

## Anti-inflammatory and antioxidant activities of cat's claw (*Uncaria tomentosa* and *Uncaria guianensis*) are independent of their alkaloid content

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### Summary

Cat's claw is an herbal medicine from the Amazon that is used widely to treat inflammatory disorders. The purpose of this study was to characterize the antioxidative and antiinflammatory properties of cat's claw, *Uncaria tomentosa* (UT) and *Uncaria guianensis* (UG). Alkaloids and flavanols were determined using reversed-phase HPLC; scavenging of 1,1-diphenyl-2-picrylhydrazyl (DPPH), hydroxyl radicals, and lipid peroxidation by spectrophotometry; and TNF $\alpha$  production by ELISA. Anti-inflammatory activity was assessed *in vitro* by inhibition of TNF $\alpha$  and nitrite production from RAW 264.7 cells exposed to LPS (50 ng/ml) and *in vivo* using the indomethacin-induced gastritis model. Apoptosis was assessed using the TUNEL technique and TNF $\alpha$  mRNA by *in situ* RT-PCR. In each of the antioxidant assays tested, UG was more potent than UT ( $P < 0.01$ ). The total oxindole and pentacyclic alkaloid content of UT was 35-fold  $>$  UG. The IC<sub>50</sub> value for inhibition of TNF $\alpha$  production was significantly ( $P < 0.01$ ) higher for UT (14.1 ng/ml) vs UG (9.5 ng/ml), yet at concentrations that were considerable lower than that required for antioxidant activity. Non-alkaloid HPLC fractions from UT decreased LPS-induced TNF $\alpha$  and nitrite production in RAW 264.7 cells ( $P < 0.01$ ) at a concentration range comparable to the parent botanical. Oral pre-treatment for 3 d with UT protected against indomethacin-induced gastritis, and prevented TNF $\alpha$  mRNA expression and apoptosis. These results indicate that while both species of cat's claw provide effective antioxidant and anti-inflammatory activities, *U. guianensis* is more potent. In conclusion, the presence of oxindole or pentacyclic alkaloids did not influence the antioxidant and anti-inflammatory properties of cat's claw.

**Key words:** Cat's claw, antioxidant, inflammation, TNF $\alpha$ , free radical, gastritis, NSAID.

### ■ Introduction

Interest in using complementary medicine to alleviate inflammatory conditions and improve health conditions is increasing in developed countries (Marshall, 2000). New medicinal plants from different parts of the world are being investigated with this purpose in mind (Budzinski et al., 2000). Although the utilization of

botanicals has increased in the Western world, there is a lack of information about mechanisms of action and potential differences among species within the same genus. To fill this need, we have established a research program focusing on medicinal plants from the Amazon, and we selected cat's claw, or uña de gato, because

of its wide use. Cat's claw is a vine that grows very rapidly in the Peruvian Amazon and has been used in traditional medicine to alleviate inflammation.

There are two prevalent species of cat's claw (*Uncaria tomentosa* and *Uncaria guianensis*), and they are used interchangeably in traditional medicine in South America for their anti-inflammatory properties. However, there is a lack of scientific data comparing the efficacy of the species *U. guianensis* with that of *U. tomentosa*. Ethnomedically, the bark and root of cat's claw are the parts of the plant that are most frequently used, and are prepared as an aqueous extraction in hot water (Obregon, 1995). Several groups have reported the presence of a wide range of chemical constituents in cat's claw (Aquino et al., 1991; Senatore et al., 1989; Stupper et al., 1992; Laus et al., 1997), although few studies have demonstrated that administration of these isolated components exerts consistent anti-inflammatory effect. Hence, cat's claw's biological value has been attributed to a variety of components (Aquino et al., 1989, 1990; Cerri et al., 1988).

