Full Papers

Effect of the Major Glycosides of *Harpagophytum procumbens* (Devil's Claw) on Epidermal Cyclooxygenase-2 (COX-2) in Vitro

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Received May 3, 2007

Harpagophytum procumbens, commonly known as Devil's Claw, is indigenous to southern Africa, and extracts of the tubers have been used for centuries in the treatment of a variety of inflammatory disorders. Its major active components, harpagoside (**1**), harpagide (**2**), 8-coumaroylharpagide (**3**), and verbascoside (**4**), are believed to interact either synergistically or antagonistically in modulating the enzymes responsible for inducing inflammation, although this has not been probed hitherto. In the current work, the ability of these compounds to inhibit the expression of COX-2 following administration to freshly excised porcine skin has been investigated. An ethanol-soluble extract of *H. procumbens* tubers and two of the pure compounds tested showed promising activity in Western blotting and immunocytochemical assays, with harpagoside (**1**) and 8-coumaroylharpagide (**3**) exhibiting greater reductions in COX-2 expression than verbascoside (**4**). Harpagide (**2**) caused a significant increase in the levels of COX-2 expression after 6 h of topical application. The data suggest that the efficacy of *H. procumbens* is dependent upon the ratios of compounds **1**–**4** present, which is inconsistent with some current official monograph specifications based solely on harpagoside (**1**) content.

Harpagophytum procumbens DC., also known as Devil's Claw, belongs to the Pedaliaceae and is a plant from the Kalahari region of southern Africa. Its dried and powdered tubers have been used in the treatment of various conditions for many years, such as against pain and complications of pregnancy, as well as to heal sores, boils, and other skin problems.[1,2] In addition to its anecdotal health benefits, there are supportive data on the clinical efficacy of both an extract and a crude powder of *H. procumbens* in inflammation and pain reduction,[3] particularly in rheumatoid arthritis and osteoarthritis.[4–6] Phytochemical screening of a *H. procumbens* extract has revealed the presence of iridoid glycosides,[7] acetylated phenolic glycosides,[8] and terpenoids.[9] The pharmacological effects of *H. procumbens* have been attributed largely to the major iridoid and phenylethanoid glycosides present in the secondary tubers of the plant, in particular harpagoside (**1**), harpagide (**2**), 8-coumaroylharpagide (**3**), and verbascoside (**4**).[10–12] Laboratory investigations on this plant go back to the 1950s and involve studies mainly from Germany,[11] where *H. procumbens* is a licensed medicine. The current European Pharmacopeia monograph for *H. procumbens* tuber extracts requires not less than 1.2% of the iridoid glycoside harpagoside (**1**), calculated with reference to the dried drug.[13]

Harpagoside (**1**) was considered for a long time to be the major active constituent of the extracted material of *H. procumbens* against inflammation,[14] although it demonstrated a less potent effect compared to the crude plant extract. Therefore, other constituents present in *H. procumbens* extract appear to exert an adjunct effect on inflammatory mediators.[15] Furthermore, other constituents such as sugars, and flavonoid and polyphenol (with antioxidant activity) derivatives may play contributory roles.

The anti-inflammatory activity of *H. procumbens*and its constituents is still not clearly understood, although inhibition of the arachidonic, cyclooxygenase (COX), and lipo-oxygenase (LOX) pathways[16] is probable. Tubers of *H. procumbens* were found to have no effect on the COX-1 subtype.[17] More recent studies have shown effects of *H. procumbens* acting on thromboxane biosynthesis.[16,18] In addition to interfering with this pathway, it has been suggested that *H. procumbens* suppresses lipopolysaccharide-stimulated expression of COX-2 and inducible nitric oxide synthase (iNOS) in fibroblast cell lines.[19] It was also shown that 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced COX-2 expression in mouse skin was inhibited by a methanol extract of *H. procumbens* by inhibiting DNA-binding sites of NF-κB.[10] Moreover, antioxidant activity was observed in a harpagoside (**1**)-free extract,[20] and most outcomes in vivo and in vitro of this extract have been concentration dependent.[21,22]

Existing in vivo and in vitro reports on the pharmacological efficacy of *H. procumbens* extracts are inconsistent, with variability depending on the extraction procedure, the geographical source of the crude drug, and the polarity of the extracts tested. Extracts of *H. procumbens* can thus differ greatly in terms of both phytochemical constituents and consequent pharmacological potency.[21,23] Although there are a great deal of data supporting anti-inflammatory effects, negative or insignificant results were obtained in several studies examining the action of *H. procumbens* extracts on leukotrienes, eicosanoids, and prostaglandins in human or murine blood cells.[17,24]

The individual effects of the major iridoid glycosides of *H. procumbens* tubers on inflammatory processes have not been determined to date. In the current work, we have examined the mechanism of a *H. procumbens* ethanol extract and its major pharmacologically active constituents, harpagoside (**1**), harpagide (**2**), 8-coumaroylharpagide (**3**), and verbascoside (**4**), in modulating the expression of COX-2 in freshly excised porcine skin.

