 min, remaining at this last condition for 10 min before the next run. The injection volume was 40 μL. ESI-MS detection was performed in the negative ion mode with mass acquisition between 100 and 1500 Da. Nitrogen was used as drying and nebulizer gas (7 L/min and

3.5 bar, respectively), and 180 °C for drying temperature. For MS/MS experiments fragmentation was achieved by using Auto MS^2 option. DAD analyses were carried out in the range between 200 and 700 nm. The compounds were identified by comparing the mass spectral data with those stored in database libraries and/or by interpretation of the UV data and mass spectra.

TOF MS of the mixtures and single compounds was carried out using a Q-tof Micro (Micromass U.K. Ltd.). The spectra showed the $[M+1]^+$ as well as the $[M+Na]^+$ and $[M+K]^+$ adducts of the ionized compounds. MS–MS analysis of selected ions allowed tentative identification of the constituents by comparison with literature data.

2.6.2. NMR analysis

^{1}H NMR spectra were recorded at 400 MHz on a Bruker Avance spectrometer (Bruker, Rheinstetten, Germany). Chemical shifts are given in δ (ppm) in reference with the residual chloroform in CDCl$_3$ or MeOH in MeOH-d$_4$.

2.7. Statistical analysis

Each experimental value is expressed as the mean $\pm$ standard deviation (S.D.). The scientific statistic software *InfoStat* was used to evaluate the significance of differences between groups. Comparisons between groups were done using a one-way ANOVA with Tukey post test at a confidence level of 95%. The criterion of statistical significance was taken as $p \leq 0.05$.

3. Results and discussion

In the present study, the traditional use of *Parastrephia lucida* to relieve inflammation was investigated using enzymatic assays. Alberto et al. (2009) revealed the *in vitro* inhibitory effect of *Parastrephia lucida* crude extract on COX-1 and COX-2. However, there was no previous data on the effect of the crude drug extracts or its fractions on LOX, sPLA2 and COX. To better understand the mechanism of action of *Parastrephia lucida* on inflammation, the whole extract and its fractions were assessed on the selected enzymes.

3.1. Inhibitory activity on sPLA$_2$ and COX-2

MeOH and DCM extracts were more effective inhibitors of sPLA$_2$ and COX-2 than the AQ extract (Table 1). Under the same experimental conditions (50 μg/mL), the reference compounds naproxen, indomethacin and acetyl salicylic acid inhibited the sPLA$_2$ activity by 12.0 ± 5.0, 16.0 ± 4.7, and $40.2 \pm 5.0\%$, respectively. The most active fractions towards sPLA$_2$ were subfractions from the DCM extract, namely E, F and G with inhibition higher than 50%, being more active than the commercial anti-inflammatory drugs tested. Fractions E and F showed the highest anti-COX-2 activity although lower than nimesulide. Fraction F was less active than E against the enzyme COX-1 (constitutive form). Moreover, the COX-1 inhibition by fraction F was 77% less effective than the control indomethacin, this is important considering the side effects derived from COX-1 inhibition.

3.2. Inhibitory activity on LOX

5-LOX inhibitors have a therapeutic potential in a variety of inflammatory and allergic diseases, such as asthma and in rheumatoid arthritis. LOX and their products play an important role in tumor formation and cancer metastasis. High expression of 5-LOX was found in prostate, lung, colon, breast, and other cancer cell lines (Nimmanapalli et al., 2010).

Table 1
Effect of *Parastrephia lucida* extracts and fractions at 50 μg/mL on the enzymes phospholipase A$_2$, lipoxygenase and cyclooxygenases.