Based on *in vitro* experiments, it has been indicated that oxindole alkaloids promote phagocytosis, leading to the claim that cat's claw has immunostimulant properties (Wagner et al., 1985), and also the ability to induce a lymphocyte-proliferation-regulating factor in endothelial cells (Wurm, et al., 1998). However, these actions are difficult to reconcile with the use of cat's claw to treat chronic inflammation (Sandoval-Chacón et al., 1998; Piscocoya et al., 2001). Hence, we have continued with our efforts to evaluate alternative explanations of the mechanisms of action of cat's claw.

There are very few scientific publications on *U. guianensis*, and those which exist provide evidence only for its quinovic acid glycoside and alkaloid content (Lee et al., 1999; Yepez et al., 1991). However, *U. guianensis* is also well-used in traditional medicine in tropical South America for the treatment of arthritis and as an anti-inflammatory agent (Piscocoya et al., 2001). Currently, standardization of commercial formulations of cat's claw (*U. tomentosa*) is based on their alkaloid content. Partly as a result of this practice *U. tomentosa* is the preferred species sold in the Western world because of its higher alkaloid content and ease of standardization. In contrast, *U. guianensis* is used interchangeably with *U. tomentosa* in the Amazon, but has a low alkaloid content. As there is a paucity of information on the biological properties of *U. guianensis*, we decided to compare the biological actions of these two species of cat's claw.

We have reported the first *in vitro* evidence that cat's claw is cytoprotective against potent oxidants such as peroxynitrite. In experimental models of intestinal inflammation, cat's claw appears to be effective in preventing the side-effects of non-steroidal anti-inflam-

matory drugs, specifically indomethacin. It is an effective inhibitor of inducible nitric oxide synthase (iNOS) gene expression through suppression of the activation of the nuclear transcription factor kappa beta (NF- $\kappa$ B) (Sandoval-Chacón et al., 1998). Recently, we have reported that cat's claw (*U. tomentosa*) is an effective antioxidant against the free radical DPPH, but more importantly a remarkable inhibitor of TNF $\alpha$  production. These data suggest that one of the mechanisms of action of cat's claw appears to be immunomodulation via suppression of TNF $\alpha$  (Sandoval et al., 2000). It is this collage of activity that formed the basis of the comparisons between the subspecies in this investigation.

## ■ Material and methods

### Chemicals

Methanol, acetonitrile (HPLC grade), 1,1-diphenyl-2-picrylhydrazyl (DPPH), molecular biology-grade reagents, flavanols (catechin, epigallocatechin, epicatechin and epigallocatechin gallate), and Polyphenon 60 were purchased from Sigma Chemical Co. (St. Louis, MO). Unless indicated, all cellular reagents and culture medium were from Life Technologies (Grand Island, NY). Cat's claw standards, Mitraphylline and Uncarine E, were purchased from ChromaDex, Inc. (Laguna Hills, CA).

### Plant material

The bark of the two cat's claw species, *Uncaria tomentosa* (UT) and *Uncaria guianensis* (UG), was collected from the experimental plots of Universidad Nacional Agraria de la Selva, Tingo Maria, Peru. An aqueous extract of the bark of each species was prepared using boiling water (50g/l w/v) for 30 min. The decanted and total solids were separated by filtration with a Whatman N° 4 filter paper. The filtrate was then freeze-dried using a Freezemobile 6 concentrator (Virtis, Gardiner, NY). For all cell culture experiments we used the freeze-dried cat's claw (UT and UG) dissolved in water (20 mg/ml) and then filtered at 0.2  $\mu$ m.

### Cell culture

RAW 264.7 cells were obtained from the American Type Culture Collection (Rockville, MD). The cells were grown in DMEM (high glucose), 10% FCS and supplemented with 25 mM HEPES, pH 7.4; 4 mM L-glutamine; 40 mg/ml penicillin; 90 mg/ml streptomycin and 1.2 g/l NaHCO<sub>3</sub>. Cell cultures were maintained in a humidified 5% CO<sub>2</sub> incubator at 37 °C. Harvested cells were plated in 96-well microplates (5 × 10<sup>4</sup> cells/well) and allowed to grow to confluence over 24 h before use.

