International Journal of Pharmaceutics 376 (2009) 63–68

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

A novel *ex vivo* skin model for the assessment of the potential transcutaneous anti-inflammatory effect of topically applied *Harpagophytum procumbens* extract

Nassima Abdelouahab Ouitas, Charles M. Heard*

Welsh School of Pharmacy, Cardiff University, Cardiff, CF10 3XF, Wales, UK

ARTICLE INFO

Article history:
Received 12 February 2009
Received in revised form 8 April 2009
Accepted 10 April 2009
Available online 19 April 2009

Keywords:
Skin
Transcutaneous delivery
Inflammation
Devil's Claw
H. procumbens
COX-2
PGE-2
5-LOX
iNOS

ABSTRACT

Using *ex vivo* skin as a model, this work tested the hypothesis that the major pharmacologically active components of topically applied *Harpagophytum procumbens* (*H. procumbens*) can elicit anti-inflammatory responses in deeper tissues post-transcutaneous delivery. Using Franz-type diffusion cells, ethanol extract of powdered *H. procumbens* tuber was dosed onto freshly excised porcine skin. After 24 h the receptor phase was recovered, analysed for the major glycosides of DC, then used directly to dose further freshly excised skin membranes. After 6 h the skin was recovered and probed for the expression of the three major enzymes involved in the inflammatory factors: cyclooxygenase (COX-2) and its product prostaglandin E2 (PGE-2), lipoxygenase (5-LOX), and inducible nitric oxide (iNOS), using immunocytochemistry and Western blotting analyses. It was found that the receptor phase at 24 h contained (0.8, 25, 1.8, 3×10^{-3}) μmol mL^{-1} of harpagoside, harpagide, verbascoside, 8-*O*-*p*-coumaroyl-harpagide, respectively. When applied to skin, this solution effectively inhibited the expression of COX-2 and its product PGE-2. However, it did not have a significant effect on either 5-LOX or iNOS compared to control samples (PBS only). These data support the hypothesis that the transcutaneous delivery of *H. procumbens* can treat inflammation in deeper tissues such as in arthritis. Moreover, a novel *ex vivo* model has been described for assessing the potential anti-inflammatory activity of permeants delivered to deeper subcutaneous regions.

1. Introduction

Harpagophytum procumbens (*H. procumbens*) is a plant which originates from the Kalahari region of Southern Africa and has been used in the treatment of various conditions for many years (Ernst and Chrubasik, 2000; Baghdikian et al., 1997). The relief of inflammation and pain has been attributed to the secondary tubers of this plant and particular inflammation and pain reduction (Chrubasik et al., 1996, 2004; Chantre et al., 2000).

The delivery into and across excised skin of *H. procumbens* has previously been reported (Abdelouahab and Heard, 2008a,b). It has been also shown that *H. procumbens* had varied effects on the inflammatory mediators in other tissues and cellular systems generating COX and LOX metabolites (Tippler et al., 1997). Existing *in vivo* and *in vitro* reports on the pharmacological efficacy of *H. procumbens* extracts are inconsistent, and differences had widely been attributed to the extraction procedure, geographical source of the crude drug and the fractions of constituents (Fiebich et al., 2001; Joubert et al., 2005).

Most of the results showed an effect of the iridoid glycosides on the arachidonic pathway and its products such as LOX in addition to free radicals produced in inflamed tissues (Benito et al., 2000; Ling et al., 2003; Lin et al., 2006). In addition, harpagoside was considered for a long time to be the most active constituent of the extracted material of *H. procumbens* against inflammation (Eich et al., 1998), although it exerts less of an effect compared to the total extract confirming the activity of other components in reducing inflammatory mediators.

In addition to interfering with the inflammatory pathways, it was suggested that *H. procumbens* suppresses lipopolysaccharide-stimulated expression of COX-2 and inducible nitric oxide synthase (iNOS) in fibroblast cell lines and in mouse skin by inhibiting DNA-binding sites of NF-κB (Kundu et al., 2005). Moreover, antioxidant activity was markedly noticeable from a harpagoside-free extract (Kaszkin et al., 2004). Although there is a great deal of pharmacological data supporting its anti-inflammatory effects, negative or non-significant results were also obtained in several studies examining the action of various extracts on leukotrienes, eicosanoids and prostaglandins in human or mice blood cells (Benito et al., 2000; Moussard et al., 1992) and most outcomes *in vivo* and *in vitro* were concentration dependent (Fiebich et al., 2001; Wegener, 2000). However, our recent research has demonstrated that one component of *H. procumbens* extract, harpagide, is pro-inflammatory and

* Corresponding author. Tel.: +44 029 20875819; fax: +44 029 20874149.
E-mail address: heard@cf.ac.uk (C.M. Heard).

