

Molecular Targets of the Antiinflammatory *Harpagophytum procumbens* (Devil's claw): Inhibition of TNF α and COX-2 Gene Expression by Preventing Activation of AP-1

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Harpagophytum procumbens (Hp) is often used in the supportive treatment of inflammatory and degenerative diseases of the skeletal system. Although the clinical efficacy in osteoarthritis has been demonstrated in clinical trials, the molecular target(s) of Hp are unclear. This study quantified the effects of the ethanol Hp extract (60% v/v ethanol, sole active ingredient of Pascoe®-Agil), on the expression and release of the major pro-inflammatory mediators in LPS-stimulated human monocytes and the intracellular signalling pathways involved in inflammation. The Hp extract dose-dependently inhibited the release of TNF α as well as that of interleukin (IL)-6, IL-1 β and prostaglandin E₂ (PGE₂). The Hp prevented TNF α and IL-6 mRNA expression in human monocytes and cyclooxygenase-2 (COX-2) in RAW 264.7 cells. Furthermore, the Hp extract inhibited LPS-stimulated AP-1-mediated gene transcription activity and binding to the AP-1 response elements. The extract had no effect on the LPS-induced binding of nuclear factor- κ B in RAW 264.7 cells, on LPS-induced degradation of I κ B α or on LPS-induced activation of mitogen-activated protein kinases (MAPK), p38MAPK and JNK in human monocytes. The data indicate that a standardized ethanol Hp extract inhibits induction of pro-inflammatory gene expression, possibly by blocking the AP-1 pathway. This is novel evidence of a possible mechanism of action of this antiinflammatory drug. Copyright © 2011 John Wiley & Sons, Ltd.

Keywords: rheumatic arthritis; cytokines; signal transduction; Nuclear factor- κ B; phytomedicine; cytokines.

INTRODUCTION

Extracts of the secondary roots of the southern African plant, *Harpagophytum procumbens*, (Hp) (Devil's claw), a member of the sesame family (Pedaliaceae), provide a herbal drug with a variety of traditional indications including, mostly recently in Europe, chronic inflammation. The European Scientific Cooperative on Phytotherapy (ESCOP) issued a monograph recommending its use in the symptomatic treatment of painful osteoarthritis, relieving lower back pain, loss of appetite and dyspepsia (Brendler *et al.*, 2006). The EMA Committee on Herbal Medicinal Products (HMPC) has issued a draft monograph for discussion in which an indication for the treatment of mild articular pain and mild digestive disorders is proposed on the basis of traditional use (EMEA, 2008).

The efficacy of Hp-containing products in the treatment of lower back pain or osteoarthritis has been tested in several clinical trials and these have been reviewed (Brien *et al.*, 2006; Denner, 2007; Gagnier *et al.*, 2007; Loew and Rietbrock, 1996). There is good evidence of the efficacy and good tolerability, including equivalence

with NSAIDs and superior tolerability (Chantre *et al.*, 2000; Chrubasik *et al.*, 1999, 2002, 2003, 2004; Gagnier *et al.*, 2007; Wegener and Lupke, 2003). One clinical study has been performed with the Hp extract (DEV4.4-5.0:1, 60% v/v ethanol; ALLYA® tablets/Pascoe®-Agil) that was used in the investigations reported here. This clinical study demonstrated that the Hp extract had similar efficacy, but superior tolerability, to that of Vioxx® and of Voltaren® resinat (Ruetten and Kuhn, 2005). Another more recent clinical trial assessed the efficacy and safety of a similar ethanol extract of Hp in the treatment of 259 patients with arthritis and other rheumatic conditions (Warnock *et al.*, 2007). The authors reported statistically significant reductions in mean pain scores for hand, wrist, elbow, shoulder, hip, knee and back pain, and a significant increase from base-line of quality of life measurements (SF-12) with 60% patients either reducing or stopping concomitant pain medication (Warnock *et al.*, 2007).

The aim of the present study was to determine the molecular pharmacological targets of Hp underlying its anti-inflammatory effects. For these investigations, macrophage-based cellular models were chosen, including primary human monocytes (Widomski *et al.*, 1997). Similar studies have been made in the past and have provided some information on the mechanism of action of Hp (Loew, 1996). Here, the effects were investigated of a standardized

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extract of Hp on the various intracellular molecular pathways that are now known to underlie the inflammatory cellular and molecular cascade. Such investigations should provide important insights in to the mode of action of this popular self-medication and provide a basis for its possible broader clinical use in treating inflammatory diseases.