Samples	% Enzyme inhibition			
	sPLA$_2$	LOX	COX-1	COX-2
MeOH extract	$44.0 \pm 2.2^{d,e}$	0.0 ± 0.0^a	$24.5 \pm 1.2^{b,c}$	31.9 ± 1.6^b
AQ extract	$14.0 \pm 0.7^{a,b}$	0.0 ± 0.0^a	$52.0 \pm 2.6^{e,f}$	10.0 ± 0.2^a
DCM extract	$33.0 \pm 1.6^{c,d}$	5.3 ± 0.3^a	31.6 ± 1.6^c	31.9 ± 1.6^b
DCM fraction pools				
A	30.0 ± 1.5^c	$8.1 \pm 1.4^{a,b}$	2.0 ± 0.1^a	5.0 ± 2.5^a
B	$32.0 \pm 1.6^{c,d}$	21.1 ± 1.4^c	4.6 ± 0.2^a	7.2 ± 0.4^a
C	$25.0 \pm 1.3^{b,c}$	43.4 ± 4.4^d	32.0 ± 3.1^c	$31.3 \pm 1.6^{b,c}$
D	$51.0 \pm 2.6^{e,f}$	$20.9 \pm 1.6^{b,c}$	$36.0 \pm 1.8^{c,d}$	$31.9 \pm 1.6^{b,c}$
E	$55.0 \pm 2.8^{e,f,g}$	$11.6 \pm 0.8^{a,b,c}$	62.0 ± 3.1^f	$40.0 \pm 2.1^{c,d}$
F	$59.0 \pm 3.0^{f,g}$	6.6 ± 0.5^a	18.0 ± 0.9^b	50.6 ± 2.5^d
G	67.0 ± 3.4^g	7.6 ± 0.1^a	$46.0 \pm 2.3^{d,e}$	25.1 ± 1.2^b
Naproxen	12.0 ± 0.6^a	95.0 ± 2.8^e	–	–
Acetylsalicylic acid	$40.2 \pm 5.0^{d,e}$	4.4 ± 2.8^a	–	–
Indomethacin	$16.0 \pm 0.8^{a,b}$	–	95.0 ± 4.8^g	–
Nimesulide	–	–	–	100.0 ± 5.0^e

Values are reported as mean $\pm$ standard deviation of triplicates. Different letters in the same column show significant differences among each treated group, according to the Tukey test ($p \leq 0.05$).

While the MeOH and AQ extracts of *Parastrephia lucida* were devoid of activity (IC$_{50}$ = 465.0 ± 32.5 and 1030.0 ± 72.1 μg/mL, respectively), the DCM extract (IC$_{50}$ = 358.0 ± 25.1 μg/mL) showed a weak effect that increased in some of the extract fractions. The most active fraction was C reducing enzyme activity by $43 \pm 4\%$ at 50 μg/mL and showing an IC$_{50}$ value of 60.0 ± 4.1 μg/mL. Fractions A, B and D presented IC$_{50}$ values of 124.0 ± 8.7, 111.0 ± 7.7 and 165.0 ± 11.4 μg/mL, respectively. These four fractions had in common a higher non-flavonoid phenolic content (D'Almeida et al., 2012). As the IC$_{50}$ value of fraction C was similar to the reference compound caffeic acid (IC$_{50}$ = 57.0 ± 0.4 μg/mL), the type of enzyme inhibition was investigated. Fraction C was found to be a non-competitive inhibitor (Fig. 2), with a Km value of 167 μM (R^2 = 0.9928) while the V_{max} values decreased from 52 μM/min (control without inhibitor) to 38, 30 and 23 μM/min with the increase of fraction C concentrations (25, 50 and 75 μ/mL, respectively). The K_i value was 60 μg/mL.

