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Antiinflammatory actions of cat's claw: the role of NF-κB

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SUMMARY

Background: *Uncaria tomentosa* is a vine commonly known as cat's claw or 'uña de gato' (UG) and is used in traditional Peruvian medicine for the treatment of a wide range of health problems, particularly digestive complaints and arthritis.
Purpose: The aim of this study was to determine the proposed anti-inflammatory properties of cat's claw. Specifically: (i) does a bark extract of cat's claw protect against oxidant-induced stress *in vitro*, and (ii) to determine if UG modifies transcriptionally regulated events.
Methods: Cell death was determined in two cell lines, RAW 264.7 and HT29 in response to peroxynitrite (PN, 300 μM). Gene expression of inducible nitric oxide synthase (iNOS) in HT29 cells, direct effects on nitric oxide and peroxynitrite levels, and activation of NF-κB in RAW 264.7 cells as influenced by UG were assessed. Chronic intestinal inflammation was induced in rats with indomethacin (7.5 mg/kg), with UG administered orally in the drinking water (5 mg/mL).
Results: The administration of UG (100 μg/mL) attenuated ($P < 0.05$) peroxynitrite-induced apoptosis in HT29 (epithelial) and RAW 264.7 cells (macrophage). Cat's claw inhibited lipopolysaccharide-induced iNOS gene expression, nitrite formation, cell death and inhibited the activation of NF-κB. Cat's claw markedly attenuated indomethacin-enteritis as evident by reduced myeloperoxidase activity, morphometric damage and liver metallothionein expression.
Conclusions: Cat's claw protects cells against oxidative stress and negated the activation of NF-κB. These studies provide a mechanistic evidence for the widely held belief that cat's claw is an effective anti-inflammatory agent.

INTRODUCTION

Among the numerous factors associated with chronic gut inflammation the enhanced production of oxidants and free radicals have become widely recognized as integral components of cell and tissue injury.[1] Agents which negate the production and/or effects of reactive metabolites of oxygen and nitrogen have displayed therapeutic benefits.[2–4] Endogenous antioxidants may be depleted during states of chronic inflammation, which may in part explain the therapeutic efficacy of mesalamine and glucocorticoids.[5, 6] Crohn's disease and ulcerative colitis are the two most common forms of inflammatory bowel disease. Although the aetiology of inflammatory bowel disease remains unclear, there is mounting evidence to suggest that oxidants, free radicals and bacterial flora may play a role in the pathogenesis of gut inflammation. Bacterial overgrowth has been associated with a range of inflammatory disorders of the gut.[7] Increased intestinal permeability to luminal contents (bacterial or dietary) may also promote mucosal inflammation as well as affecting systemic organs, particularly the liver.[8] During states of inflammation, LPS and cytokines have been reported to induce the synthesis of metallothionein in the liver.[9] Metallothioneins (MT) are sulfhydryl-rich proteins that

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bind heavy metals and oxidants and are considered as acute phase response proteins.[10]

Our current therapeutic approaches to gut inflammation remains inadequate. In developing countries many of the present therapeutic options are beyond the financial reach of the general population. For this reason we are evaluating traditional herbal remedies in gut inflammation.

In the present work we have used an aqueous extract from dried bark of cat's claw *Uncaria tomentosa* (Willd.) DC. Cat's claw is a plant belonging to the family Rubiaceae, commonly known as 'uña de gato'. It is a vine that grows wild in the Peruvian Amazon. The aqueous extract and decoctions of cat's claw are widely used in traditional Peruvian medicine for the treatment of gastritis, arthritis and as an anti-inflammatory.[11] Similarly, during the last 10 years, cat's claw in different forms (e.g. extracts, tablets and capsules) has been introduced in Europe to treat patients suffering from cancer and some viral diseases. In addition to the anti-inflammatory properties of cat's claw, its protective antimutagenic effects have also been demonstrated *in vitro* against photomutagenesis.[12] The extract contains a mixture of quinovic acid and glycosides as well as pentacyclic or tetracyclic of oxindole alkaloids such as pteropodine, isopteropodine, speciophylline, uncarine F, mitraphylline and isomitraphylline.[13, 14]

The purpose of this study was twofold: (i) to investigate whether the bark extract of cat's claw *Uncaria tomentosa* (Willd.) DC. is a cytoprotective agent *in vitro* against oxidant-induced stress in murine macrophages (RAW 264.7) and human intestinal epithelial cells (HT29), and (ii) to determine if the anti-inflammatory activity of cat's claw involved an inhibition of transcriptionally regulated genes.

MATERIALS AND METHODS

Materials

Unless otherwise stated, all chemicals were at least reagent grade and were obtained from Sigma Chemical Co. (St Louis, MO). All cellular reagents and culture media were from Gibco BRL (Gaithersburg, MD).

Plant material and aqueous extraction

The bark of cat's claw *Uncaria tomentosa* (Willd.) DC. was collected in Tingo María, Peru and identified by Eng. Raúl Araujo of the Universidad Nacional Agraria de la Selva. The extract of *Uncaria tomentosa* was prepared from the air-dried bark of cat's claw by boiling it in water (20 g/L) for 30 min and then leaving it at room temperature overnight. The extract was decanted and filtered at 10 μm. The cat's claw (UG) extract for the cell culture experiments was filtered at 0.2 μm and diluted to a final concentration of 5 mg/mL, and then refrigerated. For the *in vivo* studies the UG extract was filtered at 0.45 μm. The extract contains oxindole alkaloids such as pteropodine, isopteropodine, mitraphylline and isomitraphylline.[15, 16]

Cell culture

HT29 and RAW 264.7 cells were obtained from the American Type Culture Collection (Rockville, MD). Cells were grown in DMEM high glucose, 10% FCS and supplemented with 25 mM HEPES, pH 7.4; 4 mM L-glutamine; 40 μg/mL penicillin; 90 μg/mL streptomycin; 0.25 μg/mL fungizone and 1.2 g/L $NaHCO_3$. Cell cultures were maintained in a humidified 5% CO_2 incubator at 37 °C. Cells were plated at 1×10^6 cells/mL. Harvested cells were plated in six-well tissue culture plates and allowed to grow to confluence over 24 h before use.