Results and Discussion

Porcine skin lysates were collected from homogenized skin at 6 h following treatment with PBS (control), the main constituents **2**–**4** of *H. procumbens* extract when combined in the presence and absence of harpagoside (**1**), and each of the main constituents **1**–**4** individually. The doses given were determined to inhibit partially or completely COX-2 expression. Densitometry of the resulting bands for COX-2 enzyme at ~72 kDa (Figure 1) indicated a slight reduction of the protein expression at 6 h compared to the control for *H. procumbens*

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10.1021/np070204u CCC: $40.75
Published on Web 04/16/2008

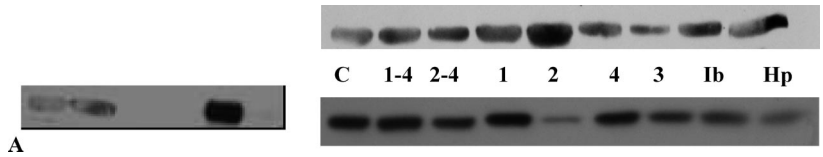


Figure 1. Effects of harpagoside (**1**), harpagide (**2**), 8-coumaroylharpagide (**3**), verbascoside (**4**), and mixtures of **1**–**4** and **2**–**4**, the *H. procumbens* ethanol extract (Hp), and ibuprofen (Ib) on COX-2 expression as shown by Western blotting at 6 h; control (**C**). Positive and negative control cells for COX-2 expression are shown in A.

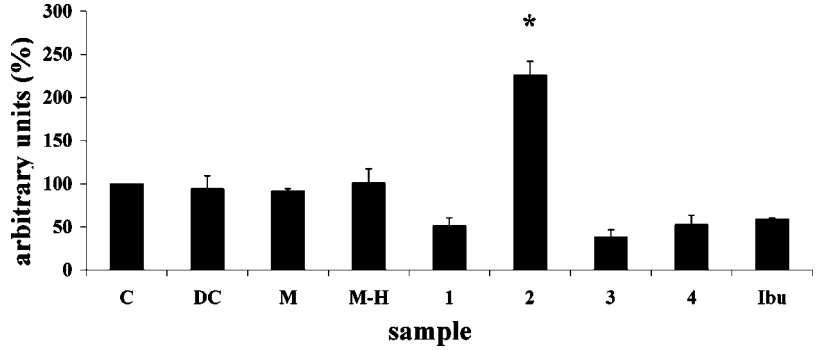
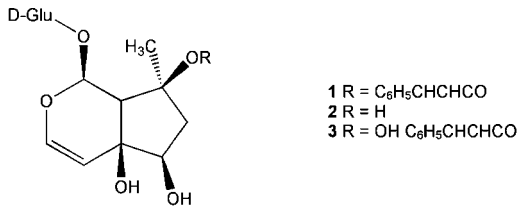
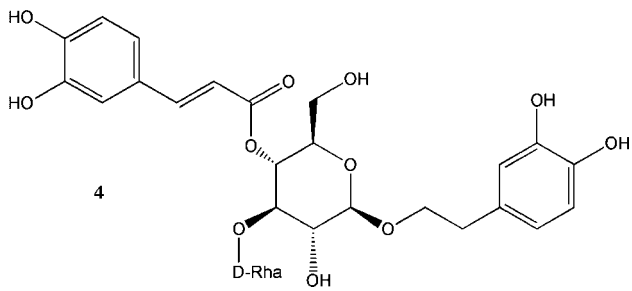


Figure 2. Effects of the components (**1**–**4**) of *H. procumbens* ethanol extract on the expression of COX-2 in porcine skin. The results were normalized using β-actin. Levels in the control were arbitrarily assigned a value of 100%. All values are means ± SD ($n = 3$), $^*P < 0.05$.