Most of the LOX inhibitors, synthetic and as well as from natural sources (*e.g.*, polyphenols, coumarins, isoflavones and quinones) act at the catalytic domain by reducing or chelating the active-site iron or simply by scavenging electrons participating in the redox cycle of iron (Werz and Steinhilber, 2005). For this, 5-LOX inhibitors can be classified into three main groups: redox-active compounds, iron-ligand inhibitors (with weak redox-active properties) and non redox-type inhibitors (Werz and Steinhilber, 2005). In order to understand the interaction as well as the nature of inhibition, we studied the iron reducing, metal chelating and radical scavenging activities of fraction C and their most active sub-fractions (C$_{IV}$–C$_{IX}$) (Table 2). Only sub-fractions C$_{IV}$ and C$_{IX}$ showed values of Fe^{+3} reducing power statistically similar to fraction C (RC$_{50}$ = 24.0 ± 1.6 μg/mL). This implies that the sub-fraction C$_{IX}$ with the concentration necessary to produce 50% of LOX inhibition (75.3 μg/mL) was able to reduce 100% of free iron. The active site of the 5-LOX enzyme contains a non-heme iron. In the resting state, the iron is in the ferrous form (Fe^{2+}). Activation of 5-LOX by hydroperoxides oxidizes the iron into the ferric form (Fe^{3+}) that allows the enzyme to enter the catalytic cycle. This cycling is interrupted by redox-type 5-LOX inhibitors which are able to reduce the active-site ferric iron (Werz and Steinhilber, 2005).

Redox-active 5-LOX inhibitors can act by keeping the active site iron in the ferrous state, thereby uncoupling the catalytic cycle of
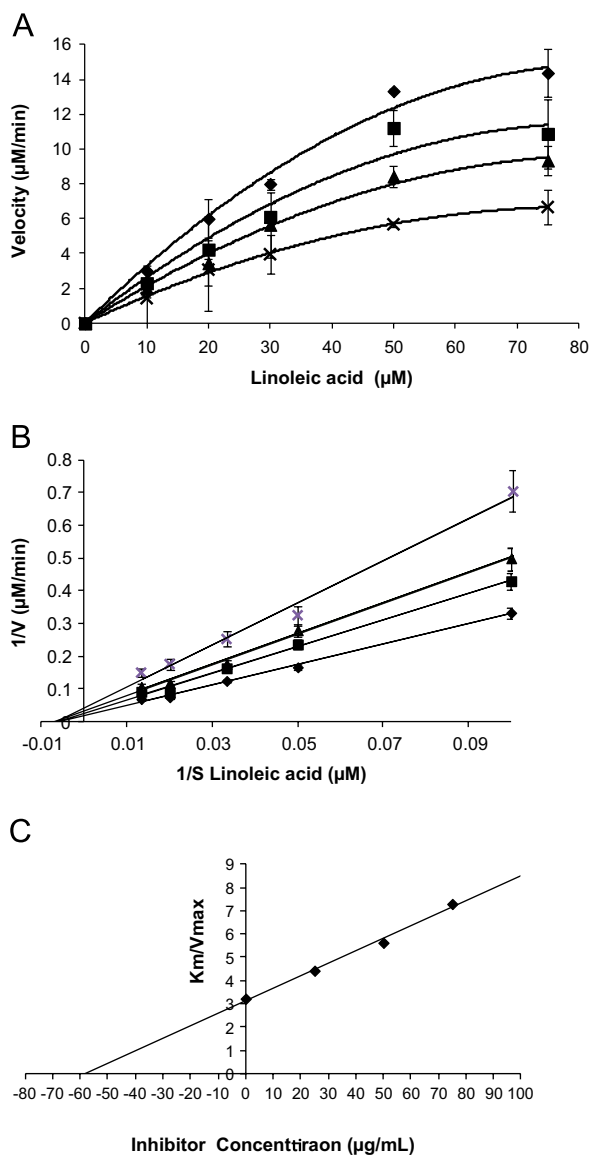
Fig. 2. (A) Hydroperoxide production kinetics of linoleic acid in presence of different concentrations of fraction C (in micrograms per milliliter): 0 (♦), 25 (■), 50 (▲) and 75 (✕). (B) Lineweaver–Burk plot analysis for lipoxygenase inhibitory activity of fraction C. (C) Determination of K_i for fraction C. The slope (K_m/V_{max}) of the lines described from the double reciprocal plot is plotted against the fraction C concentrations. All values are average of three experiments.