Peroxynitrite synthesis

Peroxynitrite (PN) was synthesized by modifying a previously reported methodology.[17] Briefly, solutions of (a) 0.7 M $NaNO_2$, 0.7 M H_2O_2 and (b) 0.6 M HCl, were pumped using a syringe infusion pump (Harvard Apparatus, South Natick, MA) at 25 mL/min, into a Y-junction and mixed in a 2 mm-diameter by 0.5 cm silica tube. The mixture was collected in a beaker containing a 1.5-M KOH solution. To destroy the excess H_2O_2, the peroxynitrite solution was filtered in a column containing MnO_2 (4 g). The prepared solution contained 35–50 mM peroxynitrite, as determined by absorbance at 302 nm ($E_{302} = 1670$/M/cm).[18] A fresh working peroxynitrite solution (5 mM) was prepared in 5 mM KOH for each experiment and filtered at 0.2 μm.

Cell viability

Aliquots of treated cells were examined for viability as determined by trypan blue dye exclusion. Briefly, HT29 cells were detached with trypsin-EDTA and RAW 264.7

cells were scraped and washed with phosphate buffered saline (154 mM, NaCl; 2.7 mM, $Na_2HPO_4{\cdot}7H_2O$; 1.3 mM, KH_2PO_4), resuspended in medium and 0.4% trypan blue stain was added. After 5 min of incubation, the number of cells excluding the dye was expressed as a percentage of total cells counted from three randomly chambers of the haemocytometer.

Measurement of nitrite/nitrate

For these experiments, HT29 and RAW 264.7 cells were treated with lipopolysaccharide (LPS, 1 μg/mL) and/or UG (100 μg/mL) and incubated for 18 h and 12 h, respectively. The stable end products of nitric oxide nitrite/nitrate (NO_2^- and NO_3^-) were assayed in HT29 and RAW 264.7 cells using Griess reagent after the conversion of NO_3^- to NO_2^- with a colourimetric assay kit (Cayman Chemical Co., Ann Arbor, MI).

Detection of Apoptosis by ELISA

HT29 and RAW 264.7 cells were either treated with 300 μM PN and/or supplemented with cat's claw extract (UG, 100 μg/mL) and incubated for 4 h. Apoptosis (DNA fragmentation) was quantified using a cell death detection ELISA (Boehringer Mannheim, Indianapolis, IN) as previously described.[19]

Electrophoretic mobility shift assay (EMSA)

RAW 264.7 cells, at 1×10^6/well, were plated in six-well clusters and treated with LPS (1 μg/mL) and/or UG (50–100 μg/mL) and incubated at 37 °C. After 2 h, the medium covering the cells was removed and replaced with ice-cold phosphate-buffered saline. RAW 264.7 cells were harvested by scraping followed by centrifugation (1000 ***g***). Preparation of the nuclear protein extracts and EMSAs were carried out as previously reported.[20, 21] The protein concentration of the nuclear extracts was determined using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA). The binding reactions were carried out with 5 μg of protein for NF-κB per reaction. The consensus sequence of the NF-κB probe (the binding site is underlined) was 5′-AGTT-GAGGGGACTTTCCCAGGC-3′ (Promega, Madison, WI).

Evaluation of peroxynitrite scavenging by cat's claw

Diluted samples from the stock solution of peroxynitrite (40 mM) were used to prepare working solutions of 300 μM peroxynitrite containing 5 mM KOH (pH 12). The final volume was 1 mL, and the absorbance at 302 nm was determined or scanned from 200 to 400 nm at 1 and 10 min, respectively. A Beckman DU 64 spectrometer (Beckman Instruments Inc., Fullerton, CA) was used to assess the change in absorbance. In separate experiments, the absorbance of UG (100 μg/mL) diluted in 5 mM KOH or reacted with 300 μM peroxynitrite was scanned and the absorbance at 302 nm was determined.

Auto-oxidation of nitric oxide by cat's claw extract

To see if the nitric oxide reacted with the UG extract, the time-dependent depletion of 30 μM nitric oxide was monitored in two solutions: (i) phosphate solution (pH 7.4) containing 5 mg/mL UG extract, and (ii) phosphate solution without the UG extract. The microelectrode experiments were performed at 25 °C. The saturated stock solution contained 160 μM nitric oxide, as determined by electrochemistry (BAS 100 B/W, Bioanalytical Systems, West Lafayette, IN). The conditions for the electrochemical experiments have been reported previously.[22]