**Scavenging effect on DPPH radical**

The DPPH radical scavenging capacity was assessed as reported previously (Sandoval et al., 2000) but modified as follows. The aqueous extract of the freeze-dried cat's claw (UT and UG) was standardized to give a stock solution (25 mg/ml), and filtered at 20 µm using a Whatman paper N° 4. Aliquots (25-µl) of either extract were placed in a cuvette and an ethanolic solution of DPPH (60 µM) was added to a final volume of 1 ml. The decrease in absorbance at 515 nm was determined continuously with data capturing at 30-sec intervals using a UV-1601 PC spectrophotometer (Shimadzu Corporation, Japan). The degree of DPPH radical scavenging activity of cat's claw was calculated as percentage of inhibition (% inhibition) by the following expression: % inhibition =  $[(A_{\text{control}} - A_{\text{sample}}) / A_{\text{control}}] \times 100$  where  $A_{\text{control}}$  is the absorbance at time = 0, and  $A_{\text{sample}}$  is the absorbance of the sample at time = 5 min.

**Scavenging effect on ABTS-radicals**

Experiments were conducted in order to measure the total radical-trapping antioxidant potential (TRAP) of cat's claw (UT and UG) by means of 2,2'-azobis(2-amidinopropane)HCl (ABAP)/2,2'-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS). The ABAP/ABTS assay is based on the scavenging of light absorbance ABTS-radicals. The relatively stable ABTS-radical has a green color and is quantified spectrophotometrically at 734 nm. The method described previously (Van Overveld et al., 2000) was modified as follows. A stock solution containing 0.225 mM ABTS, 2 mM ABAP in PBS buffer (50 mM phosphate, 0.9% NaCl, pH 7.4) was prepared and incubated at 70 °C for 20 min then cooled on ice. Inhibition of ABTS-radicals was determined by adding 10 µl of cat's claw (UT and UG) dissolved in water to 990 µl of the stock solution of ABTS-radicals at 37 °C. The decrease in absorbance at 734 nm was determined continuously with data capturing at 30-sec intervals using a DU-640 spectrophotometer (Beckman Instruments, Fullerton, CA). The degree of ABTS-radicals scavenging was calculated as percentage of inhibition (% inhibition) by the following expression: % inhibition =  $[(A_{\text{control}} - A_{\text{sample}}) / A_{\text{control}}] \times 100$  where  $A_{\text{control}}$  is the absorbance at time = 0, and  $A_{\text{sample}}$  is the absorbance of the sample at time = 30 sec.

**Deoxyribose assay for OH scavenging activity**

To further evaluate the antioxidant activity of cat's claw (UT and UG) we assessed deoxyribose protection against hydroxyl radicals (OH) generated by reacting Fe<sup>3+</sup>-EDTA, ascorbic acid and H<sub>2</sub>O<sub>2</sub> (Halliwell et al., 1987). Briefly, the reaction mixtures contained, in a final volume of 1 ml, the following reagents: deoxyribose (2 mM), KH<sub>2</sub>PO<sub>4</sub>-KOH buffer, pH 7.4 (20 mM), FeCl<sub>3</sub> (100 µM), ascorbate (100 µM), and cat's claw

(variable concentrations). Formation of malonaldehyde (MDA) as a pink MDA thiobarbituric acid (MDA-TBA) chromogen was quantified at 532 nm using a Beckman Coulter DU-640 spectrophotometer (Beckman Instruments, Fullerton, CA).