Table 2
Antioxidant activity of *Parastrephia lucida* fraction C and sub-fractions.

Samples	RC_{50} (µg/mL)	QC_{50} (mg/mL)	SC_{50} (µg/mL)
C	24.0 ± 1.6^a	0.5 ± 0.0^a	11.0 ± 1.3^a
IV	20.1 ± 1.5^a	0.5 ± 0.0^a	> 50
VI	> 50	0.7 ± 0.1^a	> 50
VII	> 50	0.8 ± 0.1^a	> 50
VIII	> 50	> 1.8	46.2 ± 4.2^b
IX	20.2 ± 1.7^a	> 1.8	42.5 ± 3.8^b
Ascorbic acid	3.1 ± 0.1^b	–	–
EDTA	–	0.022 ± 0.0^b	–
Quercetin	–	–	3.6 ± 0.4^c

Iron reducing concentration (RC), iron chelating concentration (QC), ABTS free radical scavenging concentration (SC). Values are reported as mean ± standard deviation of triplicates. Different letters in the same column show significant differences among each treated group, according to the Tukey test ($p \leq 0.05$).

the enzyme (Werz, 2007). On the other hand, the transition metal ion, Fe^{2+} possess the ability to move single electrons by virtue of which it can allow the formation and propagation of many radical reactions, even starting with relatively non-reactive radicals. One strategy to avoid ROS generation that is associated with redox active metal catalysis involves chelating of the metal ions. However, the sub-fractions C_{VIII} and C_{IX} presented low chelating activity. Other possible role for the samples constituents is to act as free radical scavengers. In the ABTS free radical scavenging assay the SC_{50} values for the sub-fractions C_{VIII} and C_{IX} (the more active inhibitors of LOX enzyme) were 46.2 ± 4.2 and 42.5 ± 3.8 µg/mL, respectively. These results lead to the conclusion that the mechanism of action of fraction C is probably a combination of the reducing power and scavenging capacity of the chemical components present in the C_{VIII} and C_{IX} sub-fractions. Compounds able to block LOX enzyme are interesting targets when looking for bioactive natural products.