Analysis of iNOS gene expression by RT-PCR

HT29 cells 2×10^6 cells/well were seeded in six-well tissue culture plates and incubated with LPS (1 μg/mL) and/or UG (50–200 μg/mL). After 12 h, the total RNA was isolated from cells by the acid guanidine thiocyanate-phenol-chloroform extraction method.[23] The integrity of RNA was assessed on a 1.2% agarose gel and the RNA was visualized by ethidium bromide. First-strand complementary DNAs were synthesized from 1 μg of total RNA using oligo dT and Superscript II Reverse Transcriptase (Gibco BRL, Grand Island, NY). The first-strand complementary DNA templates were amplified for glyceraldehyde-3-phosphate dehydrogenase and iNOS by polymerase chain reaction (PCR) using a hot start (Ampliwax, Perkin Elmer, Foster City, CA). The primers for iNOS were as follows: forward 5′-TCG AAA CAA CAG GAA CCT ACC A-3′ (a 22-mer oligonucleotide at position 529) and reverse 5′-ACR GGG GTG ATG CTC CCA GAC A-3′ (a 22-mer oligonucleotide at position 1435), giving rise to a 907 base pair PCR product. These sequence data are available from GenBank under accession number D14051. The primers for the glyceraldehyde-3-phosphate dehydrogenase

(GADPH) used as an internal standard for rats were as follows: forward 5′-ATT CTA CCC ACG GCA AGT TCA ATG G-3′ and reverse 5′-AGG GGC GGA GAT GAT GAC CC-3′ (GENBANK accession number M17701). The PCR cycle was an initial step of 95 °C for 3 min, followed by 94 °C for 30 s, 60 °C for 45 s, 72 °C for 1 min, with 30 cycles and a final cycle of 72 °C for 4 min. The negative control was a cDNA reaction that used water instead of RNA. PCR products (iNOS 12 μL, GADPH 6 μL) equalized to give equivalent signals from the GADPH mRNA, were electrophoresed through 2% agarose gels (FMC, Rockland, ME) containing 0.2 μg/mL of ethidium bromide. Gels were visualized under UV light and photographed with Polaroid film (Polaroid Corporation, Cambridge, MA).

Indomethacin-induced intestinal inflammation

Animal models of nonsteroidal anti-inflammatory drug (NSAID) induced enteropathy are associated with changes in morphology, microvascular injury and changes in epithelial permeability.[24, 25] To evaluate the anti-inflammatory activity of cat's claw against indomethacin-induced intestinal injury, male Sprague–Dawley rats (225–250 g) were purchased from Harlan Sprague–Dawley (Indianapolis, IN) and housed in stainless-steel cages in an environmentally controlled room (25 °C, 12 h/12 h light/dark cycle). Food and water were supplied *ad libitum* for 5 days before the experiment. A chronic model of the intestinal inflammation was induced by two s.c. injections of indomethacin (7.5 mg/kg) daily at a 24-h interval as described by Yamada *et al.*[24] Animals were divided into four groups: (A) vehicle control group (5% $NaHCO_3$), (B) injected s.c. with indomethacin, (C) injected s.c. with indomethacin and supplemented with cat's claw (UG, 5 mg/mL) in the drinking water (indomethacin + UG), and (D) injected s.c. twice daily with vehicle and supplemented with cat's claw (UG, 5 mg/mL) in the drinking water (UG). The rats from groups A and B were given water, and all animals were fed a standard laboratory rat chow *ad libitum*.

Tissue myeloperoxidase activity

Tissue myeloperoxidase (MPO) was quantified as an index of neutrophil infiltration. Tissue samples were weighed, frozen on liquid nitrogen and then stored at −77 °C until assayed. Tissue levels of myeloperoxidase were determined by modifying a previously described technique.[26] Briefly, 0.2 g of midjejunum was homogenized for 45 s at 4 °C with a tissue homogenizer (Virtis, Gardiner, NY) in 2 mL of ice cold 0.5% hexadecyltrimethylammonium bromide (HTAB) in 50 mM potassium phosphate buffer, pH 6.0. The homogenate was then sonicated for 10 s, freeze-thawed three times, after which the sonication was repeated; suspensions were then centrifuged at 40 000 ***g*** for 15 min. Myeloperoxidase was measured spectrophotometrically: 50 μL of the supernatant was mixed with 1450 μL of 50 mM phosphate buffer, pH 6.0 containing 0.167 mg/mL o-dianisidine dihydrochloride and 0.0005% H_2O_2. The change in absorbance at 460 nm was measured with a Beckman DU 64 spectrophotometer (Beckman Instruments, Fullerton, CA).

Determination of intestinal damage

A group of rats was used for a comparison of morphologic studies. At the end of 7 days, rats were anaesthetized and samples of the midjejunum were taken. The tissue was fixed in phosphate-buffered formaldehyde, embedded in paraffin, and 5-μm sections were prepared. The tissue was routinely stained with haematoxylin and eosin and evaluated by light microscopy.

Metallothionein protein assay

After 7 days in the study, animals were sacrificed and samples of liver and intestinal mucosal cells were collected for metallothionein (MT) protein. The metallothionein concentration of cytosolic fractions of the liver and intestinal cells was determined by ^{109}Cd-Hb affinity assay, as previously described.[27]

Statistical analysis

Each experiment was performed at least three times and results are presented as the mean ± S.E.M.. Statistical analyses were performed using one-way ANOVA. *Post hoc* comparison of means was carried out by a least significant difference test. A probability of < 0.05 was considered significant.

RESULTS

Assessment of viability and apoptosis in cell lines

Experiments to examine the cytotoxic effects of peroxynitrite (300 μM, for 4 h), with and without cat's claw

Table 1. Cell viability in HT29 and RAW 264.7 cells treated with cat's claw extract*

Group	Viability (%)	
	HT29	RAW 264.7
Control	94.5 ± 0.8	96.2 ± 0.9
UG, 25 μg/mL	98.2 ± 0.3	93.2 ± 1.1
UG, 50 μg/mL	98.3 ± 0.3	94.5 ± 1.8
UG, 100 μg/mL	93.4 ± 0.6	96.7 ± 0.5
UG, 200 μg/mL	94.2 ± 1.2	92.8 ± 1.5

*Cells 1×10^6/well were seeded in six-well clusters and incubated overnight at 37 °C.