MATERIAL AND METHODS

Materials. The *Harpagophytum* (Hp) extract (batch no. 7873/03; DEV 4.4-5.0:1) (DEV4.4-5.0:1, Pascoe®-Agil 240 mg, film-coated tablets) was obtained by hydroalcohol extraction of *Radix Harpagophyti procumbens*. The content of the marker substance harpagoside in the native extract was calculated as 2.9% according to HPLC. Harpagoside was isolated from various natural sources and was identified by NMR.

Isolation of human peripheral monocytes. Monocytes from healthy human donors were prepared following a standardized protocol (Ficoll gradient preparation, Amersham-Biosciences) using a completely endotoxin-free cultivation (English and Andersen, 1974; Noble *et al.*, 1968). By using 50 mL tubes, 25 mL Ficoll was loaded with 25 mL blood of buffy coats from healthy blood donors. The gradient was established by centrifugation at 1800 rpm, 20 °C for 40 min by using slow acceleration and brakes. Peripheral blood mononuclear cells in the interface were carefully removed and resuspended in 50 mL pre-warmed phosphate buffered saline (PBS, from Invitrogen) followed by centrifugation for 10 min at 1600 rpm and 20 °C. The supernatant was discarded and the pellet washed in 50 mL PBS and centrifuged as described above. The pellet was then resuspended in 50 mL RPMI-1640 low endotoxin-medium (from Invitrogen) supplemented with 10% human serum (PAA). After counting the amount of cells in a particle counter (Euro Diagnostics, Krefeld), the cells were seeded in 24-well plates and incubated at 37 °C/5% CO₂. The medium and the non-adherent cells (lymphocytes) were removed and fresh RPMI-1640 medium containing 1% human serum was added. The monocytes were ready for use in the experiments.

Determination of inflammatory parameters. Measurements of the cytokines IL-6, TNF α and IL-1 β and PGE₂ were performed using commercially available EIAs (PGE₂: Biotrend, Köln, Germany) and ELISA (IL-6, TNF α and IL-1 β : HISS, Freiburg, Germany) kits. The cells were seeded in 24-well plates (Falcon) for EIA-/ELISA measurements. Then 30 min prior to stimulation with LPS (10 ng/mL) the cells were incubated with Hp Pascoe®-Agil. After incubation at 37 °C and 5% CO₂ for 24 h, the supernatants were removed, centrifuged and investigated for IL-1 β , IL-6, TNF α and PGE₂ concentrations. Statistical analysis was performed by the Institut für Biochemische Analysen und Methodenentwicklung GmbH, Freiburg (Germany). Original data were expressed as a percentage of the lipopolysaccharide control values and the mean \pm SE calculated. In the figures, a regression curve was adjusted to the calculated mean values.

RNA extraction and northern blot analysis. Total RNA was extracted using the guanidine isothiocyanate method (Chomczynski and Sacchi, 1987). RNA (10 μ g) was separated by agarose-formaldehyde gel electrophoresis, blotted onto positively charged nylon membranes (Pharmacia, Freiburg) and cross-linked by exposure to 120 °C for 30 min. As a marker, 10 μ g of total RNA was run on the same gel and stained with ethidium bromide to determine the bands for 18s and 28s ribosomal RNA. The filters were prehybridized in 50% formamide, 0.25 M Na-phosphate buffer, pH 7.2, 0.25 M NaCl, 10 mM EDTA, 200 μ g/mL salmon sperm DNA and 7% SDS at 43 °C for 2 h. The cDNA probes (IL-6, TNF α , S12) were labelled with 50 μ Ci [³²P]dCTP (Amersham & Buchler, Braunschweig, Germany) using a random priming kit from Stratagene (Heidelberg, Germany). Unincorporated nucleotides were removed using a nucleotide removal kit from Qiagen. Overnight hybridization was performed at 43 °C, adding the radiolabeled probe to the prehybridization buffer. Membranes were washed in 2 \times SSC/0.1% SDS (3 \times 20 min) at 60 °C and exposed to Kodak XAR film at -80 °C for adequate periods of time for signal development. For rehybridization, the probes were desorbed by boiling the filter at 95 °C in distilled water. Experiments were carried out in triplicate.