3.3. Identification of bioactive compounds in fractions C and F

Assay-guided fractionation of the *Parastrephia lucida* extract showed that fractions were more effective inhibiting COX and sPLA$_2$ than LOX. Fraction C was more active towards LOX while fraction F inhibited sPLA$_2$ and COX-2. Based on this information, fractions C and F were selected to continue with the activity-guided isolation of anti-inflammatory agents. Silica gel column chromatography of the fraction C afforded 19 sub-fractions with the following extraction yields: I (13.12 mg/g), II (7.77 mg/g), III (119.68 mg/g), IV (53.6 mg/g), V (56.96 mg/g), VI (20.0 mg/g), VII (89.2 mg/g), VIII (27.1 mg/g), IX (13.0 mg/g), X (57.68 mg/g), XI (67.0 mg/g), XII (51.84 mg/g), XIII (3.12 mg/g), XIV (3.24 mg/g), XV (154.24 mg/g), XVI (4.1 mg/g), XVII (21.84 mg/g), XVIII (9.72 mg/g), and XIX (7.1 mg/g) (Fig. 1). All sub-fractions were evaluated for LOX inhibitory activity and were less active towards LOX than the whole fraction C (Fig. 3A). The IC_{50} values for the more active sub-fractions C_{IV}, C_{VIII} and C_{IX} were 87.7, 69.9 and 75.3 µg/mL, respectively. The compounds occurring in the more active sub-fractions were identified using spectroscopic and spectrometric means. The structure of the simple phenolics and phenylpropanoids found in the *Parastrephia lucida* fractions follow from a combination of HPLC–DAD, HPLC–DAD-MS/MS and ^{1}H NMR analysis. The UV spectra give information on the class of phenolic compound, also related to the Rt in the HPLC chromatogram. The MS and MS/MS analysis of the different peaks in the HPLC chromatograms allowed a tentative identification, using literature data and previous work. This information was verified by NMR analysis and literature data. The identified compounds included ferulic acid methyl ester, ferulic acid ethyl ester, ferulic acid prenyl ester and vanillin (fraction C_{IV}), as well as cinnamic acid (fractions C_{VIII} and C_{IX}). These compounds were previously reported from this plant (D'Almeida et al., 2012). Ferulic acid 2-phenethyl alcohol and dimethylcaffeic acid 2-phenethyl ester, close related to caffeic acid phenethyl ester (CAPE) were found in sub-fraction C_{II} (D'Almeida et al., 2012). Ferulic acid 2-phenethyl alcohol or phenethyl ferulate was responsible for the COX and 5-LOX inhibitory activity of *Notopterygium incisum* extracts, a plant used in China to treat rheumatism (Zschocke et al., 1997). Several biological and pharmacological properties, including: antioxidant, anti-inflammatory, anti-carcinogenic, antiviral, and inmunomodulatory effect were demonstrated to CAPE (Dang et al., 2010). CAPE, present in propolis, shows anti-inflammatory activity through inhibiting the release of AA from the cell membrane (Mirzoeva and Calder, 1996; Rossi et al., 2002). In propolis, caffeic acid, quercetin, naringenin, and CAPE showed anti-inflammatory activity contributing to the suppression of PGs and LTs synthesis by macrophages and have inhibitory effects on COX and LOX activity, myeloperoxidase, NADPH-oxidase, ornithine decarboxylase and tyrosine-protein-kinase (Mirzoeva and Calder, 1996). The anti-inflammatory activities of propolis have
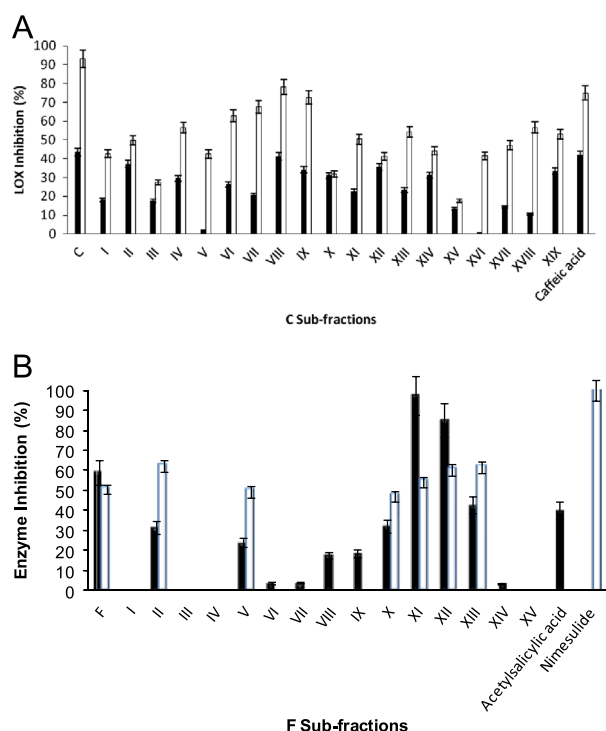
Fig. 3. (A) Effect of sub-fractions of the *Parastrephia lucida* pool C and caffeic acid at 50 (■) and 100 (□) μg/mL on lipoxygenase activity. (B). Effect of sub-fractions of the *Parastrephia lucida* pool F at 50 μg/mL on phospholipase A_2 (■) and cyclooxygenase (□). Acetylsalicylic acid (50 μg/mL) and nimesulide (50 μg/mL) were used as reference drugs.

been also explained by the presence of active flavonoids and cinnamic acid derivatives (Almeida and Menezes, 2002). Naturally occurring hydroxycinnamates are extremely potent antitumor agents and shows low toxicity in human exposure (De et al., 2011).