0378-5173/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijpharm.2009.04.017

that the relative proportion of this compared to the other glycosides within the extract determines the activity of the material towards COX-2 expression (Abdelouahab and Heard, 2008b).

Established models for probing anti-inflammatory activity includes the rat paw oedema test (Escribano et al., 2003) although such tests are an indicator of swelling and an indirect marker of inflammation. Inflammation is not always accompanied with swelling, particularly in the early stages of disease. On the other hand, up-regulation of COX, LOX and iNOS are positive indicators of an inflammatory response and, as has been found recently, of other diseases including cancer (Aggarwal et al., 2006).

We have recently used *ex vivo* skin as a model for determining inflammation. Within the skin, most metabolic and enzymatic processes take place in the viable epidermis. Inflammatory processes involved in eczema, psoriasis and psoriatic arthritis are generally proliferated by those enzymes responsible for inducing inflammatory reactions in arthritis, namely COX-2 and 5-LOX pathways by production of prostaglandins and leukotrienes, respectively (Marcouiller et al., 2005). Therefore, pharmacological analysis of the skin can provide a model for the transcutaneous activity of topically applied formulations on inflammatory diseases such as arthritis in deeper tissues, in addition to indicating the fate of the compounds as they permeate the skin (Abdelouahab and Heard, 2008b; Thomas et al., 2007).

In the current work, the potential for anti-inflammatory activity of *H. procumbens* in subcutaneous tissues was modelled using a novel approach involving *ex vivo* skin. The phytochemicals from *H. procumbens* that had permeated freshly excised full thickness porcine skin were retrieved after 24 h and re-applied to further *ex vivo* skin samples. Western blotting, ELISA and immunohistochemical techniques were then used to probe the effects on COX-2, PGE-2 in addition to the expression of 5-LOX and iNOS.

2. Materials and Methods

2.1. Materials

Powdered tuber of *H. procumbens* was obtained from Handa Fine Chemicals (Nottingham, UK)—a voucher sample was deposited in the National Museum of Wales, Cardiff, UK (ref V.2007.021.1) and was cold extracted with ethanol. High vacuum grease was obtained from Dow Corning (Barry, UK). Primary COX-2 antibody was purchased from Cell Signalling Technology, New England Biolabs (Hitchin, UK), primary antibody iNOS was purchased from Santa Cruz, Biotechnology, Inc. (Santa Cruz, USA), 5-LOX and prostaglandin E_2 (PGE-2) ELISA Kit which includes pre-coated (goat anti-mouse IgG), EIA 96-well strip plates, 96-well cover sheets, Tween 20, EIA tracer dye, EIA antiserum dye, Ellman's reagent, EIA buffer concentrate (×10), wash buffer concentrate (×400), prostaglandin E2 AChE tracer, prostaglandin E2 EIA monoclonal antibody and prostaglandin E2 EIA standard were purchased from Cayman Chemical Europe (Tallinn, Estonia).