A fresh extract of cat's claw (UG) was prepared for each experiment and filtered at 0.2 μm. Values are the mean ± S.E.M. of three experiments, each with three samples.

(UG, 100 μg/mL) were conducted to delineate the protective effect of UG extract. Cell viability was not affected by the experimental conditions (Table 1). However, the results indicated that HT29 and RAW 264.7 cells receiving 300 μM peroxynitrite showed a significant increase ($P < 0.05$) in cytosolic DNA fragments compared to their decomposed/peroxynitrite (DC/PN) control group. In both cell lines, the simultaneous administration of 100 μg/mL UG extract and peroxynitrite resulted in a significant ($P < 0.05$) reduction in apoptosis (Figures 1 and 2). Table 2 shows the cytoprotective effect of the UG extract in RAW 264.7 cells treated with LPS (1 μg/mL). Similarly,

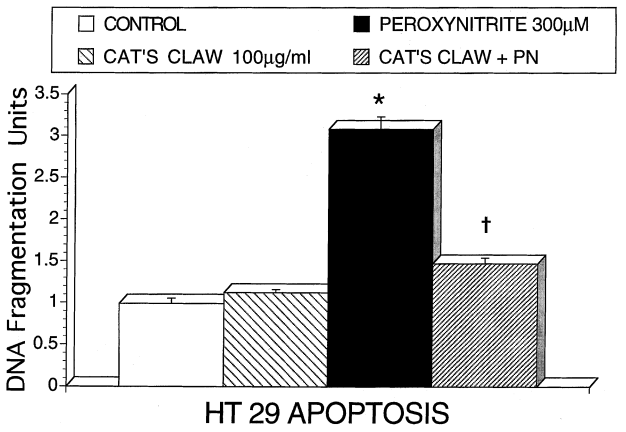

Figure 1. Apoptosis in HT29 cells after treatment with peroxynitrite (PN, 300 μM) and/or simultaneous administration of cat's claw extract (UG, 100 μg/mL). Cells were seeded at a density of 1×10^6 cells/well. Bars represent enrichment of cytosolic DNA fragments of cells incubated for 4 h. Values represent mean ± S.E.M. from three samples. *Significant increase ($P < 0.05$) in apoptosis compared with DC/PN; †significant decrease ($P < 0.05$) in apoptosis compared to peroxynitrite.

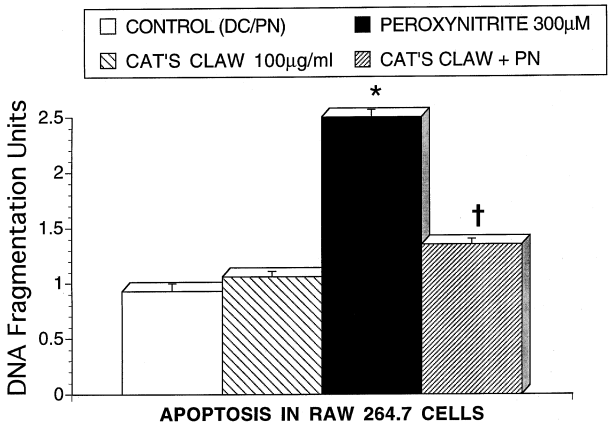

Figure 2. Apoptosis in RAW 264.7 cells after treatment with peroxynitrite (PN, 300 μM) and/or simultaneous administration of cat's claw extract (UG, 100 μg/mL). Cells were plated at a density of 1×10^6 cells/well. Bars represent enrichment of cytosolic DNA fragments of cells incubated for 4 h. Values represent mean ± S.E.M. from three samples. *Significant increase ($P < 0.05$) in apoptosis compared with DC/PN; †significant decrease ($P < 0.05$) in apoptosis compared to peroxynitrite.

the concentration of nuclear protein was greater ($P < 0.05$) in cells treated simultaneously with LPS and UG than in cells exposed to LPS alone.

Levels of nitrite/nitrate

HT29 cells treated with LPS produced higher ($P < 0.05$) levels of NO_2^-/NO_3^- than cells simultaneously treated with LPS and UG extract (Figure 3). In another set of experiments with RAW 264.7 cells, the simultaneous administration of cat's claw and LPS caused a significant ($P < 0.05$) inhibition of 60% nitrite formation (Figure 4).

Table 2. Cytoprotective effect of cat's claw in RAW 264.7 cells against oxidative stress induced by lipopolisaccharide*

Group	Cell viability (%)	Nuclear protein (μg/mL)
Control	95.9 ± 0.9	172 ± 1.6
UG, 100 μg/mL	96.7 ± 0.6	162 ± 3.0
LPS, 1 μg/mL	80.1 ± 1.8[a]	115 ± 3.2[a]
LPS + UG	87.0 ± 0.7[b]	150 ± 2.5[b]

*Cells 1×10^6/well were plated in six-well clusters and incubated overnight at 37 °C. Values represent mean ± S.E.M. Data are from three separate experiments, each performed in triplicate. [a]Significant decrease ($P < 0.05$) compared to control and LPS + UG; [b]significant increase ($P < 0.05$) compared to LPS.