The fraction F was chromatographed on silica gel affording 15 subfractions as follows: I (114.7 mg/g), II (106.5 mg/g), III (132.8 mg/g), IV (1.64 mg/g), V (5.74 mg/g), VI (6.56 mg/g), VII (4.92 mg/g), VIII (67.2 mg/g), IX (3.28 mg/g), X (189.34 mg/g), XI (36.0 mg/g), XII (262.3 mg/g), XIII (2.46 mg/g), XIV (43.44 mg/g) and XV (12.3 mg/g). All F sub-fractions were tested for sPLA2 and COX-2 inhibitory activity (Fig. 3B). In the sPLA2 assay, the sub-fractions F_{XI} and F_{XII} were more active than fraction F, with inhibition percentages of 97.5 ± 2.1 and $85.3 \pm 6.0\%$, respectively. In the COX-2 assay the sub-fractions F_{II}, F_{XI}, F_{XII} and F_{XIII} were more effective than the fraction F. The compounds identified in the bioactive sub-fractions from Fraction F were mainly flavonoid and benzofurans while in fraction C there were caffeic, ferulic and cinnamic acid derivatives, previously reported from the plant (D'Almeida et al., 2012).

The compounds occurring in the active fractions were identified by NMR techniques, MS and UV data. For the complex mixtures, QTOF-MS was carried out to identify the constituents. The information was compared with the ^{1}H NMR spectra of the mixtures as well as with the UV data of the HPLC–DAD analyses. The main compound of F_{II}, with a Rt of 23.01 min and a molecular formula of $C_{16}H_{14}O_5$ was unambiguously identified as 5,4′-dihydroxi-7-methoxyflava-none (sakuranetin). Sakuranetin: ^{1}H NMR (MeOH-d_4, 400 MHz): δ: 7.22 (2H, d, $J=8.8$ Hz), 6.71 (2H, d, $J=8.8$ Hz), 5.96 (1H, d, $J=2.4$ Hz), 5.94 (1H, d, $J=2.4$ Hz), 5.27 (1H, dd, $J=13.0, 3.0$ Hz), 3.71 (3H, s), 3.05 (1H, dd, $J=17.0, 13.0$ Hz), and 2.63 (1H, dd, $J=17.0, 3.0$ Hz). QTOF-MS: $[M+H]^+$: 287 (57), 167 (100), and 147 (76). UV maxima (nanometer): 325 sh, 302, and 278.

Traces of the following compounds were also found in the fraction: 3,7,4′-trimethoxy-5-hydroxyflavone $(C_{18}H_{16}O_6)$ $[M+H]^+$: 329, 167;

$C_{18}H_{16}O_5$ $[M+H]^+$: 313. The F_X, according to HPLC–MS/MS analysis contained quercetin methyl ether, quercetin dimethyl ether, kaemp-ferol methyl ether (main compound), apigenin, apigenin methyl ether and apigenin trimethyl ether. The complex mixture of F_{XI} contained mainly methoxy flavones and flavonols bearing two oxygen functions in the B-ring. The main compound was assigned as luteolin-7-methylether. Luteolin-7-methylether: ^{1}H NMR (MeOH-d_4, 400 MHz): δ 7.27 (1H, m), 7.27 (1 H, brs), 6.80 (1H, dd, $J=8.4, 2$ Hz), 6.41 (1H, s), 6.32 (1H, s), 6.09 (1H, s), and 3.78 s (3H, s). QTOF-MS: $[M+H]^+$: 301 $(C_{16}H_{12}O_6)$. UV maxima (nanometer): 349, 290 sh, 270 sh, 255, and 228.

Other compounds detected were $C_{16}H_{12}O_7$ $[M+H]^+$: 317, compatible with a tetrahydroxy methoxy flavone, 5,4′-dihydroxi-7-methoxyflavanone (MS in agreement with the same compound occurring in F_{II}) and a tremetone derivative in trace amounts. F_{XII} presented a similar flavonoid pattern than F_{XI} but differing in the relative proportion of the minor compounds. The TOF MS of the fraction F_{XIII} showed a constituent with $[M+H]^+$ of 203.0769, compatible with tremetone $(C_{13}H_{14}O_2)$.