Western blot analysis. Monocytic cells were exposed to LPS in the presence or absence of Hp extract. The cells were washed with phosphate-buffered saline (PBS) and lysed in SDS sample buffer (sodium dodecyl sulfate buffer containing 100 μ M orthovanadate (Laemmli 1970)). Lysates were homogenized through a 26-gauge-needle and measured for protein content using the bicinchoninic acid method (BCA protein kit, Pierce, distributed by KFC Chemikalien, München, Germany). For western blotting, 60 μ g of cell protein from each sample for phosphor-(p38)-MAPK, phosphoJNK and I κ B α were subjected to SDS-PAGE (polyacrylamide gel electrophoresis) on a 12% gel under reducing conditions. Proteins were then transferred onto a polyvinylidene fluoride (PVDF) membrane (Millipore, Bedford, MA, USA) by semi-dry blotting. The membrane was blocked overnight at 4 °C using Rotiblock (Roth, Karlsruhe, Germany) and another hour at room temperature before incubation with the antibody. The quantity of phosphoJNK, and active p38 MAPK in each sample was assessed using rabbit polyclonal antibodies, the quantity of I κ B α by using a goat polyclonal antibody. Anti-active p38 MAPK, recognizing both phosphorylated residues T180 and Y182 (NEB, Schwalbach, Germany), was diluted 1:500 in TBS-T + 1% BSA, anti-I κ B α (Santa Cruz, Heidelberg, Germany) was diluted 1:1000 in TBS-T + 1% BSA and anti-phosphoJNK (NEB, Schwalbach, Germany) was diluted 1:1000 in TBS-T + 1% BSA. Subsequent detection was performed using the ECL western blotting system (Amersham) according to the manufacturer's instructions.

Plasmids. The AP-1-Luc plasmid used here was constructed by inserting three copies of an SV40 AP-1 binding site into the Xho site of pGL-2 promoter vector (Promega, MA, USA) while the employed KBF-Luc plasmid contained three copies of the MHC enhancer kB site upstream of the conalbumin promoter, followed by the luciferase gene. The employed COX-2-Luc plasmid was a gift from M. Fresno (CMB-UAM, Madrid, Spain)

and the DNA construct has been described previously (Iniguez *et al.*, 2000).

Transient transfection cells and gene reporter assays.

The RAW264.7 cells (10^6) were transfected with the indicated plasmids in Opti-MEM (Invitrogen) by using Lipofectin™ reagent (Gibco BRL) according to the manufacturer's instructions. Twenty-four hours after transfection, the cells were pre-incubated with different concentrations of *Harpagophytum* for 30 min and then treated as indicated for 6 h. Then, the cells were lysed in 25 mM Tris-phosphate pH 7.8, containing 8 mM MgCl₂, 1 mM DTT, 1% Triton X-100 and 7% glycerol. Luciferase activity was measured using an Autolumat LB 9501 (EG&G Berthold, USA) according to the manufacturer's instructions (Promega, Madison, WI, USA). The background obtained with the lysis buffer was subtracted from each test value and the specific transactivation expressed as the total RLU induction. All the experiments were repeated at least three times.

Isolation of nuclear extracts and mobility shift assays.

Following LPS (10 ng/mL) treatment with and without the various antagonists, RAW264.7 cells (10^6 /mL complete medium) were washed twice with cold PBS, and proteins from total cell extracts were isolated as described previously (Sancho, 2004). The protein concentration was determined as described above (Bio-Rad, Richmond, CA, USA).

For the electrophoretic mobility shift assay (EMSA), double-stranded oligonucleotides containing the consensus sites for AP-1 (Promega, Madison, WI, USA), were end-labeled with [γ -³²P]ATP. The binding reaction mixture contained 3 μ g of nuclear extract (or 15 μ g of

total extracts), 0.5 μ g poly(dI-dC) (Pharmacia Fine Chemical, Piscataway, NJ, USA), 20 mM Hepes pH 7, 70 mM NaCl, 2 mM DTT, 0.01% NP-40, 100 μ g/mL BSA, 4% Ficoll and 100000 cpm of end-labeled DNA fragments in a total volume of 20 μ L. Where indicated, 0.5 μ L of rabbit anti-NF-AT1 or pre-immune serum was added to the standard reaction before the addition of the radio-labeled probe. After 30 min incubation at 4 °C, the mixture was electrophoresed through a native 6% polyacrylamide gel containing 89 mM Tris-borate, 89 mM boric acid and 1 mM EDTA. Gels were 'pre-electrophoresed' for 30 min at 225 V and then for 2 h, after loading the samples. After electrophoresis, the gels were dried and exposed to an x-ray film at -80 °C.

