

Full Length Research Paper

Direct inhibition of cyclooxygenase-2 enzyme by an extract of *Harpagophytum procumbens*, harpagoside and harpagide

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Accepted 8 November, 2011

A methanolic extract of *Harpagophytum procumbens* as well as harpagoside and harpagide were tested as direct inhibitors of cyclooxygenase-2 enzyme (COX-2). The *H. procumbens* extract demonstrated direct inhibition (68%) of COX-2 enzyme. The concentration of harpagoside and harpagide equivalent to that found in the extract (3 and 1% respectively) contributed 1.5 and 13% to this inhibition. Results indicated direct COX-2 enzyme inhibition by the *H. procumbens* extract due to possible synergistic activity of active components in the extract, which include harpagide and harpagoside.

Key words: *Harpagophytum procumbens*, harpagoside, harpagide, cyclooxygenase-2, devils claw, synergism.

INTRODUCTION

Devil's claw (*Harpagophytum procumbens*) is a plant geographically located in many regions throughout Southern Africa. Its typical habitat is open savannahs and arid regions. The plant root has been used for the treatment of osteoarthritic conditions and has analgesic properties (Van Wyk et al., 1997). Devil's claw contains iridiode glycosides, sugars, triterpenes, sterols and fats (Wichtl, 2004). The iridiode glycosides (harpagoside and harpagide) are believed to have medicinal properties (Van Wyk et al., 1997).

H. procumbens extract and its various active components have anti-inflammatory properties expressed by different mechanisms of activity (Fiebich et al., 2001; Loew et al., 2001; Benito et al., 2000). The clinical efficacy of *H. procumbens* for inflammatory conditions has been reported (Chrubasik et al., 2002). These reveal favourable results for the relief of osteoarthritic conditions with *H. procumbens* and harpagoside as compared to anti-arthritic drugs, rofecoxib and diacerein (Gagnier et al., 2004; Chantre et al., 2000). Questions have been raised about the clinical assessment criteria used in some

studies (McGregor et al., 2005).

Studies involving COX-2 inhibition by *H. procumbens* extract in animals indicate significant reduction in pain and inflammation induced by experimental test procedures. These include Freund's adjuvant induced arthritis and carrageenan-induced rat paw oedema (Anderson et al., 2004; Lanhers et al., 1992). However, animal studies tend to indicate that isolated components, such as harpagoside do not demonstrate anti-inflammatory properties (McGregor et al., 2005; Lanhers et al., 1992).

Two forms of cyclooxygenase are known, namely, cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2). COX-1 is a constitutive enzyme and COX-2 is induced in inflammatory cells by inflammatory stimuli (Rang et al., 2003). Anti-inflammatory properties of *H. procumbens* extract and its active components have been attributed to the inhibition of activities related to the pathways of COX-1 (Jang et al., 2003) and COX-2 (Abdelouhab and Heard, 2008), the latter being more frequently investigated.

Cyclooxygenase enzyme inhibition studies may be categorized by (a) measuring direct inhibition of enzyme activity by the inhibitor; (b) measuring the inhibition of enzyme activity through reduced enzyme expression; or (c) measuring other biochemical activities due to reduced enzyme output. The last two are categorized as indirect

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measurements of inhibition as demonstrated by McGregor et al. (2005) and Kundu et al. (2005) with the *H. procumbens* extract, respectively.

In vitro studies investigating constituents of *H. procumbens* extract, such as harpagoside and harpagide reveal opposing results in its ability to inhibit COX-2 activity (Abdelouahab and Heard, 2008; Fiebich et al., 2001). This may be related to differences in the method of inhibition assessment and plant extract composition (McGregor et al., 2005; Loew et al., 2001). The *H. procumbens* extract with all its constituents seem to be the most effective in the inhibition of COX-2 as compared to its individual constituents, which may be related to synergy (Abdelouahab and Heard, 2008).

Synergistic interactions of compounds may play an important role in improved therapeutic activity of medicinal plants. In addition, crude extracts may also offer better stability of various active compounds and may include the presence of unknown constituents (Williamson, 2001).