Numerous experimental data have shown that luteolin pos-sesses a wide range of biological activities like antioxidant (Choi et al., 2007), anti-inflammatory (Hu and Kitts, 2004; Chen et al., 2007), antimicrobial (Tshikalange et al., 2005) and anticancer (Manju and Nalini, 2007). The presence of luteolin derivates in fraction F may contribute with its activity on pro-inflammatory enzymes. Methoxytremetone and tremetone were identified in *Parastrephia lepidophylla* (Bohlmann et al., 1979; Ayma et al., 1995; Benites et al., 2012) and p-coumaroyloxy-tremetone, was reported in *Parastrephia lepidophylla* and *Parastrephia quadrangularis* (Bohlmann et al., 1979; Loyola et al., 1985). Tremetone derivatives were also found in South American *Ophryosporus* species (Aster-aceae). Seven acetophenone derivatives from *Ophryosporus axilliflorus* were investigated for anti-inflammatory activity by the carrageenan-induced mouse paw edema. Tremetone showed a strong anti-inflammatory activity (Favier et al., 1998) and a morphine-like analgesic property (Benites et al., 2012).

Our results showed the presence of flavonoids (Fraction F) and phenolic acid (Fraction C) in *Parastrephia lucida*. The effect of flavo-noids on a variety of inflammatory processes has been extensively investigated and it has been demonstrated that they are able to inhibit a series of enzymes which are activated in the course of the inflammatory process (Kim et al., 2004; González-Gallego et al., 2007). Sakuranetin was reported as the major flavonoid phytoalexin in rice (*Oryza sativa*) (Kodama et al., 1992) and can be induced by UV radiation or phytopathogenic infection in the plant. Recent studies have shown that sakuranetin is a plant antibiotic and has potential as a pharmaceutical agent (Shimizu et al., 2012). Moreover, sakuranetin showed considerable inhibitory activity against COX (Zhang et al., 2006) and allergy-preventive effects (Ogawa et al., 2007). Toledo et al. (2013) suggested a potential anti-inflammatory activity in an experi-mental asthma model, which may be related to modulatory effects of sakuranetin concerning pro-inflammatory cytokines. Kim et al. (2004) reported that plant flavonoids show anti-inflammatory activity *in vitro* and *in vivo* and one of their action mechanisms is the inhibition of eicosanoid generating enzymes including PLA2, COXs, and LOXs, thereby reducing the concentrations of prostanoids and LTs.

Structure-activity relationships studies of flavonoids have shown that several flavones such as apigenin are COX inhibitors, while some flavonols such as quercetin are preferential LOX inhibitors (Kim et al., 2004). The optimum chemical structures for these effects are the C-2,3-double bond, A-ring 5,7-hydroxyl groups, and B-ring 4′- or 3′,4′-hydroxyl groups. The C-3 hydroxyl group as in flavonols is favorable for LOX inhibition. On the other hand, while flavanones showed less inhibition, flavonols were found to considerably inhibit sPLA2 indicating the importance of the C-ring-2,3-double bond (Kim et al., 2004).

4. Conclusions

The traditional indication of use of *Parastrephia lucida* as an anti-inflammatory agent was assessed using enzyme inhibition assays and bio-guided fractionation of the active extract and fractions. The crude drug was shown to have effect on different enzymes associated with the eicosanoid metabolism and several compounds were identified in the active fractions. *Parastrephia lucida* acts via a variety of mechanisms to inhibit prostanoids biosynthesis making it an interesting crude drug to relieve inflammation disorders. Other mechanisms for the anti-inflammatory activity as well as other pharmacological activities of *Parastrephia lucida* are under investigation.

Acknowledgments

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