RESULTS

Effects of Pascoe®-Agil 240 mg *Harpagophytum* extract (DEV4.4-5.0:1) on LPS-induced release of inflammatory parameters in primary human monocytes

Using non-toxic doses, the effects of the Hp extract on LPS-induced release of the cytokines TNF α , IL-1 β , IL-6 and PGE₂ were investigated (Fig. 1A–D). All these parameters were inhibited by Hp in a dose-dependent manner. Maximal effects were achieved using 500 μ g/mL of the extract (Fig. 1). The release of TNF α and IL-6 were most potently inhibited by Hp extract with IC₅₀ of approx. 100 μ g/mL and lower (Fig. 1A, B).

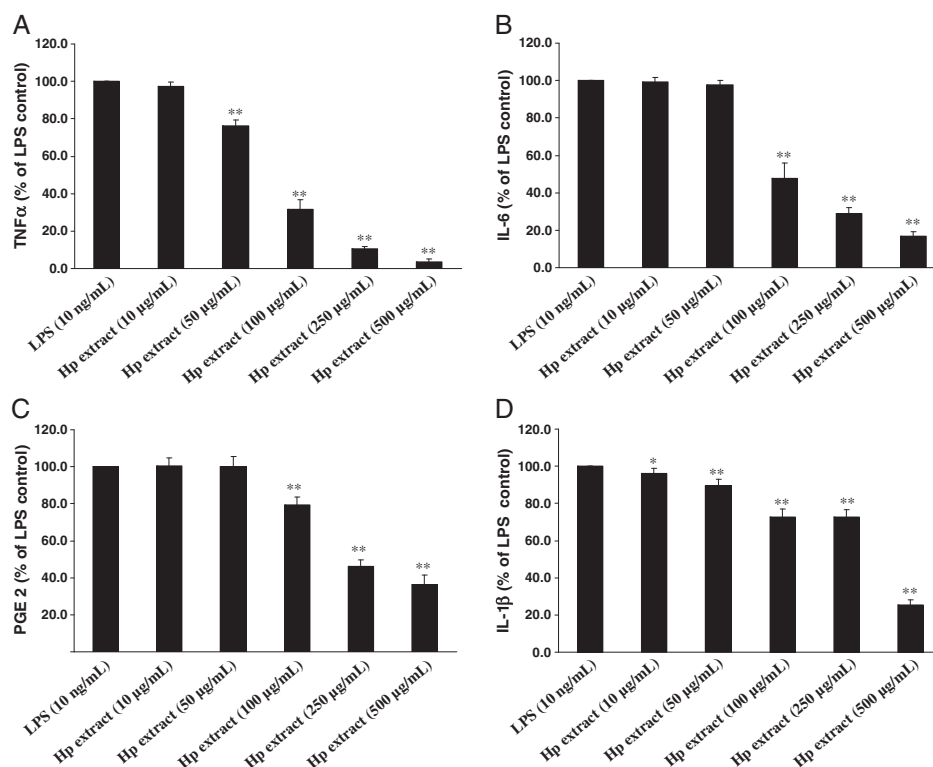


Figure 1. *Harpagophytum* extract Pascoe®-Agil inhibits the LPS-induced release of TNF α (A), IL-1 β (B), IL-6 (C), PGE₂ in primary human monocytes. Cells were unstimulated (control) or stimulated with LPS (10 ng/mL) and LPS + Hp for 24 h. Supernatants were removed and analysed by ELISA/EIA. $n = 9$ from three different blood donors. Data are provided as percent of LPS-stimulated cells. * $p < 0.05$, ** $p < 0.01$ with respect to LPS control (Student's t -test).

Effects of Pascoe®-Agil 240 mg *Harpagophytum* extract on TNF α , IL-6 and COX-2 gene expression

To establish whether Hp exerts its antiinflammatory effects at the transcriptional levels, northern blot analysis was performed using cDNA probes for TNF α and IL-6. As shown in Fig 2, incubation with Hp decreased TNF α mRNA levels, with detectable inhibition at 50 $\mu\text{g}/\text{mL}$ and maximal inhibition at 500 $\mu\text{g}/\text{mL}$. The IL-6 mRNA levels were also decreased by Hp but to a lesser extent than TNF α (line 2). To determine whether there was equal loading of the RNA gels, hybridization with an S12 cDNA was performed (lane 3). A reduction of TNF α mRNA levels by Hp was confirmed by RT-PCR analysis (not shown).