Hanks balanced buffered salt solution (HBBSS), gentamycin sulphate, sodium bicarbonate, de-ionised and phosphate buffered saline (DPBS, PBS), PBS + 0.02% Tween, sodium citrate, methyl green, white paraffin wax pellets, 30% hydrogen peroxide solution, sodium hydroxide, hydrogen chloride, HEPES (*n*-[2-hydroxyethyl] piperazine-*N*′-[2-ethanesulphonic acid]), bovine serum albumen (BSA), radioimmunoprecipitation assay (RIPA) buffer, glycerol, aprotinin, leupeptin, phenylmethyl sulphonyl fluoride (PMSF), acrylamide/bis-acrylamide 29:1 (30% v/v solution), ammonium persulphate (APS), di-thiothreitol (DTT), ethylene diamine tetraacetic acid (EDTA), ethylene glycol-bis(2-amino-ethylether)-*N*,*N*,*N*′,-tetraacetic acid (EGTA), glycine, *N*,*N*,*N*′,*N*′-tetramethylene-diamine (TEMED), polyoxyethylene-sorbitan monolaurate (Tween 20), β-actin primary antibody, ponceau S solution (0.1% w/v in 5% acetic acid, uranyl acetate, sodium azide, sodium chloride (NaCl), sodium dodecyl sulphate (SDS), Tris HCl Trizma® Base were from Sigma–Aldrich Company Ltd. (Poole, UK). Horseradish peroxidase (HRP)-labelled anti-rabbit polymer EnVision™ system, peroxidise, liquid DAB and substrate chromagen system, were from Dako (Ely, UK). Trans-Blot electrophoretic Transfer Cell (Bio-Rad Laboratories), Western blocking reagent was from Roche Diagnostics, Gmbh (Mannheim, Germany). Rainbow Marker, anti-mouse primary antibody, (HRP)-linked secondary antibody were obtained from Amersham Biosciences Ltd. (Amersham, UK), chemiluminescence from Perbio (Cheshire, UK). Freshly excised porcine (*Sus scrofa domesticus*, large white) ears were obtained from a local abattoir prior to steam cleaning and immersed in iced Hank's buffer before being subjected to laboratory experimentation within 1 h of slaughter.

2.2. In vitro skin penetration I

The porcine ear sections were cut into 2 cm^2 pieces after the full thickness skin was liberated from the underlying cartilage by blunt dissection, whilst being continually bathed in Hanks buffer. The membranes were then placed on the pre-greased flanges of glass Franz type diffusion cell receptor compartments and the donor compartments affixed using metal clamps. Skin viability was maintained throughout using a receptor phase of Hanks balanced buffered solution modified with the addition of 25 μM HEPES and adjusted to pH 7, sodium bicarbonate (0.35 g L^{-1}) and 50 μg mL^{-1} gentamycin sulphate, following the data sheet provided with the product. The whole cells were placed onto a submersible stirrer plate set up in a water bath maintained at 37 °C, to provide a skin surface temperature of 32 °C by heat dissipation, before being dosed with 1 mg mL^{-1} of *H. procumbens* extract reconstituted in PBS. After 24 h the cells were dismantled and the receptor phases recovered before being freeze dried overnight.

2.3. HPLC analysis

The four major glycosides were determined using an Agilent 1100 automated chromatograph fitted with a Gemini, 5 μm, 250 × 4.6 mm C18 column (Phenomenex, Macclesfield, UK). A gradient elution was used involving a binary mobile phase composed of: de-ionised water (A) and MeOH (B), with the time programme: 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 @ 278 nm. Chemstation software (version A.09.01) was used to acquire data and integrate peak areas. Retention times for harpagoside, harpagide, verbascoside, 8-*O*-*p*-coumaroyl-harpagide were 25, 6.1, 17.3 and 22.7 min, respectively and calibration curves were linear over the ranges of (0.002–0.516, 0.021–0.686, 0.002–0.440, 0.008–0.509 μg mL^{-1}), respectively, each providing r^2 of ≥0.999. The limits of detection for harpagoside, harpagide, verbascoside and 8-*O*-*p*-coumaroyl-harpagide were (0.104, 1.129, 0.006, 1.260 nmol mL^{-1}).

2.4. In vitro skin penetration II

The results (Table 1) demonstrate the amounts obtained in the receptor phase. Q_{24} was chosen as the amount of major glycosides permeating at steady state and applied to the skin in the donor phase following freeze drying. Using the method described for penetration, the phytochemicals from *H. procumbens* saturated solutions in PBS that had permeated freshly excised full thickness porcine skin were retrieved after 24 h and re-applied to further *ex*

Table 1
Data for the permeation of the major glycosides of *H. procumbens* across full thickness porcine ear skin from saturated aqueous solution of extract: 1. harpagoside 2. harpagide 3. *8-O-p*-coumaroyl-harpagide 4. verbascoside (J_{ss} = steady state flux, Q_{24} = cumulative permeation after 24 h). $n = 6 \pm$ S.D.

Analyte	J_{ss} ($\times 10^{-5}$ μmol cm^{-2} h^{-1})	Q_{24} ($\times 10^{-3}$ μmol mL^{-1})
1	20.0 ± 0.50	1.0 ± 0.4
2	129.7 ± 34.4	28.0 ± 9.0
3	30.6 ± 10.5	3.3 ± 0.8
4	26.7 ± 0.50	3.7 ± 1.6

vivo skin samples after being reconstituted with 1 mL PBS, with PBS serving as a control. After 6 h the cells were dismantled and the skin membranes recovered for Western blot and ELISA analysis.