and for the mixture of pure standards. However, a compound mixture in the absence of **1** indicated up-regulation in COX-2 expression and supported previous findings that harpagoside has a key role in inhibiting COX-2 expression.[10,19] This mode of action might be related to some effects on transcription factors such as NF-κB and AP-1 that were previously found to be responsible for the induction of COX-2 in murine skin.[10,25]

Unlike the total glycoside mixture and the crude extract of *H. procumbens*, compounds **1**, **3**, and **4** as well as ibuprofen (a positive control) exhibited substantial inhibition of COX-2 synthesis by about <50%. Previous work on human blood platelets and isolated mouse peritoneal macrophages showed harpagoside (**1**) and harpagide (**2**) did not attain statistical significance in inhibiting COX and LOX pathways by reducing PGE-2 or LTC-4.[24] In contrast, the present study has shown not only that is **1** responsible for the anti-inflammatory effect observed, but also that 8-coumaroylharpagide (**3**) is important in the reduction of COX-2 expression. The discrepancies suggest that the ability of individual iridoid glycosides to attenuate COX-2 expression or pathways, as well as the whole plant ethanol extract, depends on the cell stimulus as well as the extract type used.[26,27] In light of the current study, the ability of *H. procumbens* to attenuate the cytokine COX-2 activity is likely to be related to both the expression and pathway of this particular enzyme. Clearly, 8-coumaroylharpagide (**3**) is one of the most effective constituents of *H. procumbens* tubers, as it led to the lowest level of COX-2 produced.

The glycoside mixture (**1**–**4**) was shown to achieve a greater COX-2 knockdown than the whole crude ethanol extract of *H. procumbens*. In addition, individual iridoid glycosides were more effective compared to the whole extract. These results partially contradict previous findings that harpagoside (**1**) is less effective than the whole extract.[2,18,21] Interestingly, harpagide (**2**) resulted in a 2-fold increase in COX-2 expression (p 0.0033), suggesting that this compound promotes expression of COX-2 and thus could result in increased inflammatory responses. In addition, the decreased and varied potency of *H. procumbens* extract (as well as the mixture of compounds **1**–**4** could be due to the fact that **2** was able to promote higher expression of COX-2. The results also confirmed that harpagoside (**1**) is a key anti-inflammatory constituent of *H. procumbens*, as its absence in the mixture led to higher COX-2 expression in porcine skin. A pharmacopeial standardization of an extract from crude *H. procumbens* powder is based upon harpagoside (**1**) only. However, it is clear from the current data that this is inadequate as a yardstick for the potential effect of the extract on inflammation. Other iridoid glycosides also play an important role in modulating the inflammatory mechanisms (either as anti- or pro-inflammatory mediators), and variability in their presence in the total extract will lead to variability in therapeutic response.

Figure S1 (Supporting Information) shows immunoreactive COX-2 (as denoted by the red staining) for epithelial tissues of porcine skin from control and 6 and 10 h after dosing with *H. procumbens* ethanol extract, ibuprofen (Ibu), harpagoside (**1**), harpagide (**2**), 8-coumaroylharpagide (**3**), verbascoside (**4**), a mixture of **1**–**4** (M), and a mixture in the absence of harpagoside (**2**–**4**, M−H). It can be seen that the staining increased over time when the skin was treated with PBS solution. Hence, following removal of the skin from the ear it is apparent that an inflammatory response had been triggered from the initiation of the inflammatory metabolic pathway of COX-2.

There was no apparent difference in the amount of COX-2 expressed at 3 h, indicating that the permeation of the compounds was within the lag phase. However, in most samples immunostaining for COX-2 was minimal at 6 and 10 h with a subtle expression within regions of the differentiated epidermis. By comparison, harpagoside (**1**), 8-coumaroylharpagide (**3**), verbascoside (**4**), and ibuprofen as well as the mixture dramatically decreased staining at 6 h. This indicates the desired knockdown in COX-2 enzyme expressed in the skin.

The skin dosed with harpagide (**2**) showed heavy staining, demonstrating enhanced levels of expressed COX-2, especially in the more biologically active basal layer of the epidermis. The staining appears in the epidermis rather than the lower dermis, which is almost transparent due to the lack of enzymically active cells. Overall, these data represent a qualitative confirmation of the Western blotting results.

In conclusion, a previously unknown relationship has been demonstrated in the interaction of *H. procumbens* tuber extract and its glycoside constituents on COX-2, whereby overall activity is dependent upon the precise proportions of the compounds present in the extract. There is evidence for interplay between the anti-inflammatory glycosides [harpagoside (**1**), 8-coumaroylharpagide (**3**), and verbascoside (**4**)] and the pro-inflammatory harpagide (**2**) that, given the natural variability within sourced materials, may help explain the variable therapeutic responses to extracts of *H. procumbens* and its generally low potency compared to conventional anti-inflammatory drugs. Selectively removing harpagide (**2**) from *H. procumbens* extracts may make this a more potent natural medicine.