The present study demonstrates direct inhibition of COX-2 enzyme (in contrast to others which focus on indirect inhibition) by an extract of *H. procumbens* as well as pure harpagoside and harpagide. DuP-697 [5-bromo-2-(4-fluorophenyl)-3-(4-(methylsulfonyl)-thiophene)], a member of the diaryl heterocyclic group of selective COX-2 inhibitors, which includes MK-966 rocoxib and celecoxib, was used as the reference inhibitor. Copeland et al. (1994) demonstrated significant direct COX-2 enzyme inhibition by DuP-697.

Harpagoside was identified in the *H. procumbens* extract by thin layer chromatography (TLC) and after purification by UV-spectroscopy. Harpagide was identified in the extract by TLC after spraying with a visualization reagent. Weak UV absorption characteristics of harpagide rendered confirmative UV spectroscopy unfeasible. Quantification of both compounds was done by quantitative TLC.

METHODOLOGY

Preparation of *H. procumbens* extract

Dried *H. procumbens* (A. White Chemist, South Africa) was powdered using a hammer mill (Cullati, Germany). Methanol (Sigma-Aldrich, South Africa) was added to a final powder concentration of 100 mg/ml. The samples were vortexed for 5 min followed by shaking at 400 rpm (Infors HT, Switzerland) for 5 min at room temperature. Thereafter, it was centrifuged at 8000 rpm for 5 min (Hettich, Germany) and the methanol phase was carefully removed from the pellet. This supernatant was concentrated by the removal of the methanol at 35°C under a gentle stream of nitrogen resulting in a resinous *H. procumbens* extract which was stored (-20°C).

Identification of the extract components

Thin layer chromatography analysis was performed on methanol

reconstituted solutions of the resinous crude extract (400 mg/ml) with a mobile phase of butanol (Merck, South Africa), acetic acid (Merck, South Africa) and water (12:3:5). Five microlitres of the sample was applied to the TLC plates (silica gel 60, F₂₅₄, Merck, South Africa) and chromatographed over a plate length of 8 cm with pure harpagoside and harpagide (PhytoPlan, Germany) in methanol as reference chemical standards. The TLC plates were air dried, observed under UV light (254 nm), compounds marked, and they were sprayed with a solution containing 1% (w/v) vanillin (Sigma-Aldrich, South Africa) in ethanolic sulfuric acid, by means of an aerosol spraying device. It was then heated (5 min, 80°C) in an oven yielding identifiable visible spots. The chromatographed compounds on the plate were quantified using a scanner connected to a laptop computer with specialized Quantiscan software version 3 (Biosoft, England) with the ability to quantify and differentiate between chromatographed compounds.

In order to perform confirmative UV-spectroscopy (Cintra 202, Australia) on the chromatographed harpagoside, further purification was necessary. This was achieved by following preparative TLC methods under the same conditions. Chromatographed harpagoside bands on the TLC plates were removed from the plates, extracted with methanol and the UV spectrum of the TLC purified harpagoside was determined by UV spectroscopy in phosphate buffered saline solution.

Determination of cyclooxygenase-2 inhibition

COX-2 inhibition experiments were based on the methods reported by Copeland et al. (1994) and Corazzi et al. (2005) with minor changes. Twenty-five units of purified COX-2 (ovine) enzyme (Cayman Chemical, USA) were added to 700 µl of enzyme reaction buffer. The buffer consisted of 100 mM sodium phosphate pH 6.5, 0.5 µM hematin (Sigma-Aldrich, South Africa) and 1 mg/ml gelatin (Sigma-Aldrich, South Africa). The enzymatic reaction was initiated by adding 100 µM of arachadonic acid (Sigma-Aldrich, South Africa) and 50 µM N,N,N',N'-tetramethyl-p-phenylenediamine (TMPD) (Sigma-Aldrich, South Africa) to a final reaction mixture of 1 ml.

Various concentrations of COX-2 inhibitors, namely; *H. procumbens* extract, harpagoside and harpagide were dissolved in dimethylsulphoxide (DMSO) (Merck, South Africa) and added to the reaction solution buffer 15 min before arachadonic acid and TMPD were added. DuP-697 was dissolved in dimethylformamide (Merck, South Africa) instead of DMSO. All determinations were performed in triplicate.