2.5. Western blotting analysis

The skin membranes were gently cleaned with de-ionised 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 clarified by centrifugation at 14,000 rpm for 2 × 15 min and the supernatant stored at −80 °C for subsequent protein analysis.

Aliquots of 30 μg total protein were separated by SDS-PAGE, transferred to nitrocelullose membranes using the Trans-Blot electrophoretic Transfer Cell 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, 5-LOX at 1:1000 and iNOS at 1:500 in (1:20 and 1:100 western blocking reagent and sodium azide solution, respectively made up to volume with TBS tween). Membranes were then incubated for 1 h with HRP-conjugated anti-rabbit at 1:10,000. For β-actin, membranes were probed with anti-actin and anti-mouse for 1 h each at room temperature at 1:10,000 in TBS tween. After 3 × 10 min washes in TBS Tween, they were finally exposed to freshly prepared Dura Substrate for chemiluminescence for appropriate time before performing autoradiography.

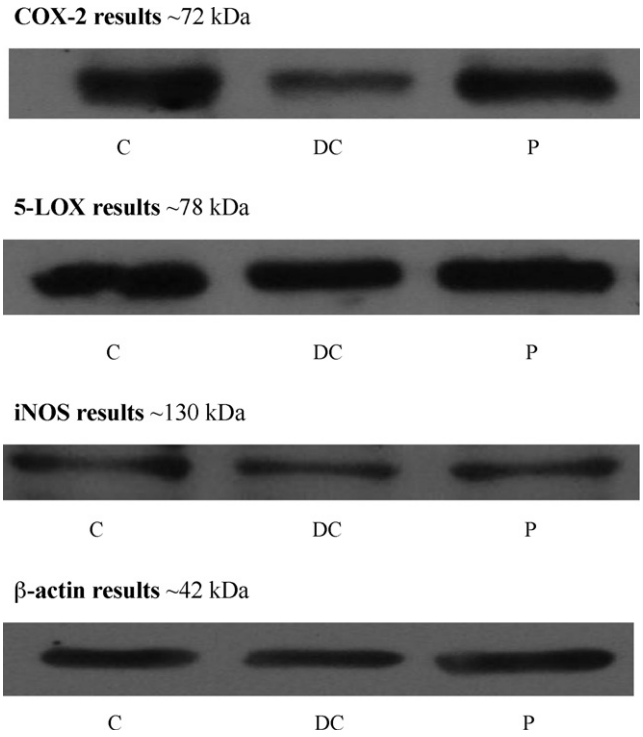

Fig. 1. Effects *H. procumbens* (DC) and receptor phase containing permeated constituents (P) on COX-2, 5-LOX and iNOS expressions as shown by Western blotting at 6 h control (C).

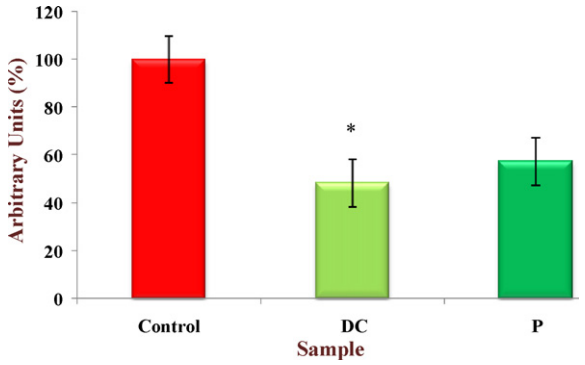

Fig. 2. Effects of *H. procumbens* saturated extract solution in PBS (DC), and its permeated components (P) on the expression of COX-2 in porcine skin. Control = PBS. The results were normalised using β-actin. Levels in control were arbitrarily assigned a value of 100% ($n = 3$, ±S.D.), ($^*p < 0.05$).

2.6. ELISA analysis

The supernatant obtained from skin lysates was also used for ELISA analysis of PGE-2 (the main product of COX-2 pathway). Amounts of PGE-2 were measured using ELISA kit as described by the information manual obtained from Cayman Chemical Europe (Tallinn, Estonia). The colorimetric readout was performed at 405 nm on an automated microELISA plate reader (TECAN Sunrise, NC, USA).