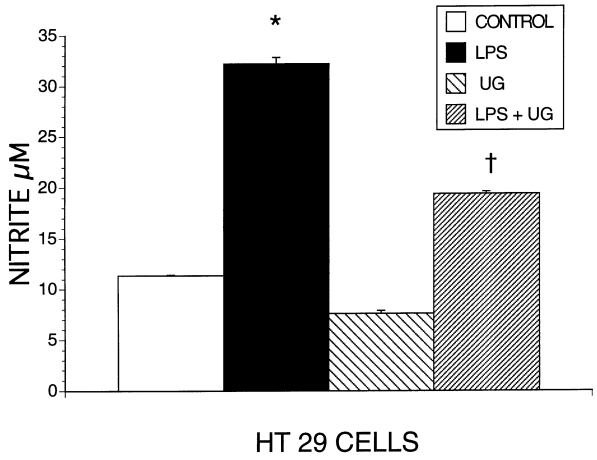

Figure 3. Effect of cat's claw extract on LPS-mediated nitrite formation. HT29 cells 1×10^6 cells/well were incubated for 18 h. In all experiments LPS (1 μg/mL) and/or UG (100 μg/mL) was simultaneously added to the culture medium. Nitrite was quantified as described in Materials and Methods. All data represent means ± S.E.M. for triplicate determinations from three different experiments. * $P < 0.05$ compared to all other groups; †significant decrease ($P < 0.05$) in nitrite formation compared with LPS.

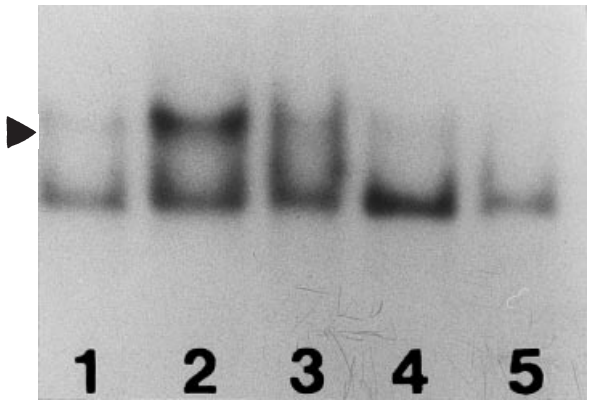

Figure 5. Effect of cat's claw extract (UG) on the activation of NF-κB by LPS in RAW 264.7 cells. Cells were treated with the indicated concentrations of LPS and/or UG and incubated for 2 h. Nuclear extracts were analysed by EMSA, as described in Materials and Methods. The filled arrowhead indicates the position of a NF-κB–DNA complex. Lane 1 control; lane 2 LPS (1 μg/mL); lane 3 LPS + UG, 50 μg/mL; lane 4 LPS + UG, 100 μg/mL; lane 5 (UG, 100 μg/mL). Only the shifted bands are shown.

Inhibition of NF-κB activation by cat's claw extract

Figure 5 shows the effect of LPS (1 μg/mL) and UG extract on NF-κB activation in RAW 264.7 cells. In the presence of LPS as a source of oxidative stress, the activation of NF-κB was markedly enhanced, consistent with previous reports.[28] On the other hand, RAW 264.7 cells treated with LPS and UG extract (100 μg/mL) caused an inhibition of NF-κB.

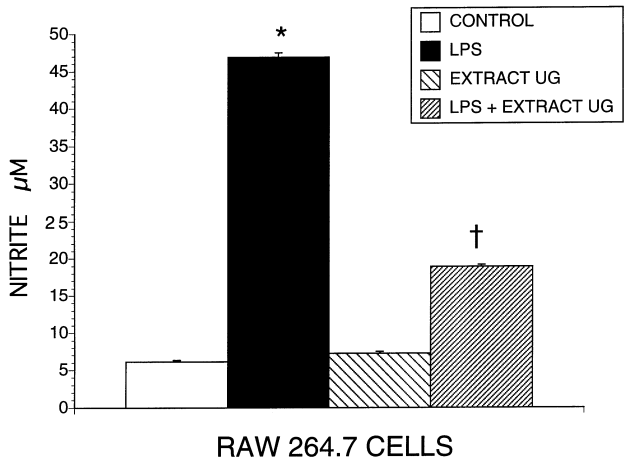

Figure 4. Effect of cat's claw extract on LPS-mediated nitrite formation. RAW 264.7 cells 1×10^6 cells/well were incubated for 12 h. In all experiments LPS (1 μg/mL) and/or UG (100 μg/mL) was simultaneously added to the culture medium. Nitrite was quantified as described in Materials and Methods. All data represent means ± S.E.M. for triplicate determinations from three different experiments. * $P < 0.05$ compared to all other groups; †significant decrease ($P < 0.05$) in nitrite formation compared with LPS.

Regulation of iNOS mRNA in HT29 cells induced by LPS

Figure 6 shows the levels of iNOS mRNA expression from HT29 cells treated with LPS (1 μg/mL) and/or UG extract (50–200 μg/mL). The expression of iNOS mRNA was increased in LPS treated cells, as was evident after 12 h incubation. However, the simultaneous administration of UG extract and LPS significantly decreased the levels of iNOS mRNA. While the expression of the house-keeping gene, GAPDH, was variable in this gel, the reduction in iNOS gene expression with UG was evident, and was supported by the reduced production of nitrite/nitrate (Figures 3 and 4).

