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Effect of Isolated Fractions of *Harpagophytum procumbens* D.C. (Devil's Claw) on COX-1, COX-2 Activity and Nitric Oxide Production on Whole-Blood Assay

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The present study evaluates the effect of isolated fractions of *Harpagophytum procumbens* (devil's claw) on cyclooxygenase (COX-1 and COX-2) activities and NO production using a whole blood assay. The activity of COX-1 was quantified as platelet thromboxane B_2 production in blood clotting and COX-2 as prostaglandin E_2 production in LPS-stimulated whole blood. Total NO_2^-/NO_3^- concentration was determined by Griess reaction in LPS stimulated blood. Assays were performed by incubation of isolated fractions obtained by flash chromatography monitored with HPLC, TLC and identified by ^{1}HNMR, containing different amounts of harpagoside with blood from healthy donors. Indomethacin and etoricoxib were the positive controls of COX-1 and COX-2 Inhibition. Data shows that fraction containing the highest concentration of harpagoside inhibited indistinctively COX-1 and COX-2 (37.2 and 29.5% respectively) activity and greatly inhibited NO production (66%). In contrast the fraction including iridoid pool increased COX-2 and did not alter NO and COX-1 activities. The fraction containing cinnamic acid was able to reduce only NO production (67%). Our results demonstrated that the harpagoside fraction is the main responsible for the effect of devils claw on these enzyme activities. However, other components from devil's claw crude extract could antagonize or increase the synthesis of inflammatory mediators.

Keywords: Harpagophytum procumbens; COX-1; COX-2; nitric oxide.

INTRODUCTION

The extract of *Harpagophytum procumbens* (Burchell De Candolle) Ex Meissn (Pedaliaceae), usually called devil's claw, is an herbal substance commonly used by patients with osteoarthritis (OA) as a complementary therapy (Chantre *et al.*, 2000; Churubasik *et al.*, 2003a, 2003b). This perennial plant grows mainly in Africa, especially in the Namibian steppes and the Kalahari Desert. Depending on the extraction process, the resulting products contain different fractions of constituents with analgesic and antiinflammatory properties (Loew *et al.*, 2001). The leading constituent, harpagoside, belongs to the iridoid glycoside family and has been described as being responsible for many of the therapeutic effects of the devil's claw extracts. There is evidence that extracts of devil's claw interact with both cyclooxygenase (COX) and lipooxygenase, as well as with the release of cytokines and nitric oxide production (Spelman *et al.*, 2006).

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In experimental studies, extracts of devil's claw promoted significant reduction in pain and inflammation (Lanhers *et al.*, 1992; Andersen *et al.*, 2004). The effect of devil's claw on different inflammatory parameters has also been demonstrated in *in vitro* studies. In fibroblast cell lines, devil's claw extracts inhibited the lipopolysaccharide (LPS)-stimulated expression of COX-2 and inducible nitric oxide synthase (iNOS) (Jang *et al.*, 2003). In human monocyte cultures, in addition to these effects, concentrations higher than 100 μg/mL may inhibit the release of tumor necrosis factor-alpha (TNF-α), IL-6 and IL-1β (Fiebich *et al.*, 2001). Devil's claw crude extracts also suppressed nitrite formation in renal mesangial cells, due to the inhibition of iNOS expression at the transcriptional level (Kaszkin *et al.*, 2004). Huang *et al.* (2006), using human HepG2 hepatocarcinoma cells and RAW 264.7 macrophage cell lines, showed that harpagoside (200 μM) inhibited COX-2 activity and nitric oxide synthase expression in LPS-stimulated cells. No inhibition of the COX-1 enzyme activity was verified under this condition. The inhibition of the enzyme synthesis is likely to be due to its modulator effect on transcription factor NF-be activity. This is, in fact, supported by the finding that *H. procumbens* extracts inhibit cytokine production, particularly TNF-α (Fiebich *et al.*, 2001), suggesting it

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does interfere with a common target in the NF-κB pathway.