2.7. Immunohistochemical analysis

Skin sections were dosed with 1 mg mL^{-1} *H. procumbens*, receptor phase obtained from permeated saturated solution of *H. procumbens* in water and reconstituted with PBS. Three replicates were prepared for each formulation, i.e. control (PBS), 1 mg mL^{-1} *H. procumbens* and receptor phase. The diffused areas were excised at 6 h, fixed in formaldehyde and embedded in paraffin wax blocks. Microtomed sections of 5 μm were then sliced and subjected to immunohistochemical analysis using a previously described method (Abdelouahab and Heard, 2008b). The primary antibodies were as used in the Western blotting analysis. Presence of COX-2, 5-LOX and iNOS was indicated by a brown-red stain, with reduced

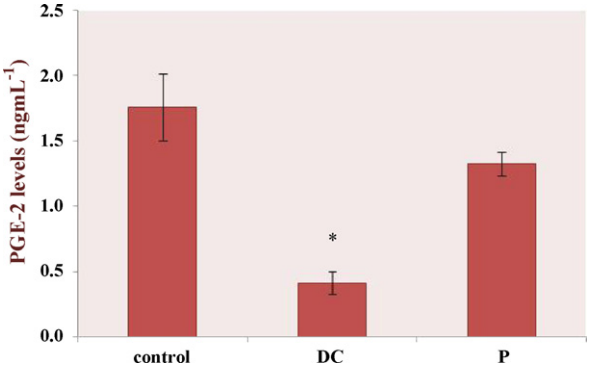

Fig. 3. Inhibition of COX-2 by reduction of PGE-2 production. DC: *H. procumbens* saturated solution, P: receptor phase containing the permeated constituents of *H. procumbens* extract, control: PBS ($n = 3 \pm$ S.D.).

levels of the enzyme determined qualitatively by a reduction in the intensity of the staining.

2.8. Data analysis

Statistical analysis was performed by a one-way ANOVA, Kruskal Wallis non-parametric test followed by Dunn's post-test. Probabilities of ≤ 0.05 were defined as significant.

3. Results

Previous work has shown the successful transcutaneous delivery of the major glycosides of *H. procumbens* from a range of different vehicles across skin (Abdelouahab and Heard, 2008a). It can be seen from Table 1 that, despite their water solubility and high molecular weights, all the glycosides permeated skin. Harpagide levels in the receptor phase were considerably higher than glycosides 1, 3, 4 as shown in Table 1.

The effect of the permeated constituents of *H. procumbens* was analysed by determining the relative inhibition of COX-2, 5-LOX and iNOS expressions. The resulting bands at ~72 kDa, ~78 kDa, ~130 kDa (Fig. 1) indicate a fairly significant reduction of the protein expression at 6 h compared to the control for *H. procumbens* and for the permeated components in the case of COX-2. However, there was no effect noticed on the amounts of produced 5-LOX and the results obtained for iNOS were not significant, the bands