**Lipid peroxidation in mouse brain homogenates**

The capacity of cat's claw (UT and UG) to inhibit lipid peroxidation was determined by measuring the quantity of thiobarbituric acid-reactive substances (TBARS) (Keum et al., 2000). Brain homogenates from young adult male C57 BL6 mouse (The Jackson Laboratory, Bar Harbor, ME) were prepared in 0.15 M KCl (10% w/v) and centrifuged at 1000 × g for 10 min, and the supernatant was used for *in vitro* lipid peroxidation assays. The incubation mixture, in a final volume of 1 ml, contained brain homogenate (0.5 ml), 0.15 M KCl, and varying amounts of cat's claw (UT and UG). Lipid peroxidation was initiated by addition of ferric chloride (100 µM) alone or in combination with ascorbic acid (100 µM). After incubation for 20 min at 37 °C the reaction was terminated by addition of 0.5 ml cold trichloroacetic acid (5.5%) and an equal volume of thiobarbituric acid (TBA, 2% in 0.05 M NaOH), followed by heating at 100 °C for 10 min. The mixtures were then centrifuged at 1000 × g for 10 min and the absorbance of the supernatant was measured at 532 nm using a Beckman Coulter DU-640 spectrophotometer (Beckman Instruments, Fullerton, CA).

**Nitrite measurements**

Experiments were conducted to evaluate whether nitric oxide production is decreased by the non-alkaloid HPLC fractions of *Uncaria tomentosa* separated from the most polar peak, which were given to RAW 264.7 cells treated with lipopolysaccharide (LPS). RAW 264.7 cells were either pretreated with non-alkaloid fractions of UT (0.1–100 ng/ml) for 2 h and/or treated with 50 ng/ml for 1 h, the media replaced, and cells incubated for 16 h at 37 °C. Nitric oxide release was determined spectrophotometrically by the accumulation of nitrite in the medium (phenol red free) with Griess reagent as described previously (Sandoval et al., 2000).

**TNFα production**

The capacity of freeze-dried cat's claw (UT and UG) to inhibit TNFα production *in vitro* was determined by stimulating RAW 264.7 cells with lipopolysaccharide (LPS). Cells were either pretreated with freeze-dried cat's claw (0.001–1 µg/ml) for two hours and/or treated with LPS (0.5 µg/ml) for 1 h, the media replaced, and cells incubated for 16 h at 37 °C overnight. Culture medium was collected for determination of TNFα levels using the Quantikine M mouse TNFα Immunoassay kit (R & D Systems, Inc., Minneapolis, MN). Sam-

ples were processed for ELISA determinations following the manufacturer's recommendations. In a separate set of experiments, the non-alkaloid HPLC fractions (separated from the polar peak) of *Uncaria tomentosa* were evaluated for their ability to inhibit TNF $\alpha$  synthesis/release in RAW 264.7 cells after administration of LPS. Cells were either pretreated with the non-alkaloid fractions for 2 h and/or treated with LPS (50 ng/ml) for 1 h, the media replaced, and cells incubated for 16 h at 37 °C. Culture medium was collected for determination of TNF $\alpha$  levels using the Quantikine M mouse TNF $\alpha$  Immunoassay kit (R & D Systems, Inc., Minneapolis, MN) as indicated above.

- **HPLC sample preparation:** For the alkaloid determination, 1 g of freeze-dried cat's claw (UT and UG) was extracted using 10 ml of methanol-water-1.2 M HCl (50:50:1) for 4 h at room temperature. The extract was then centrifuged at 5000 rpm and filtered at 0.2- $\mu$ m prior to injection. To quantify the concentration of flavanols in UT and UG, samples were dissolved in hot water (100 mg/ml), centrifuged at 5000 rpm and filtered at 0.2- $\mu$ m prior to injection.

- **Apparatus:** All separations were conducted at ambient temperature by reversed-phase HPLC on a 25 cm C18 column, using isocratic elution. The HPLC system consisted of a GBC LC 1150 series pump, a GBC 1650 advanced autosampler (GBC Scientific Equipment Pty. Ltd., Dandenong, Australia), a 25-cm  $\times$  4.6-mm. Discovery® C18, 5- $\mu$ m analytical column (SUPELCO, Bellefonte, PA), and a Waters 490E programmable multi-wavelength detector (Waters, Milford, MA). Alkaloids (Mitrephylline and Uncarine E) were detected at 245 nm and flavanols [Catechin (CAT), epigallocatechin (EGC), epicatechin (EC) and epigallocatechin gallate (EGCG)] were detected at 280 nm.