The effect of Hp on COX-2 expression was assessed using a COX-2 promoter construct in transiently transfected RAW cells. The COX-2 promoter activity was induced by PMA but inhibited in the presence of Hp, starting at a concentration of 25 $\mu\text{g}/\text{mL}$ with a maximal effect at 50 $\mu\text{g}/\text{mL}$ of the extract (Fig. 3).

Effects of Pascoe®-Agil *Harpagophytum* extract on signal transduction pathways

The data suggest that Hp exerts its antiinflammatory effects via effects on specific signal transduction pathways leading to inhibition of pro-inflammatory gene

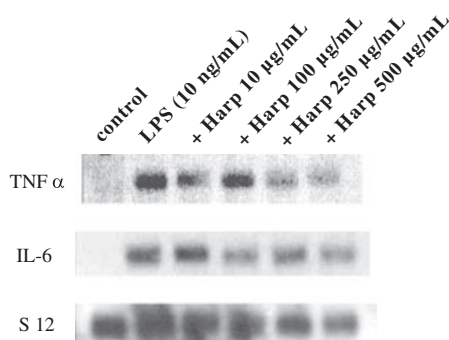


Figure 2. *Harpagophytum* extract Pascoe®-Agil inhibits the LPS-induced mRNA levels of TNF α and IL-6 in primary human monocytes. Cells were unstimulated (control) or stimulated with LPS and LPS + Hp for 4 h. Total RNA was then extracted and 10 μg was used in northern blot analysis. After hybridization with a cDNA probe coding for TNF α /IL-6 (upper panels), filters were hybridized with β -actin cDNA for standardization (lower panel).

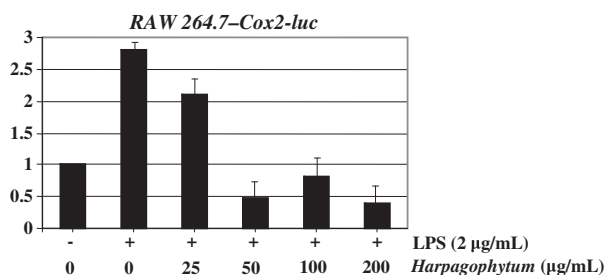


Figure 3. *Harpagophytum* extract Pascoe®-Agil inhibits LPS-induced COX-2 mRNA levels in mouse RAW 264.7 macrophages using a COX-2 luc plasmid. Cells were unstimulated (control) or stimulated with LPS (2 $\mu\text{g}/\text{mL}$) and LPS + Hp for 4 h. Luciferase activity was measured and results are the mean \pm SE of at least three determinations and expressed as the fold induction over non-stimulated cells.

transcription. LPS-induced TNF α , IL-6 and COX-2 expression depends on several signal transduction pathways including p38 MAPK, NF- κ B/I κ B, JNK, PKC and AP-1. It was of interest to determine which of those LPS-induced signal transduction pathways are targeted by the Hp extract.

The Hp did not affect LPS-induced activation of the p38 MAPK and JNK (Fig. 4) activation. I κ B α degradation was not prevented (Fig. 4) suggesting that NF- κ B activation (Fig. 5) is not affected by the extract used. To further exclude possible effects of the Hp extract on the NF- κ B pathway, gene reporter assays were performed demonstrating that NF- κ B is not the target of the Hp extract used in our cell cultures (Fig. 5).

The effects of the Hp extracts on the DNA-binding of AP-1 transcription factor were further evaluated. The THP-1 cells were treated with increasing concentrations of Hp (10–200 $\mu\text{g}/\text{mL}$) for 30 min, after which the cells were stimulated with LPS (2 $\mu\text{g}/\text{mL}$) for 60 min and the nuclear proteins isolated as described above. In Fig. 6, it is shown that Hp at the lower concentrations was able to increase the binding of AP-1 to DNA but at the higher doses (100–200 $\mu\text{g}/\text{mL}$) it clearly inhibited the DNA binding activity of this factor. In addition, it was found that AP-1 transcriptional activity was inhibited by this extract in a concentration-dependent manner in the RAW cell line (Fig 6, right panel).