Regarding clinical efficacy, devil's claw crude extracts have demonstrated good results and tolerability for the treatment of low-back pain (Chrubasik *et al.*, 2004; Warnock *et al.*, 2007). The suppression of the synthesis of COX-2 and NOS by harpagoside could explain, at least in part, the effectiveness of devil's claw when used in the treatment of pain in inflammatory conditions (Huang *et al.*, 2006). Confirming this fact, fractions without harpagoside had no inhibitory effect on leukotriene and TXB_2 synthesis in whole blood stimulated with ionophore A23187 (Loew *et al.*, 2001). However, the effect of devil's claw on COX-1 and COX-2 activity in whole blood of humans who took 500 mg of *H. procumbens* daily during 21 days was not verified. No significant differences in the production of $PGF_{1\alpha}$, PGE_2, TXB_2 and LTB_4 were observed with this treatment schedule before and after devil's claw intake (Moussard *et al.*, 1992). The observed discrepancies could be attributed to the lack of standardization of the *H. procumbens* extracts and insufficient chemical profiling of the plant material. The aim of this study was to test fractions of crude extract of *H. procumbens* with different concentrations and compounds of harpagoside and cinnamic acid on TXB_2 and PGE_2 and NO production in a whole-blood assay.

MATERIAL AND METHODS

Plant material. The crude hydroalcohol extract of *Harpagophytum procumbens* (Burch) DC. Ex Meissn. (Pedialiaceae) (devil's claw) was obtained through the mixture of powder containing 1.78% of harpagoside (Pro-formula, Sao Paulo, Brazil) with ethanol (60% v/v).

Immediately before the assays, the crude hydroalcohol extract was dissolved in distilled water to produce final concentrations of *H. procumbens* extract in the assays of 10, 5, 2.5, 1.25 and 0.625 μg.

Fractionation of devil's claw extract. The crude hydroalcohol extract of *H. procumbens* (5 g) was fractionated by a short column vacuum chromatographic method (flash chromatography, FC) using a normal phase silica bed 60 (200–500 mm, Merck) and a mixture of chloroform/methanol ($CHCl_3$:CH_3OH, 200 mL, ratio 100.0% chloroform, 99:01,98:02, 96:04, 92:08, 90:10, 88:12, 85:15 and 80:20, 70:30, 50:50 and 100% methanol) to elute the components, it resulted in 12 fractions (F1–F12), (Zarate *et al.*, 1992). The 12 fractions obtained were monitored by thin-layer chromatography (TLC silica gel 60 F_{254} Merck) eluted with a mixture of $CHCl_3$:CH_3OH (75:25 v/v). Plates were observed under UV light at 254 nm and 366 nm and sprayed with 3% sulfuric acid/vanillin reagent to stain the iridoids. The quantitative high performance liquid chromatography (HPLC) was carried for classification of different fractions of iridoids according to Chantre *et al.* (2000). The FC was performed several times to get fractions for the bioassays and chemical analysis. Based on the analytical TLC profile of the fractions combined with HPLC, the mixture of the fractions 5–12, which showed the presence of two main chemical markers of crude extract, harpagoside and cinnamic acid with 56.1% and 25.6%, respectively, were pooled and named fraction B. Fraction 7 was described as fraction A, which had 88.8% of harpagoside. Fraction 5 from one of the FCs was also analysed separately and named fraction C. This fraction showed a light spot of iridoid after vanillin staining and contained the lowest concentration of harpagoside (2.7%) plus 85.1% of cinnamic acid. The purity degree of the fractions A, B and C (%) was evaluated by HPLC and their compounds identified by hydrogen nuclear magnetic resonance (^{1} HNMR) (Clarkson *et al.*, 2006).

Measurement of COX-1 activity in human whole blood. The crude hydroalcohol extract was dissolved as described above and 10 μL of each solution was added to the vials. The final concentrations of *H. procumbens* extract in the assays were 10, 5, 2.5, 1.25 and 0.625 μg, a control test tube with 10 μL of the diluents was also included.

Crude extract and the fractions A, B and C were tested *in vitro* in whole blood from five healthy donors who had not taken NSAIDs for at least 2 weeks before donating blood. Normal human blood was collected without anticoagulant, and 500 μL was immediately added to each tube, containing 10 μL dose test. The tubes were mixed and placed in an incubator at 37°C for 60 min; serum was separated by centrifugation (10 min at 3000 rpm) and 100 μL was removed and added to 400 μL of methanol. After another centrifugation step, the supernatant was kept at –70°C until assayed for thromboxane B_2 (TXB_2) (Patrignani *et al.*, 1994; Mello *et al.*, 2000). The Committee for Human Ethics of the University of Sao Paulo approved this study protocol.