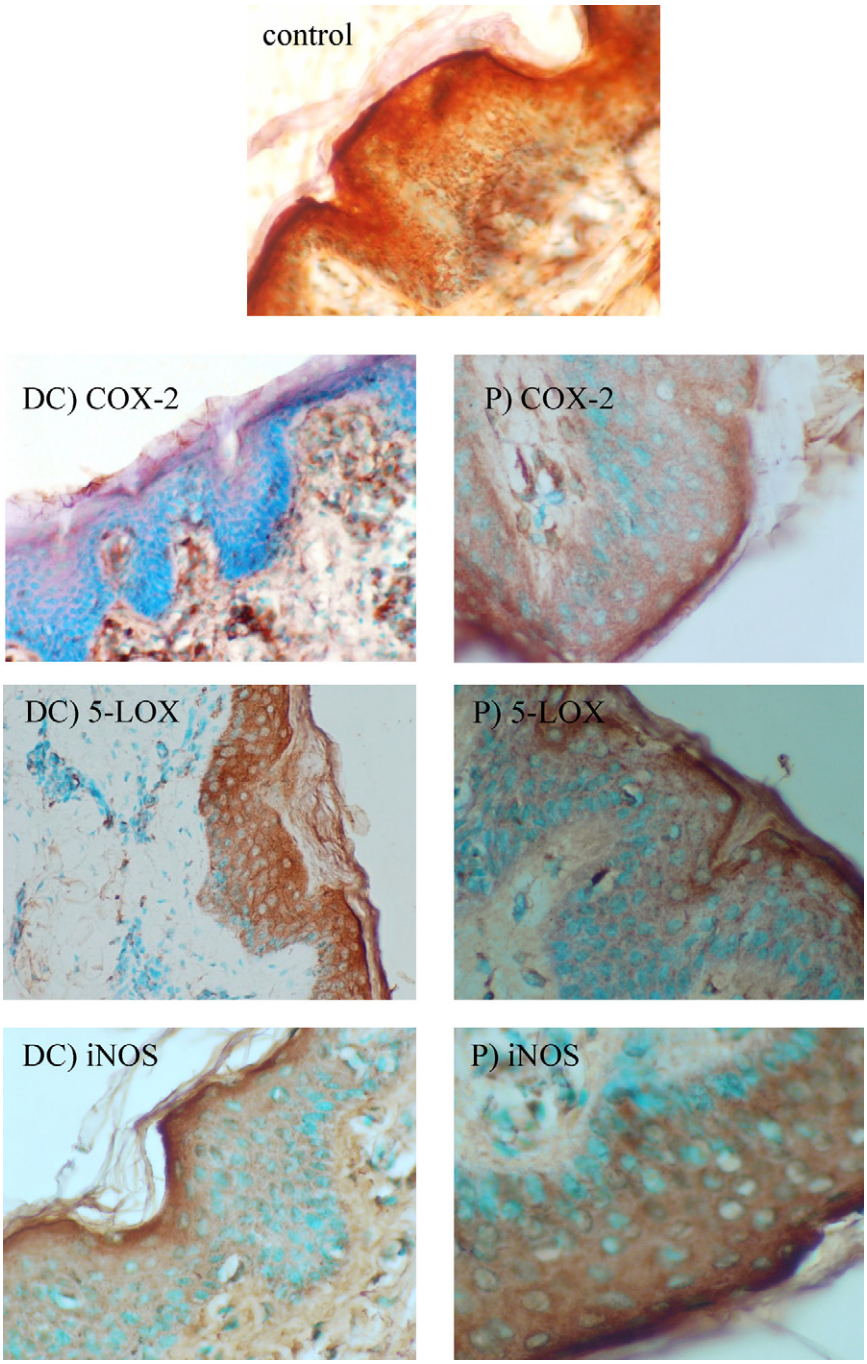

Fig. 4. Immunocytochemical analysis of COX-2, 5-LOX and iNOS. Immunostaining in porcine skin after 6 h of administration of PBS (control), *H. procumbens* (DC), freeze dried receptor phase reconstituted in 1 mg mL^{-1} in PBS (P) ($n = 3$).

were barely detectable and quantification using densitometry was not attempted. This was confirmed in the densitometric analysis of the Western blotting data, and the results were normalised against β-actin and calculated as a ratio from the control as shown in Fig. 2.

An ELISA assay was performed to determine the effects of the same samples used in Western blotting on the product of COX-2 pathway, namely PGE-2. Fig. 3 shows that PGE-2 levels were reduced very significantly (p 0.0074) by a saturated solution of *H. procumbens* while the permeated amounts of constituents did not have a significant effect on PGE-2 levels. Immunocytochemistry results probing for the effects of *H. procumbens* saturated solutions and the permeated solution (receptor phase) through porcine skin layers were demonstrated in Fig. 4.

H. procumbens saturated solution led to a considerable decline in immunoreactive enzymes (as denoted by the red staining) within epithelial tissues of porcine skin sections compared to control at 6 h. This could be seen with COX-2 and less impact with samples probed for iNOS. However, ICC results confirmed the fact that *H. procumbens* did not have any effect on the expression of 5-LOX. Similarly, when dosed with *H. procumbens* receptor phase, the staining obtained was reduced compared PBS alone but less considerably than when *H. procumbens* saturated solutions were used.

4. Discussion

The results showed an effect on COX-2 for both *H. procumbens* applied on skin and the permeated amounts. However the lack of effects in 5-LOX might suggest that *H. procumbens* does not interfere with its expression, although this does not exclude its possible action on the pathways of 5-LOX, i.e. leukotrienes. The recovery of receptor phase, containing compounds permeated across skin for use in further experimentation is, we believe, a novel method for assessing the transcutaneous anti-inflammatory potential. This procedure was preferred to the combination of the glycosides in amounts known to permeate from earlier work because it would also contain other permeants from the *H. procumbens* extract (not assayed for), which may be of significance, and better reflects an in-use scenario. These data suggest that by reducing COX-2 amounts obtained in skin, *H. procumbens* may be responsible for the knockdown of the transcription factors responsible for COX-2 expression (Chun et al., 2003). Therefore, this study agrees with previous work where *H. procumbens* successfully inhibited NF-κB for COX-2 (Kundu et al., 2005). However, it can also interfere with the enzymatic pathway of COX-2, and hence reduce the production its metabolites, mainly PGE-2.