Scavenging of oxidants by cat's claw extract

The decomposition of peroxynitrite (pH 12) in the presence and absence of cat's claw (UG extract) was monitored spectrophotometrically at 302 nm. The addition of UG extract (100 μg/mL) to peroxynitrite-containing 5 mM KOH resulted in a significant ($P < 0.05$) decrease in peroxynitrite concentration (Figure 7). The decomposition of peroxynitrite was

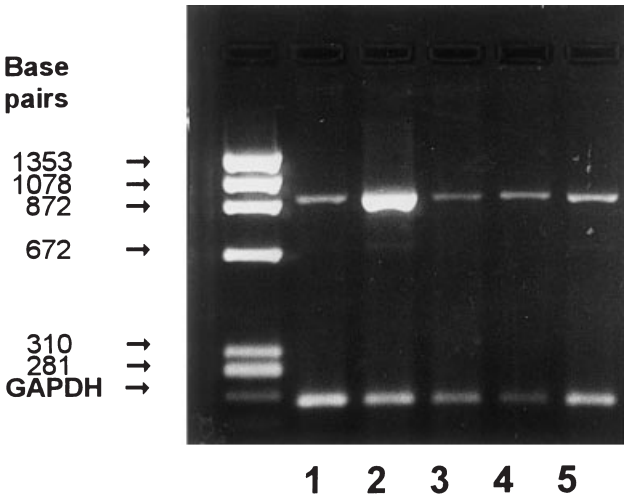

Figure 6. Expression of iNOS mRNA in HT29 cells treated with LPS and/or cat's claw extract. A representative 2% agarose gel RT-PCR products detected. Base pair markers denoting DNA size are shown in the far left lane. Reaction products for GADPH (internal marker) and iNOS RT-PCR (906 base pair product) are shown for control (1); LPS, 1 μg/mL (2); LPS + UG, 50 μg/mL (3); LPS + UG, 100 μg/mL (4); LPS + UG, 200 μg/mL (5).

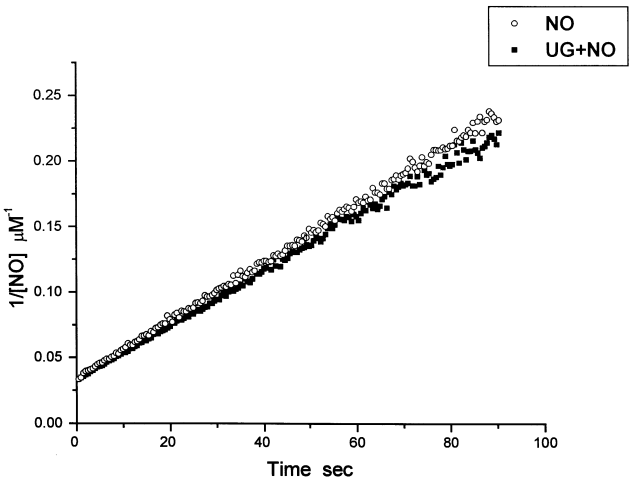

Figure 8. Auto-oxidation of 30 μM nitric oxide (NO) in PBS in the absence or presence of cat's claw extract (UG, 5 mg/mL) pH 7.4 at 25 °C. Open circles indicates NO concentration degradation. Closed squares (UG + NO) degradation of NO in the presence of cat's claw extract. Nitric oxide was recorded continuously as described in Materials and Methods. Slope of the two lines indicates that half-life of NO was not affected significantly. Data are from three experiments, each performed in triplicates.

evaluated at pH 12 because peroxynitrite degrades rapidly at pH 7.4. The absorbance of the UG extract at pH 12 did not vary during the time it was evaluated (10 min). Because of the time constraints encountered for quantifying PN at pH 7.4 (peroxynitrite has a very short half-life at physiological pH) we elected to follow the depletion of the UG extract absorbance determined at 245 nm. As expected, absorbance of the UG extract was reduced by the presence of peroxynitrite ($P < 0.05$), suggesting a decomposition or consumption of the oxindole alkaloids present in the UG extract by peroxynitrite. Figure 8 shows the *in vitro* reaction of NO, 30 μM with UG, 5 mg/mL and from these results it was clear the half-life of nitric oxide was not affected significantly.

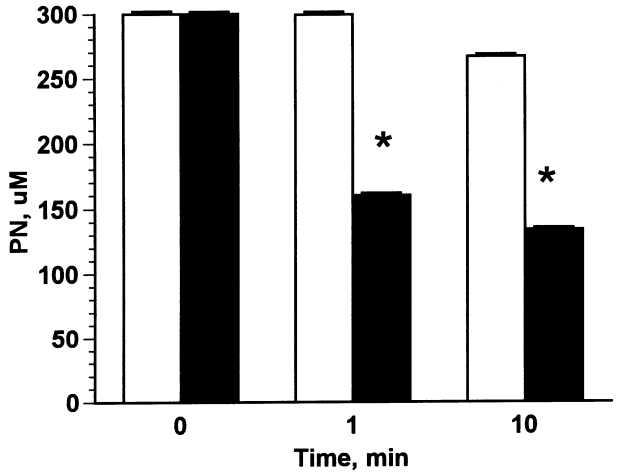

Figure 7. Peroxynitrite decomposition in the presence of cat's claw extract. In this study peroxynitrite (PN, 300 μM) was mixed with UG (100 μg/mL), pH 12. Open bars represent decomposition of peroxynitrite *per se*. Solid bars represent PN + UG. The change in peroxynitrite concentration was determined by measuring the change in absorbance at 302 nm. Values are the mean ± S.E.M. of three experiments, each with three samples. *Significant decrease ($P < 0.05$) in peroxynitrite concentration compared with peroxynitrite.