Measurement of COX-2 activity in human whole blood. Human fresh venous blood was collected without anticoagulant and 500 μL was immediately added to each tube, containing 10 μL dose test extracts (described above), containing heparin (10 UI/mL) and lipopolysaccharide from *Escherichia coli* (LPS 100 μg/mL). To assess the basal activity of the enzyme, control tubes were incubated in the absence of LPS. The production of PGE_2 under LPS stimulation was also evaluated. The tubes were mixed and placed in an incubator at 37°C for 24 h. Plasma was separated, and PGE_2 was extracted and quantified by the same procedure as that described for thromboxane B_2 (TXB_2).

Testing the fractions of devil's claw. Peripheral whole blood was collected from five healthy female donors for the measurement of COX-2 and COX-1 activity and NO production. 10 μL of *H. procumbens* extracts, previously prepared as described above, was added to the vials. The final concentrations of *H. procumbens* in the assays were: fraction A (88.8% harpagoside) – dose 1 (30 μg/mL), dose 2 (100 μg/mL) and dose 3 (300 μg/mL), fraction B (56.1 % harpagoside + pool of iridoids + 25.6% of cinnamic acid) and fraction C (2.7% harpagoside + 85.1% free cinnamic acid) dose 1 (30 μg/mL), dose 2 (100 μg/mL) and dose 3 (300 μg/mL).

Positive controls of COX-1 and COX-2 inhibition were performed with indomethacin (40 μM) and etoricoxib (300 μM) (Tacconelli *et al.*, 2002). The COX-1 and COX-2 activity and NO formation assays were performed as described above.

Analyses of TXB_2 and PGE_2. Concentrations of PGE_2 and TXB_2 were measured by radioimmunoassay using commercial kits (prostaglandin E_2 [^{125}I] biotrak assay system with magnetic separation and thromboxane B_2 [^{125}I] assay with magnetic separation from Amersham Biosciences UK Ltd (2002).

Total NO_2^-/NO_3^- determination. The measurement of NO production under LPS (100 μg/mL) stimulation was estimated through the total NO_2^-/NO_3^- concentrations in the plasma, determined by the colorimetric Griess reaction for nitrite (Ding *et al.*, 1988; Gilliam *et al.*, 1993) following nitrate reductase-mediated nitrate ion reduction. The absorbance of the chromophore was read at 504 nm and under these conditions, the method has a sensitivity of 30 pmol for each anion.

Statistical analysis. Results of parametric values are expressed as mean ± SEM within group comparisons were analysed by ANOVA test, followed by Newman–Keul's test (normal distribution was detected). For the analysed measurements, values of $p < 0.05$ were considered significant.

RESULTS

In vitro effects of crude hydroalcohol extract of devil's claw on COX-1, COX-2 activities and NO production

The effects of the co-incubation of crescent doses of crude hydroalcohol extract of devil's claw on COX-1 and COX-2 are presented in Fig. 1. Basal production of PGE_2 represents COX-2 activity without LPS stimulation. Data were compared with values obtained in blood incubated with LPS and 10 μL of diluents (tube 0). The results showed no direct action of the crude extract of devil's claw on COX-1 and COX-2 activities. No effect of the crude extract was also observed on NO production (data not shown).

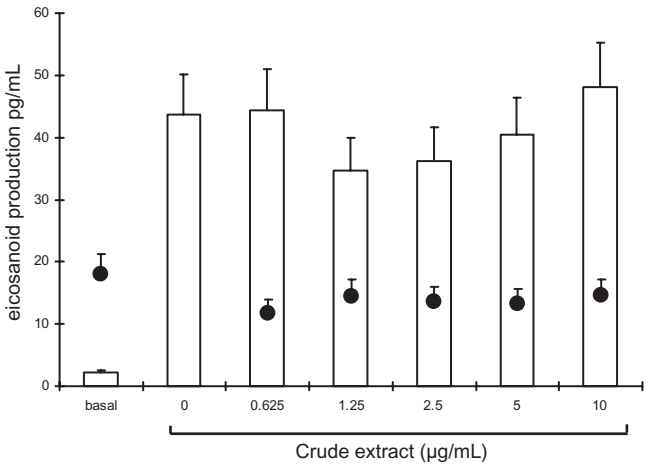

Figure 1. Effect of crescent doses of crude extract of devil's claw on COX-1 and COX-2 activity on whole blood assay. Bars represent values of PGE_2 production as a result of COX-2 activity of monocytes and the circles represent TxB_2 production as an indicator of COX-1 activity. The production of each eicosanoid at basal condition (without stimulation) is also shown. Data are presented as mean ± SEM. * $p < 0.05$ by comparison with basal values.