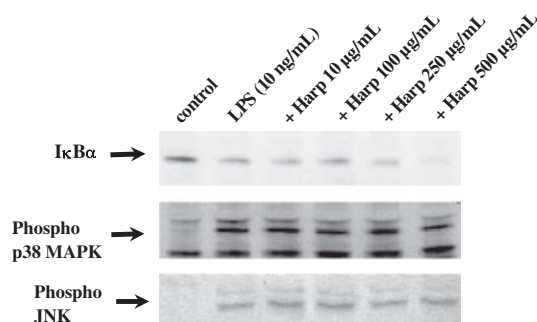


Figure 4. LPS-induced phosphorylation of p38 MAPK and JNK and the degradation of I κ B α is not inhibited by *Harpagophytum* extract Pascoe®-Agil. Total cell protein prepared from control and LPS (10 ng/mL) and LPS + Hp-treated cells was subjected to SDS-PAGE followed by western blotting using polyclonal antibodies that recognize the phosphorylated (and thus active) form of p38 MAPK/JNK and I κ B α .

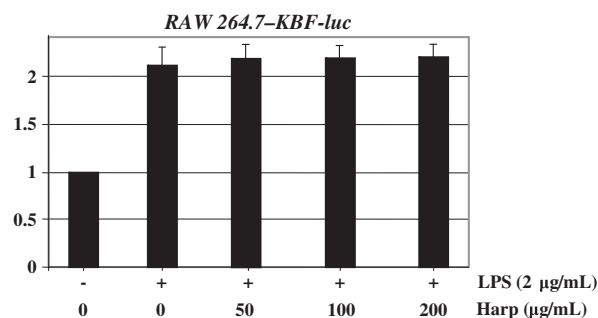


Figure 5. *Harpagophytum* extract Pascoe®-Agil does not affect LPS-induced NF- κ B activity in RAW 264.7 macrophages. RAW 264.7 cells were transiently transfected with the luciferase reporter plasmid KBF-Luc and stimulated with LPS (2 $\mu\text{g}/\text{mL}$) and LPS + Hp for 8 h. Luciferase activity was determined and transactivation is given as fold induction over the untreated cells. The mean values from three independent experiments are shown, bars indicate standard deviations.

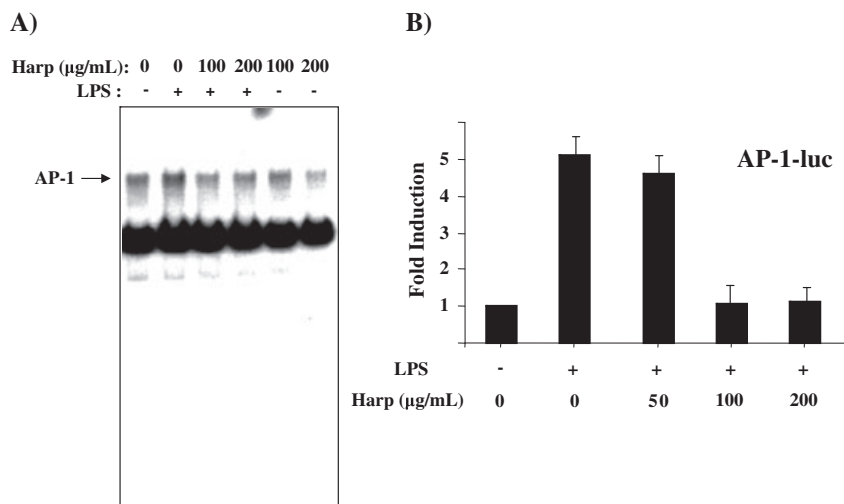


Figure 6. *Harpagophytum* extract Pascoe®-Agil inhibits LPS-induced AP-1 activity in RAW 264.7 macrophages. (A) RAW 264.7 cells were pre-treated with Hp in the presence and absence of LPS (2 µg/mL) in the indicated concentrations. Nuclear proteins were extracted and the binding to ³²P-labeled AP-1 probes studied by electrophoretic mobility shift assays. (B) RAW 264.7 cells were transiently transfected with the luciferase reporter plasmid AP-1-Luc and stimulated with LPS (2 µg/mL) and LPS + Hp for 8 h. Luciferase activity was determined and transactivation is given as fold induction over the untreated cells. The mean values from three independent experiments are shown, bars indicate standard deviations.