COX-2 activity was determined by measuring UV absorption change of the reaction mixture at wavelength 610 nm exactly 8 min after initiating the reaction against the appropriate blank. No change of initial absorption values indicated 100% inhibition and maximum absorption change in the reaction without added inhibitors indicated zero inhibition. Data was fitted into GraphPad Prism version 5 software for the calculation of mean, standard deviation (SD) and IC₅₀ values.

RESULTS AND DISCUSSION

TLC of harpagoside present in the *H. procumbens* extract showed an R_f value of 0.68 when viewed under the UV light, which corresponded to the harpagoside standard. UV-spectrometry of the purified compound was identical to that of the harpagoside standard with the ratio of peak maxima to minima at wavelength 280 and 233 nm of 1.87. TLC of harpagide present in the extract could not be visualized on the plates under UV light. It was however



Figure 1. Quantitative thin layer chromatography of *H. procumbens* extract with harpagoside and harpagide reference standards. Harpagoside R_f 0.68; Harpagide R_f 0.37; Mobile Phase: butanol, acetic acid and water (12:3:5). Harpagide spots appeared reddish upon spraying with 1% vanillin in ethanolic sulfuric acid spray reagent.

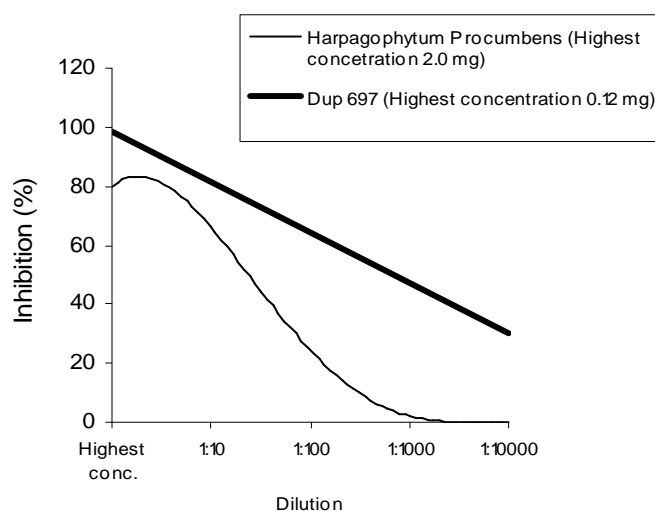


Figure 2. DuP-697 and *H. procumbens* extract COX-2 inhibition curves at various dilutions.

identified after spraying the plate with the 1% vanillin in ethanolic sulfuric acid spray reagent. It yielded a reddish spot, which correlated in colour and R_f value (0.37) with the harpagide standard (Figure 1). The TLC plates containing the harpagoside and harpagide were quantitatively analyzed using the Biosoft® Quantiscan software as described by Nikolova et al., (2004). Bruneton (1999) reported the quantity of iridiode glycosides, which include harpagoside and harpagide, present in the crude extract of *H. procumbens* to be between 0.5 and 3%. Quantitative analyses of the compounds screened on the developed TLC plates showed that the tested *H. procumbens* extract contained 3% harpagoside and 1%

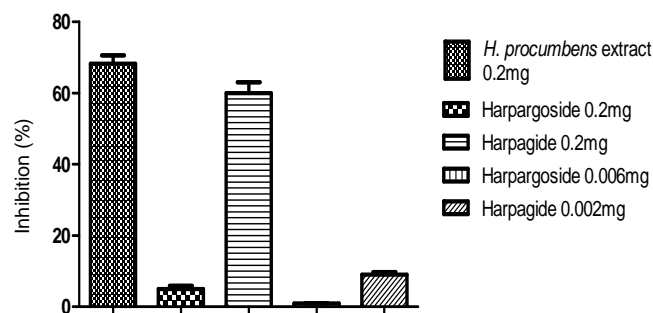


Figure 3. Direct inhibition (%) of COX-2 (25 units) activity by *H. procumbens* extract, harpagoside and harpagide. (n = 3; mean \pm S.D.).

harpagide.