Effect of isolated fractions of devil's claw

The *in vitro* assay was performed with normal blood to evaluate the effect of FC isolated fractions of devil's claw on COX-1 and COX-2 activities, as well as on NO production. Indomethacin and etoricoxib were used as positive controls for enzyme inhibition. The effectiveness of fractions A, B and C were tested on three progressive doses.

The results presented in Fig. 2(A) show the alteration promoted by each fraction on COX-1 activity. The lowest dose of fraction A (30 μg/mL) promoted a reduction of 37.2% ($p = 0.012$ vs basal) on TxB_2 production. Increasing the dose of fraction A did not promote additional changes in COX-1 activity.

Significant inhibition of COX-2 (panel B) activity and NO production (panel C) was also achieved when whole blood was incubated with fraction A (30 μg/mL), which contained the highest concentration of harpagoside (88.8%). Regarding COX-2 activity fraction A (30 μg/mL) promoted inhibition of 29.5% ($p = 0.015$) of PGE_2 production when compared with whole blood under LPS stimulation. Fraction A (30 μg/mL) also reduced NO metabolites in 66% ($p = 0.037$) when compared with values obtained in whole blood under LPS stimulation.

Fraction C (30 μg/mL), which contained a concentration of 2.7% of harpagoside plus 85.1% cinnamic acid promoted only NO metabolite reduction (67%, $p = 0.005$ vs LPS stimulated).

The accuracy of the method was confirmed, as indomethacin reduced both COX-1 and COX-2 activity in 93% and 84%, respectively, although etoricoxib greatly reduced PGE_2 production (85%) and lesser TxB_2 (54%). Neither NSAIDs affected nitric oxide production.

It is important to emphasize that results obtained with fraction B showed that it was the only one that exhibited a stimulatory effect on COX-2 activity, without affecting COX-1 and NO production. This fraction is the most complex and represents the iridoid pool.

DISCUSSION

The present study was designed to evaluate the efficacy of fractions from *H. procumbens* on COX-1 and COX-2 activity and NO production. The fractioning of this crude extract was monitored through TLC and HPLC and resulted in 12 fractions (F1–F12) that contained almost all the active compounds identified through ^{1}HNMR. The effect of the crude extract and fractions on the enzymatic activity was evaluated in whole-blood assay. The advantage of the present study design is that it simulated the physiological response of humans to drug administration, thereby resulting in a more accurate measurement of the ability of a substance to inhibit COX-1 and COX-2 activities (Patrignani *et al.*, 1994). It is important to note that the control of our assay was made with two classical non steroid antiinflammatory drugs, indomethacin and etoricoxib.