Assessment of indomethacin-induced intestinal inflammation

Rats treated with two daily s.c. injections of indomethacin produced mucosal ulcerations on the mesenteric side of the mid-small intestine, and numerous white nodules located along the serosal side of the intestine were also observed by day 7 following the injection. The degree of inflammation, in the midjejunum, was associated with significant increase of MPO in this section of the gastrointestinal tract (Figure 9). Histological sections of the midjejunum of rats that received indomethacin (7.5 mg/kg) showed a

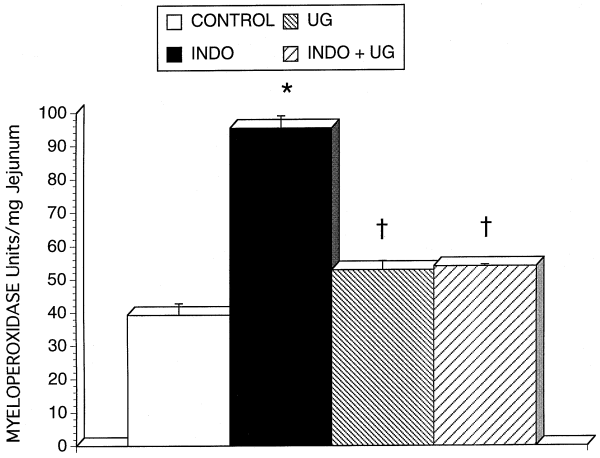

Figure 9. Effect of cat's claw on myeloperoxidase activity after induction of intestinal inflammation with indomethacin. Rats received two injections of either indomethacin or vehicle as described in Materials and Methods. Bars represent MPO activity in control, indomethacin treated (indomethacin), cat's claw in the drinking water (UG), and indomethacin treated + UG (indomethacin + UG). * Significant increase ($P < 0.05$) compared to control; †significant decrease ($P < 0.05$) in MPO activity compared to indomethacin. Values are means ± S.E.M. for three rats/group from two different experiments.

pronounced disruption of the mucosal architecture, with loss of villi and a pronounced inflammatory cell infiltrate. On the other hand, rats receiving UG (5 mg/mL in the drinking water, Figure 10) had a normal mucosal architecture.

Table 3 shows the hepatic metallothionein concentration, an index of inflammation. Metallothionein was

Table 3. Effect of chronic intestinal inflammation on metallothionein protein concentration in liver and intestine of rats*

Group	Liver MT† μg/g tissue	Jejunum MT† μg/g tissue
Control	30.5 ± 0.8	23.1 ± 3.7
UG, 5 mg/mL	38.0 ± 4.1	18.4 ± 4.8
Indomethacin, 7.5 mg/kg	216.6 ± 35[b]	20.0 ± 4.5
Indomethacin + UG	69.6 ± 19[a]	22.9 ± 4.3

*Chronic intestinal inflammation was induced with indomethacin as described in Materials and Methods. The cat's claw extract (UG) was administered in the drinking water. Data from two experiments ($n = 6$).

†Metallothionein (MT) levels represent mean ± S.E.M. measured by ^{109}Cd-Hb affinity assay 7 days after induction of intestinal inflammation with indomethacin. [a]Significant decrease ($P < 0.05$) in liver MT compared to indomethacin; [b]significant increase ($P < 0.05$) in liver MT compared to all other groups.

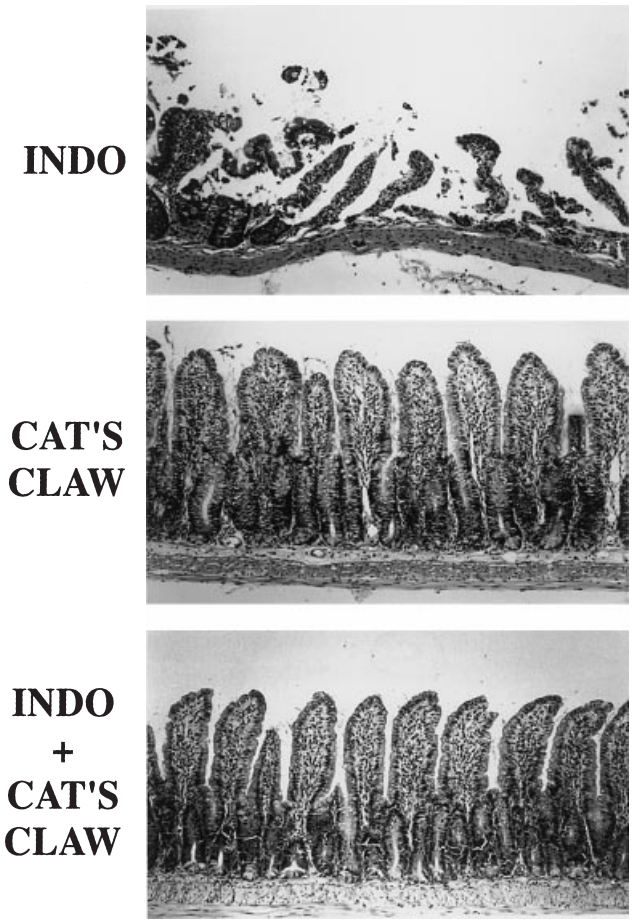

Figure 10. Histological sections of the midjejunum after induction of intestinal inflammation with indomethacin. Upper panel: Treated with indomethacin (INDO), vehicle + cat's claw in the drinking water (CAT'S CLAW), and indomethacin + drinking cat's claw (INDO + CAT'S CLAW). Tissues were fixed in paraffin, microtoned, and stained with haematoxylin and eosin.