Experimental Section

General Experimental Procedures. Commercial *H. procumbens* powdered tuber was purchased in December 2005 from Handa Fine Chemicals (Nottingham, UK). A voucher sample was deposited at the National Museum of Wales, Cardiff, UK (ref V.2007.021.1). Harpagoside (**1**) (99.88%), harpagide (**2**) (99%), 8-coumaroylharpagide (**3**) (90%), and verbascoside (**4**) (95%) were obtained from PhytoLab GmbH & Co. KG, Vestenbergsgreuth, Germany. Reversed-phase HPLC was carried out using an Agilent 1100 automated system fitted with a Gemini 5 μm, 250 × 4.6 mm column (Phenomenex, Macclesfield, UK). A gradient elution was used involving a binary mobile phase composed of deionized water (A) and methanol (B), with the time program 0–9.0 min 30% A:70% B, flow 0.5 mL min^{-1}; 9.0–9.5 min to 50% A:50% B, flow 0.5 mL min^{-1}; 9.5–18 min 50% A:50% B, flow 0.8 mL min^{-1}; 18.0−18.5 min to 70% A:30% B, flow 0.8 mL min^{-1}; 18.5−30 min 70% A:30% B, flow 1 mL min^{-1}. Detection was by UV at 278 nm. Primary COX-2 antibody (#4842) was purchased from Cell Signaling Technology (Boston, MA). Horseradish peroxidase (HRP)-labeled antirabbit polymer, DAB chromagen plus substrate was obtained from Dako UK (Ely, UK). HPLC-grade ethanol and chloroform were purchased from Fisher Scientific (Loughborough, UK). Western blocking reagent was from Roche Diagnostics Gmbh (Mannheim, Germany). Rainbow Marker (10–250 K_d), antimouse HRP and (HRP)-linked antibody were obtained from Amersham Biosciences Ltd. (Amersham, UK). Hanks balanced buffered salt solution, HEPES (*n*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid]), gentamycin sulfate, sodium bicarbonate, PBS, PBS + 0.05% Tween, sodium citrate, methyl green, paraffin wax pellets, 30% hydrogen peroxide solution, DPX mountant, NaCl, EDTA, TRISMA HCl, TritonX100, deoxycholic acid, SDS 10%, and Ponseau S were all obtained from Sigma-Aldrich (Poole, UK). Microslides were from Surgipath (Peterborough, UK). Freshly excised porcine ears were obtained from a local abattoir prior to steam cleaning and, immediately following slaughter, placed on ice and immersed in Hank's buffer before being subjected to laboratory experimentation within 1 h. No institutional approval was required for this procedure.

Extraction of *Harpagophytum procumbens* Tubers. A sample of the powdered *H. procumbens* tubers was used to make an ethanolic extract by dissolving 75 g gradually into 250 mL of ethanol and stirring it overnight under light exclusion at room temperature. The solution was then filtered and evaporated. The extract obtained was used for subsequent HPLC testing. Figure S2 (Supporting Information) shows the chromatogram obtained using an isocratic mobile phase consisting of 50:50 methanol–water. Retention times for glycosides **1**–**4** were 3.05, 35.5, 17.5, and 4.6 min, respectively.

In Vitro Skin Penetration. The dorsal porcine ear epidermis was liberated from the underlying cartilage by blunt dissection before being cut into 2 cm^2 pieces, while being continually bathed in Hank's buffer. The membranes were then placed on the flanges of glass Franz-type diffusion cell receptor compartments and the donor compartments affixed using metal clamps.[28] The whole cells were placed onto a submersible stirrer plate set up in a water bath maintained at 37 °C, thus providing a skin surface temperature of 32 °C by heat dissipation, before being dosed with 500 μM harpagoside (**1**), harpagide (**2**), 8-coumaroylharpagide (**3**), and verbascoside (**4**). Also examined were a mixture of **1**–**4**, a mixture of **2**–**4**, the *H. procumbens* ethanol extract (1 mg mL^{-1}), and ibuprofen. All samples were prepared in PBS. For a Western blotting experiment, 200 μL of 1 mg mL^{-1} *H. procumbens* extract and ibuprofen, 11.4% harpagoside (**1**), 1.6% harpagide (**2**), 8.6% 8-coumaroylharpagide (**3**), and 3.1% verbascoside (**4**) were evaluated at 6 h, in addition to a mixture of **1**–**4** and a mixture of **2**–**4**. Skin viability was maintained throughout using a receptor phase of Hank's balanced buffered solution modified with the addition of 25 μM HEPES, adjusted to pH 7 with sodium bicarbonate (0.35 gL^{-1}) and gentamicin sulfate (50 μg mL^{-1}) added, in accordance with the data sheet provided with the product from Sigma (Poole, UK).