The PG2 data imply that by achieving higher delivery of *H. procumbens* and increasing the anti-inflammatory components that cross the skin layers, an apparent knockdown PGE-2 which has a key role in inflammatory response will be achieved.

The staining increased over time when the skin was treated with PBS solution. It can be suggested that after removal of the skin from the ear it is apparent that an inflammatory response had been triggered from the initiation of inflammatory metabolic pathways of COX-2, 5-LOX and iNOS. By comparison, the receptor phase was found to have a weaker effect on the enzymes relative to *H. procumbens* and this may be due to the low levels of anti-inflammatory glycosides that permeate through the skin. In addition, this may be explained by the presence of miscellaneous constituents in the extract that are crucial in inhibiting inflammatory mechanisms but do not permeate through or get degraded within skin. However, there was still an apparent effect compared to the control as the stain was decreased at 6 h in sections treated with the receptor phase.

In the light of the current experiment, the ability of *H. procumbens* to modulate COX-2 and iNOS activities is likely to be related to both the expression and pathway of this particular enzyme. The increase in 5-LOX expression might be due to a negative feedback loop which is caused by reduction in COX-2 leading to an up-regulation of 5-LOX pathways. These data also agree with previous findings that other constituents, in addition to the major constituent glycosides, may contribute to the total efficacy of the whole extract. They can work synergistically by being involved in inhibiting inflammatory pathways and mediators.

Overall, in addition to its ability to inhibit inflammatory enzymes topically, this study showed that even the small amounts of glycosides as well as other components that permeate transcutaneously may have an important role in reducing the expression of inflammatory enzymes.

5. Conclusion

A novel model, involving *ex vivo* skin and Franz diffusion cell methodology, has been described which can be used to indicate that anti-inflammatory activity is retained once topically applied permeants have been delivered to deeper subcutaneous regions. The current data support the transcutaneous efficacy of *H. procumbens* against COX-2 and its product, PGE-2, which are key factors in the arachidonic acid inflammatory cascade..

References

Abdelouahab, N., Heard, C.M., 2008a. Dermal and transcutaneous delivery of the major glycosides constituents of *H. procumbens* (Devil's Claw) in vitro. Planta Med. 74, 527–531.

Abdelouahab, N., Heard, C.M., 2008b. Effect of the major glycosides of *Harpagophytum procumbens* (Devil's Claw) on epidermal cyclooxygenase-2 (COX-2) in vitro. J. Nat. Prod. 71, 746–749.

Aggarwal, B., Shishodia, S., Sandur, S.K., Pandey, M.K., Sethi, G., 2006. Inflammation and cancer: how hot is the link? Biochem. Pharm. 72, 1605–1621.

Baghdikian, B., Lanhers, M.C., Fleurentin, J., Ollivier, E., Maillard, C., Balansard, G., Mortier, F., 1997. An analytical study: anti-inflammatory and analgesic effects of *Harpagophytum procumbens* and *Harpagophytum zeheri*. Planta Med. 63, 171–176.

Benito, P.B., Lanza, A.M.D., Sen, A.M.S., Galindez, J.D., Matellano, L.F., Gomez, A.S., Martinez, M.J.A., 2000. Effects of some iridoids from plant origin on arachidonic acid metabolism in cellular systems. Planta Med. 66, 324–328.

Chantre, P., Cappelaere, D., Leblan, D., Guedon, J., Vandermander, J., Fournie, B., 2000. Efficacy and tolerance of *Harpagophytum procumbens* versus diacerhein in treatment of osteoarthritis. Phytomedicine 7, 177–183.

Chrubasik, S., Zimper, C., Schuett, U., Ziegler, R., 1996. Effectiveness of *Harpagophytum procumbens* in treatment of acute low back pain. Phytomedicine 3, 1–10.