DISCUSSION

This study shows that the Hp extract (the sole active ingredient of Pascoe®-Agil 240 mg film-coated tablets) inhibits inflammatory processes by inhibiting cytokine and PGE₂ release as well as COX-2, IL-6 and TNFα mRNA expression. This confirms previous studies that indicated that the herbal antiinflammatory drug inhibits endotoxin (LPS)-stimulated cytokines from human monocytes (Fiebich *et al.*, 2000) and mouse macrophages (Inaba *et al.*, 2010). The present study extended these findings and obtained evidence suggesting that Hp acts via inhibition of AP1-mediated transcription of pro-inflammatory genes without affecting the NFκB and the MAP kinase pathway.

The release of pro-inflammatory cytokine TNFα by immune cells such as monocytes and macrophages is a very early event in activation of the innate immune system. TNFα activates and initiates a cascade of parallel pro-inflammatory processes, including the induction of other pro-inflammatory molecules, and is considered a central target in treating chronic inflammation (Westacott and Sharif, 1996). Injectable biological TNFα-blocking drugs have been introduced in recent years as effective treatments of rheumatoid arthritis, with effects in reducing clinical inflammation and skeletal damage (Reimold, 2002). Our data suggests that the Hp extract, by inhibiting the biosynthesis of TNFα has a similar pharmacology to these modern antiinflammatory drugs.

There is increasing evidence that activation of the innate immune system is part of the pathophysiology of osteoarthritis (Westacott and Sharif, 1996). Thus, there is increased synthesis of the key pro-inflammatory cytokines, TNFα and IL-1β, which induce inflammatory processes, including the induction, by chondrocytes, of matrix metalloproteinases (MMP), which, in turn, are responsible for connective tissue degeneration. Schulze-Tanzil and colleagues have already demonstrated that two standardized (35% and 65%) ethanol Hp extracts attenuated IL-1β-induced MMP activity in isolated primary (non-rheumatic) human chondrocytes (Schulze-Tanzil *et al.*, 2004).

The Hp extract also inhibited LPS-induced PGE₂ and COX-2 expression. A previously published report similarly described the inhibition by an aqueous extract of Hp of COX-2 induction and PGE₂ synthesis in the LPS-treated mouse fibroblast cell-line (L929) (Jang *et al.*, 2003). Also, Kundu and colleagues reported that a Hp extract inhibited phorbol ester-induced COX-2 gene expression in mouse skin (Kundu *et al.*, 2005).

One key intracellular promoter of inflammation is NF-κB which activates several pro-inflammatory genes (Calzado *et al.*, 2007). The present study did not find evidence that Hp inhibits NF-κB in our cellular models. In mouse fibroblasts, an extract of Hp, but not harpagoside, was shown to inhibit the activated NF-κB pathway (Jang *et al.*, 2003), whereas in human macrophage cell-lines harpagoside was reported to be affective on NF-κB activation (Huang *et al.*, 2006). As shown by our group, harpagoside and harpagide are not the constituents in our Hp preparation responsible for the inhibition of TNFα (Fiebich *et al.*, 2000). Differences between species or cell types might be responsible for these divergent findings. Methanol extracts of Hp have been shown to reduce the levels of two gene transcriptional proteins (AP-1 and CREB) that mediate the TPA-induced up-regulation of the COX-2 gene. This study also provided evidence that Hp inhibited the activation of the protein kinase, ERK, but did not activate NF-κB, which supports our findings (Kundu *et al.*, 2005). However, no evidence was found of an inhibition by Hp of the LPS-activated MAP kinase pathway. This difference might be due to the different extraction methods (ethanol versus methanol) and the different cell types used. It still remains to be elucidated at which point the Hp extract acts in the signal transduction pathway downstream of the LPS receptor (the toll-like receptor 4) leading to activation of AP-1. The protein kinase C (PKC) pathway and one or more of the PKC isoforms are interesting candidates and further studies are needed to elucidate the kinase that is the specific target of Hp.

In summary, this study demonstrates that a standardized extract of Hp (and sole active ingredient of Pascoe®-Agil) influences the synthesis and release of pro-inflammatory

factors by affecting intracellular signal transduction pathways such as the activation of the transcription factor AP-1.

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Conflict of Interest

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

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