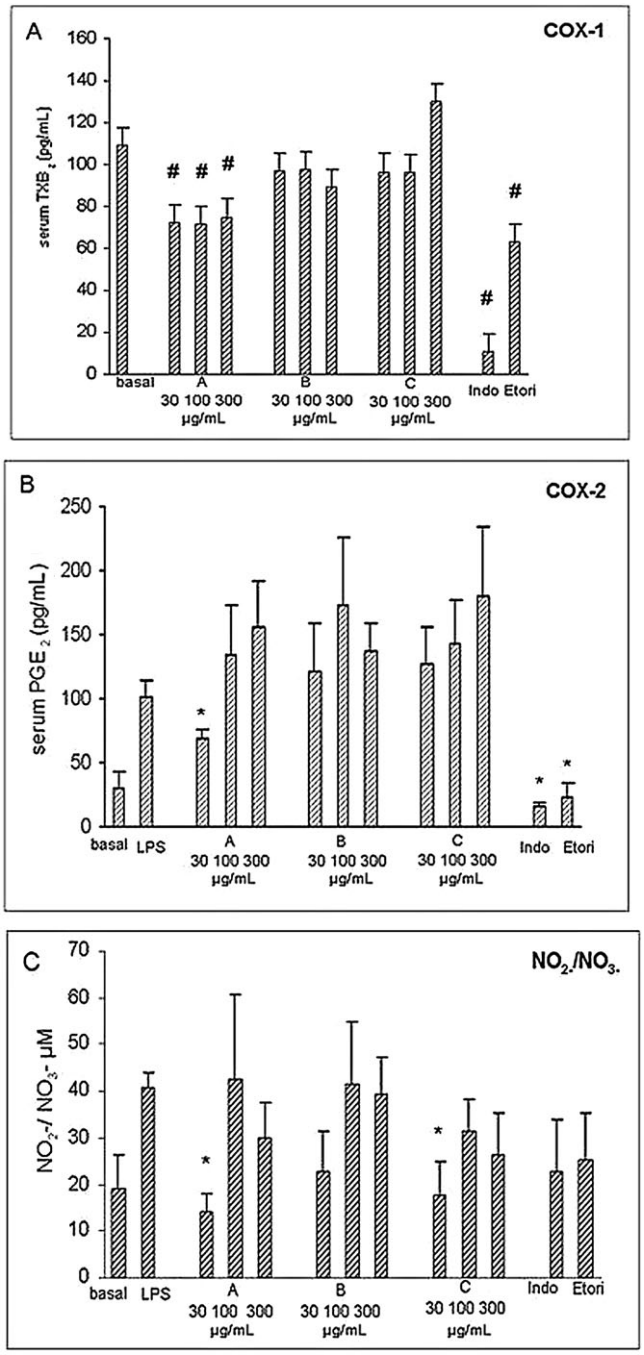

Figure 2. Effects of crescent doses (30, 100 and 300 µg/mL) of fraction A (88.8% harpagoside), fraction B (56.1% harpagoside + pool of iridoids + 25.6% of cinnamic acid) and fraction C (2.7% harpagoside + 85.1% free cinnamic acid) on COX-1 (panel A) and COX-2 activity (panel B) and NO_2-/NO_3- production (panel C). Positive controls of COX-1 and COX-2 inhibition were indomethacin (40 µM) and etoricoxib (300 µM). Basal production means the production of each mediator without stimulation, and LPS the production of PGE_2 and NO metabolites in the presence of lipopolysaccharide from *Escherichia coli*. Results are expressed as mean ± SEM. * $p < 0.05$ by comparison of values with LPS stimulation and $^{\#}p < 0.05$ vs basal values.

In this study the FC method of fractionation monitored by HPLC, TLC and ^{1}HNMR (FC, TLC, HPLC and ^{1}HNMR) were used to better characterize the constituents of *H. procumbens* isolated fractions, Three fractions of the crude extract containing different proportions of harpagoside (A, B and C) were tested. Regarding PGE_2 production, a significant reduction in COX-2 activity was observed only with 30 µg/mL of fraction A that contains the highest concentration of harpagoside. On the other hand, fraction B, which is the most complex of the fractions (containing the iridoid pool), promoted a slight increase of PGE_2 production, suggesting that the presence of iridoids in the extract could increase COX activity and possibly reduce the antiinflammatory effect of the crude extract. Confirming this assertion, a recent study with porcine skin described the effectiveness of harpagoside and 8-courmaroyllharpagide in inhibiting COX-2 expression, whereas the injection of harpagide caused a significant increase in the levels of COX-2 expression suggesting that the efficacy of *H. procumbens* is dependent upon the ratios of the two compounds (Abdelouahab and Heard, 2008).

These results reinforce the concept that the efficacy of *H. procumbens* is the result of the synergistic and antagonist actions of multiple compounds and is a possible explanation for the difficulty to obtain dose-effect in this kind of assay. In this regard, harpagoside has been considered the main responsible for the antiinflammatory properties of devil's claw crude extract. However, harpagoside alone was not sufficient to explain the effect (Ouitas and Heard, 2009). In this context, fraction A, which had the highest concentration of harpagoside (88.8%) promoted a great inhibition in NO production. On the other hand, this effect was also observed after incubation of blood with fraction C, which has the lowest content of harpagoside (2.7%) and a great amount of cinnamic acid (85%). In both cases, a similar reduction in NO metabolites (about 67%) was detected suggesting that this effect cannot be solely based on harpagoside action, but emphasizes the synergistic antiinflammatory effect of each compound. Regarding the cinnamic acid effect on NO production, data are controversial. It has been described as a potent enhancer of endothelial nitric oxide synthase expression but is associated with impairment of their activity (Wallerath *et al.*, 2005). Using LPS-stimulated RAW 264.7 cells, Lee *et al.* (2002) showed little or no activity of free cinnamic acid on NO production, probably as a consequence of the extraction process. Cinnamic acid was also described as a powerful antioxidant which could enhance harpagoside action (Kaskin *et al.*, 2004). Thus, the testing of a single compound, using a specific cell culture may not be a fair estimation of the efficacy of the 'crude' extracts used by the patients. In this context our study in whole blood would be helpful to clarify this issue.

Further studies identifying additional constituents are important to evaluate the contribution of each compound of the crude extracts of devil's claw to better characterize its clinical effect.

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Conflict of Interest

The authors have declared that there is no conflict of interest.

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