Chrubasik, S., Conradt, C., Roufogalis, B.D., 2004. Effectiveness of Harpagophytum extracts and clinical efficacy. Phytother. Res. 18, 187–189.

Chun, K.S., Keum, Y.S., Han, S.S., Song, Y.S., Kim, S.H., Surh, Y.J., 2003. Curcumin inhibits phorbol ester-induced expression of cyclooxygenase-2 in mouse skin through suppression of extracellular signal-regulated kinase activity and NF-κB activation. Carcinogenesis 24, 1515–1524.

Eich, J., Schmidt, M., Betti, G., 1998. HPLC analysis of iridoid compounds of *Harpagophytum* taxa: quality control of pharmaceutical drug material. Pharmacol. Lett. 8, 75–78.

Ernst, E., Chrubasik, S., 2000. Phyto-anti-inflammatories: a systematic review of randomised, placebo-controlled, double-blind trials. Rheum. Dis. Clin. North Am. 26, 13–27.

Escribano, E., Calpena, A.C., Queralt, J., Obach, R., Doménech, J., 2003. Assessment of diclofenac permeation with different formulations: anti-inflammatory study of a selected formula. Eur. J. Pharmacol. Sci. 19, 203–210.

Fiebich, B.L., Heinrich, M., Hiller, K.O., Kammerer, N., 2001. Inhibition of TNF-a synthesis in LPS-stimulated primary human monocytes by Harpagophytum extract SteiHap 69. Phytomedicine 8, 28–30.

Joubert, E., Manly, M., Gray, B.R., Schulz, H., 2005. Rapid measurement and evaluation of the effect of drying conditions on harpagoside content in *Harpagophytum procumbens* (Devil's Claw) root. J. Agric. Food Chem. 53, 3493–3502.

Kaszkin, M., Beck, K.F., Koch, E., Erdelmeier, C., Kusch, S., Pfeilschifter, J., Loew, D., 2004. Downregulation of iNOS expression in rat mesangial cells by special extracts of *Harpagophytum procumbens* derives from harpagoside-dependent and independent effects. Phytomedicine 11, 585–595.

Kundu, J.K., Mossanda, K.S., Na, H.K., Surh, Y.J., 2005. Inhibitory effects of the extracts of *Sutherlandia frutescens* (L.) R. Br. and *Harpagophytum procumbens* DC. on phor-

bol ester-induced COX-2 expression in mouse skin: AP-1 and CREB as potential upstream targets. Can. Lett. 218, 21–31.
Lin, L.C., Wang, Y.H., Hou, Y.C., Chang, S., Liou, K.T., Chou, Y.C., Wang, W.Y., Shen, Y.C., 2006. The inhibitory effect of phenylpropanoid glycosides and iridoid glucosides on free radical production and beta 2 integrin expression in human leucocytes. J. Pharm. Pharmacol. 58, 129–135.
Ling, S.K., Tanaka, T., Kouno, I., 2003. Effects of iridoids on lipoxygenase and hyaluronidase activities and their activation by beta-glucosidase in the presence of amino acids. Biol. Pharm. Bull. 26, 352–356.
Marcouiller, P., Pelletier, J.P., Guevremont, M., Martel-Pelletier, J., Ranger, P., Laufer, S., Reboul, P., 2005. Leukotriene and prostaglandin synthesis pathways in osteoarthritic synovial membranes: regulating factors for interleukin 1 beta synthesis. J. Rheum. 32, 704–712.
Moussard, C., Alber, D., Toubin, M.M., Thevenon, N., Henry, J.S., 1992. A drug used in traditional medicine, *Harpagophytum procumbens*: no evidence for nsaid-like effect on whole blood eicosanoid production in human. Prostag. Leukotr. Ess. 46, 283–286.
Thomas, C.P., Davison, Z., Heard, C.M., 2007. Probing the skin permeation of fish oil/EPA and ketoprofen 3. Influence of fish oil/ketoprofen on epidermal COX-2 and LOX. Prostag. Leukotr. Ess. 76, 357–362.
Tippler, B., Syrovets, T., Plaza, N., Loew, D., Simmet, T., 1997. *Harpagophytum procumbens* DC used in traditional medicine inhibits eicosanoid biosynthesis in human whole blood. Int. J. Tissue React. 19, 101–102.
Wegener, T., 2000. Devil's claw: from African traditional remedy to modern analgesic and anti-inflammatory. Herbal Gram. 50, 47–54.