increased ($P < 0.05$) in rats that received indomethacin after 7 days compared to control rats. Administration of UG (5 mg/mL) in the drinking water to rats treated with indomethacin resulted in lower ($P < 0.05$) liver metallothionein. In contrast to the liver metallothionein, either indomethacin or the UG extract did not affect the content of intestinal metallothionein. The induction of metallothionein synthesis in the intestine is not inflammation dependent but rather occurs in response to trace metals such as Zn and Cu.[29]

DISCUSSION

Inflammatory disorders are characterized by an excessive production of free radicals and reactive oxygen and

nitrogen species. Currently used therapeutics often modify the actions or production of the reactive species, and in so doing reduce the degree of tissue injury.[30] However, for the developing world, access to these therapeutic agents may be limited due to financial constraints. These populations tend to use traditional medicines, often of plant origin, for the therapeutic management of disease. While the Amazon river basin has proven to be a rich source of valuable pharmacological agents, a great deal of potential for ethnomedically driven drug discovery still remains. In this study we have evaluated the potential benefits of a widely used herbal medicine, cat's claw. An aqueous extract of the cat's claw bark negated the cellular toxicity associated with peroxynitrite, a powerful oxidant formed from the interaction of nitric oxide and superoxide (the radical precursors to a family of potent, reactive species). These results demonstrated that the aqueous extract of cat's claw elicited similar beneficial effects as an antioxidant, consistent with previous findings using a bark methanol extract.[31] In addition, cell death induced by bacterial endotoxin was attenuated by cat's claw.

To date there have been few studies evaluating the mechanisms for the proposed beneficial effects of cat's claw. Here we have defined the potential loci. Firstly, cat's claw directly degrades peroxynitrite and attenuates peroxynitrite-induced cell death, similar to that which we have recently described for mesalamine.[22] Mesalamine does not modify nitric oxide oxidative degradation, implying that its anti-inflammatory properties are not mediated by direct effects on nitric oxide, a relatively weak free radical.[22] Rather, peroxynitrite, which is highly reactive, is a site of action. Similar results were noted with cat's claw. However, cat's claw slowed the rate of oxidative degradation of nitric oxide, while directly degrading peroxynitrite. This is a pattern of effects that we have seen also with ascorbic acid.[32]

We and others have demonstrated the contributions of reactive nitrogen oxides to inflammatory bowel disease and gastritis.[33, 34] Indeed these species may be critical components in the development of gastritis and gastric cancer in response to *Helicobacter pylori* infection which is endemic in South America.[35, 36]

The second mechanism by which cat's claw may afford benefit appears to be unique amongst natural products. Cat's claw, by preventing the activation of the transcriptional factor NF-κB, inhibits the expression of inducible genes associated with inflammation, specifically, cat's claw negated the expression of inducible nitric oxide synthase, thereby attenuating nitric oxide production. Together with the direct degradation of peroxynitrite once it is formed, the cytotoxic consequences of this pathway will be greatly diminished. Other pro-inflammatory pathways regulated by NF-κB, but not investigated directly here (cytokines, adhesion molecules) should also be modified.

Suppression in the liver by cat's claw of the indomethacin-induced acute phase response protein, metallothionein, demonstrates that these transcription-dependent responses are registered *in vivo* as well as *in vitro*. Metallothionein levels in the intestine were not altered by indomethacin-induced inflammation, nor by cat's claw. This is not an unexpected finding, as intestinal metallothionein is regulated almost exclusively by heavy metals, whereas the hepatic expression of metallothionein is also influenced by the cytokines released in states of inflammation.[37] Glucocorticoids can negate the induction of iNOS expression through transcriptional mechanisms, e.g. by the inhibition of NF-κB,[38] as seen in this study with cat's claw. As NF-κB controls the expression of a wide range of pro-inflammatory signals, including the adhesion molecules and cytokines which were not evaluated here,[39] it is reasonable to assume that the anti-inflammatory effects of cat's claw involves a generalized reduction in pro-inflammatory mediators and effectors.

The anti-inflammatory actions of cat's claw were registered at doses that are consistent with the practice of traditional medicine. Indeed, rats evaluated in the indomethacin enteritis model were treated with a 'tea' made from cat's claw prepared in a manner that was identical to the ethno-medical use of cat's claw in Peru and neighbouring regions. This oral administration of cat's claw 'tea' had an impressive protective effect on indomethacin-induced enteritis in rats; normalizing mucosal architecture and attenuating granulocyte infiltration. This tea has a palatable taste and is widely consumed in South America, and is becoming quite accessible in North America. Anecdotal reports have indicated that it is useful in the treatment of refractory gut inflammation. It is also important to note that the beneficial effects observed in the present study were at doses that did not compromise cellular function or viability. Thus there was no suggestion of toxicity.

There have been reports that the active ingredients of cat's claw may be subject to regional and seasonal variability.[15] In addition, there are differences between

the use of bark and roots.[40] However, it is also appreciated that the proposed active ingredients cannot account for the known efficacy of cat's claw.[41] Thus, it is not clear if the present report of antioxidant properties and transcriptional inhibition with this Peruvian extract of cat's claw are due to the proposed active ingredients or to novel chemical entities. The possibility that novel chemical structures participate in these anti-inflammatory effects warrants a continued evaluation of this herbal medicine. However, beyond the search for new chemical leads, this study offers definitive evidence that the anecdotal reports of anti-inflammatory properties of cat's claw has a basis in fact and are sufficiently diverse to be considered an important therapeutic entity. Cat's claw is available in most Western countries and further research in other models of inflammation (gastrointestinal and systemic) including clinical studies, should be evaluated. For developing countries, where health care funds are stretched, herbal medicines like cat's claw deserve serious consideration.

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