Figure 2 illustrates the comparison of COX-2 inhibition between DuP-697 and Harpagophytum procumbens extract at 10 fold dilutions, starting at 0.12 mg (98% inhibition) and 2 mg (80% inhibition) respectively. In addition to the fact that the DuP-697 concentrations were 17 times lower than that of the extract, it exerted a greater inhibitory effect (40%) at lower concentrations (1:1000 dilution) compared to the extract. This difference in inhibition decreases to less than 20% at higher extract concentrations (1:10 dilution). Compared to the DuP-697, the Harpagophytum procumbens extract inhibition curve indicated a non-linear COX-2 inhibition at high and low concentrations, which may be explained by possible synergistic activity of compounds in the extract.

Under these experimental conditions, higher concentrations of Harpagophytum procumbens extract could not be tested because of solubility limitations of the extract in the reaction buffer. Figure 3 illustrates direct COX-2 inhibition by *H. procumbens* extract as well as harpagoside and harpagide at 0.2 mg. This was the highest concentration at the linear part of the extract's inhibition curve (Figure 2). At a concentration of 0.2 mg, *H. procumbens* extract showed direct enzyme inhibition of 68.3%, harpagoside 5% and harpagide 60% (Figure 3). The inhibition activity of the crude extract is similar to the results obtained by Jang et al. (2003) and Kundu et al. (2005). Their studies evaluated indirect inhibitory activity through COX-2 expression. Abdelouahab and Heard (2008) reported that harpagoside had higher inhibitory activity than harpagide and that the latter increased COX-expression.

When enzyme inhibition of harpagoside and harpagide were tested at 0.006 and 0.002 mg, respectively, which is the quantity that these compounds are represented in 0.2 mg of the extract, only 1 and 9% direct inhibition were demonstrated, respectively. Fiebich et al. (2001) demonstrated a similar trend where no COX-2 inhibitory effects (TNF α synthase inhibition) by harpagoside and harpagide were observed at concentrations of up to 0.01 mg.

The contrasting COX-2 inhibitory activity reported may be

Table 1. IC₅₀ values of DuP-697, *H. procumbens* extract, harpagide and harpagoside as COX-2 inhibitors.

Compound	IC ₅₀ (mg/ml)
DuP-697	0.000134
<i>H. Procumbens</i> extract	0.1046
Harpagide	0.1186
Harpagoside	104.1

as a result of the total extract containing different fractions of components resulting in synergistic, complimentary or antagonistic effects (Loew et al., 2001).

The calculated IC₅₀ values derived from inhibition studies of the inhibitors used are listed in Table 1. It confirms higher inhibition activity of harpagide than harpagoside as direct inhibitor but less than *H. procumbens* extract and DuP-697. Combined COX-2 inhibition of harpagoside and harpagide at the quantities present in 0.2 mg extract was 10% as compared to 68.3% in the *H. procumbens* extract. This greater COX-2 direct inhibition exerted by the *H. procumbens* extract may be as a result of other compounds in the extract possibly with synergistic effects as compared to the combined inhibition of the harpagide and harpagoside. This effect was demonstrated in Fiebich et al (2001) study where the inhibition is attributed to *H. procumbens* extract and not to the individual components, such as the harpagoside. Abdelouahab and Heard (2008) also suggested that the inhibition of COX-2 expression could be attributed to interplay of the active compounds present in the *H. procumbens* extract. This phenomenon was described by Williamson (2001) with plant extracts *Ginkgo biloba* and *Cannabis sativa*.

Conclusion

H. procumbens extract produced direct inhibition of COX-2 enzyme, but less than DuP-697. COX-2 inhibition by *H. procumbens* extract is greater than that of harpagoside and harpagide at the same concentrations. Harpagide has up to 12 times more inhibition activity as direct COX-2 enzyme inhibitor than harpagoside. The calculated combined inhibition of harpagoside and harpagide at the concentrations as they appear in the extract is 10% as compared to 68% by the *H. procumbens* extract. However, the combination of these two components, with other compounds, which form part of the extract, has an even greater direct inhibition of COX-2 enzymes. This difference in COX-2 inhibition may be attributed to interplay expressed by other components in the extract.

Future studies could combine the direct and indirect mechanisms of COX-2 inhibition in order to determine the total effect on COX-2 activity by the *H. procumbens* extract which will be a combination of direct and indirect enzyme activity inhibition.

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