Preparation of Skin Lysates. After 6 h skin samples were recovered from the diffusion cells and gently cleaned with deionized water before being homogenized (Silverson, Chesham, UK) in a RIPA lysis buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM PMSF, 1 mM EDTA, 5 μg mL^{-1} aprotinin, 5 μg mL^{-1} leupeptin, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS]. After 15 min incubation on ice, the lysates were centrifuged at 14000*g* for 2 × 15 min, and the supernatant was stored at −80 °C for subsequent protein analysis.

Western Blotting Analysis. The protein content was determined using the Biorad D/C protein assay kit (Bio-Rad Laboratories, Hercules, CA). Positive and negative carcinoma control cells used for COX-2 were A549 and LNCaP and DU-145, respectively.[29,30] Aliquots of 30 μg of proteins were separated by SDS-PAGE, transferred to nitrocellulose membranes using the Trans-Blot electrophoretic transfer cell (Bio-Rad Laboratories), and briefly stained with Ponceau S to verify effective transfer. Immunoblots were incubated for 1 h in a blocking solution [tris-buffered saline (TBS)-Tween 20 containing 5% (w/v) commercial skimmed milk powder (Marvel)] at room temperature. After washing, the membrane was probed overnight at 4 °C with COX-2 antibody at 1:1000 in (1:20 and 1:100 Western blocking reagent (Roche) and sodium azide, respectively, made up to volume with TBS-Tween). Membranes were then incubated for 1 h with HRP-conjugated antirabbit. For β-actin, membranes were probed with antiactin (Sigma, UK) and antimouse (Neomarkers Ab-10) for 1 h each at room temperature. After 3 × 10 min washes in TBS-Tween, they were finally exposed to freshly prepared Dura Substrate for chemiluminescence (Perbio, Cramlington, UK) for 20 min before performing autoradiography.

Immunocytochemistry. Skin samples were cut into 5 × 2 mm sections after 3, 6, and 10 h (n = 3) and placed in 4% formaldehyde, then left to fix for 24 h. The skin was immersed sequentially in 70% to 90% to 100% ethanol solutions to displace all the water from the tissue, then into four chloroform baths to displace the alcohol. The skin sections were subjected to immersion in a series of three molten wax baths attached to a vacuum to eliminate the remaining chloroform, before being embedded in a paraffin wax block. The skin was then cut using a Shandon Finesse microtome, and sections were transferred onto precleaned regular microslides. After rehydration with ethanol and equilibration in PBS, specimens were blocked with 0.2% PBS-Tween for 15 min. The COX-2 primary antibody was then applied diluted 1:50 in PBS and stored overnight at 25 °C in a humidified chamber. The slides were then washed with PBS for 2 × 5 min and incubated in HRP-labeled antirabbit polymer for 2 h at 25 °C. Slides were washed in PBS for 3 min followed by 2 × 5 min washes in 0.05% PBS-Tween. An aliquot (0.75 μL) of visualization solution (DAB chromagen-AB substrate) was applied to the specimens for 10 min. Following another washing in H_2O, the slides were counterstained with 0.5% methyl green for 3 min. Finally, sections were rinsed in distilled water and dried in an oven at 40 °C. DPX mountant was used on coverslips ready for microscope analysis.

Data Analysis and Statistics. The data obtained represent at least three individual experiments with similar results. Statistical analysis

was performed by a two-way Kruskal–Wallis nonparametric ANOVA followed by a Dunnet's test, comparing the values obtained for COX-2 for all samples.

Acknowledgment. The research described herein was supported by a grant from the Algerian government. We are also particularly grateful to L. Goddard of the Tenovus group at the Welsh School of Pharmacy for her assistance with Western blotting.

Supporting Information Available: Figures showing COX-2 immunostaining of porcine skin after treatment and a HPLC chromatogram of the *H. procumbens* tuber ethanol extract. This information is available free of charge via the Internet at http://pubs.acs.org.

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NP070204U