- **Chromatographic conditions:** A set of experiments was carried out to determine the concentration of oxindole alkaloids in cat's claw (UT and UG). The calibration curves were obtained from commercial oxindole alkaloid standards – Mitrephylline and Uncarine E (ChromaDex Inc., Laguna Hills, CA). Stock solutions of Mitrephylline and Uncarine E (200  $\mu$ g/ml) were prepared by dissolving them in methanol-water-HCl, 1.2 M (50:50:1). Less concentrated solutions of Mitrephylline and Uncarine E were prepared, as needed, by dilution in HPLC mobile phase. Stock solutions of the two standards, Mitrephylline and Uncarine E, were stored at -20 °C to preserve their stability. Quantitative levels of total alkaloids in the freeze-dried cat's claw were determined using a mobile phase (A) 10 mM buffer phosphate Na<sub>2</sub>HPO<sub>4</sub>, K<sub>2</sub>HPO<sub>4</sub>, pH 7.0, and (B) acetonitrile. The mobile phase was programmed linearly as follows: A (55%) and B (45%) from 0 to 30 min at a flow rate of 1 ml/min. Sample injection was 10  $\mu$ l (prior to injection samples were diluted with buffer

phosphate, 1:1). To obtain non-alkaloid HPLC fractions from *Uncaria tomentosa*, a separate protocol was used. For this analysis, the most polar fraction from the alkaloid separation (elution time 2–4 min) was collected, and then concentrated for further HPLC fractionation. The mobile phase was 0.1% trifluoroacetic acid (TFA) in water (solvent A) and 0.1% TFA in acetonitrile (solvent B). The linear gradient of solvent B was increased from 10–90% from 0 to 40 min at a flow rate of 1 ml/min, and the sample injection was 20  $\mu$ l. For flavanol quantification, the standard solution Polyphenon 60 (Sigma, St. Louis, MO) was dissolved using HPLC-grade water (0.24 mg/ml) and aliquots were stored at -20 °C to preserve stability; the mobile phase contained acetonitrile:ethyl acetate: 0.05% H<sub>3</sub>PO<sub>4</sub> (12:2:86). The HPLC run was programmed for 30 min at a flow rate of 1 ml/min, and the sample injection was 10  $\mu$ l.

- **Indomethacin-induced gastritis:** Male Sprague-Dawley rats (250–300 g each) were used in this study. The rats were deprived of food but not water for 20–22 h before the experiment. Gastric damage was induced by oral administration of Indomethacin (IND) at a dose of 20 mg/kg body wt. (10 mg/ml in 5% NaHCO<sub>3</sub> solution). Micropulverized cat's claw (*Uncaria tomentosa*) was administered in the drinking water (5 mg/ml) for three days prior to indomethacin. Three hours after indomethacin administration, the rats were anesthetized with ketamine (40 mg/kg body wt.) and xylazine (4 mg/kg body wt.), the stomach was removed and opened along the greater curvature. After flushing with cold normal saline, the gastric erosions were measured and assigned a score based on the following criteria: 1 = petechia of < 2 mm in length, 2 = lesion of 2–4 mm, 3 = lesion of > 4 mm. The scores for each stomach were summed to give the overall damage index as described previously (Wallace and Whittle, 1985).

#### **In Situ RT-PCR**

- **Preparation of tissue section:** Samples from the stomachs of rats treated with indomethacin or pretreated with cat's claw were used in this study. All tissues were fixed in Formalin immediately after collection and subsequently embedded in paraffin. Four-micron paraffin-embedded sections were cut from the tissue blocks and processed as described previously (Zhang et al., 1998).

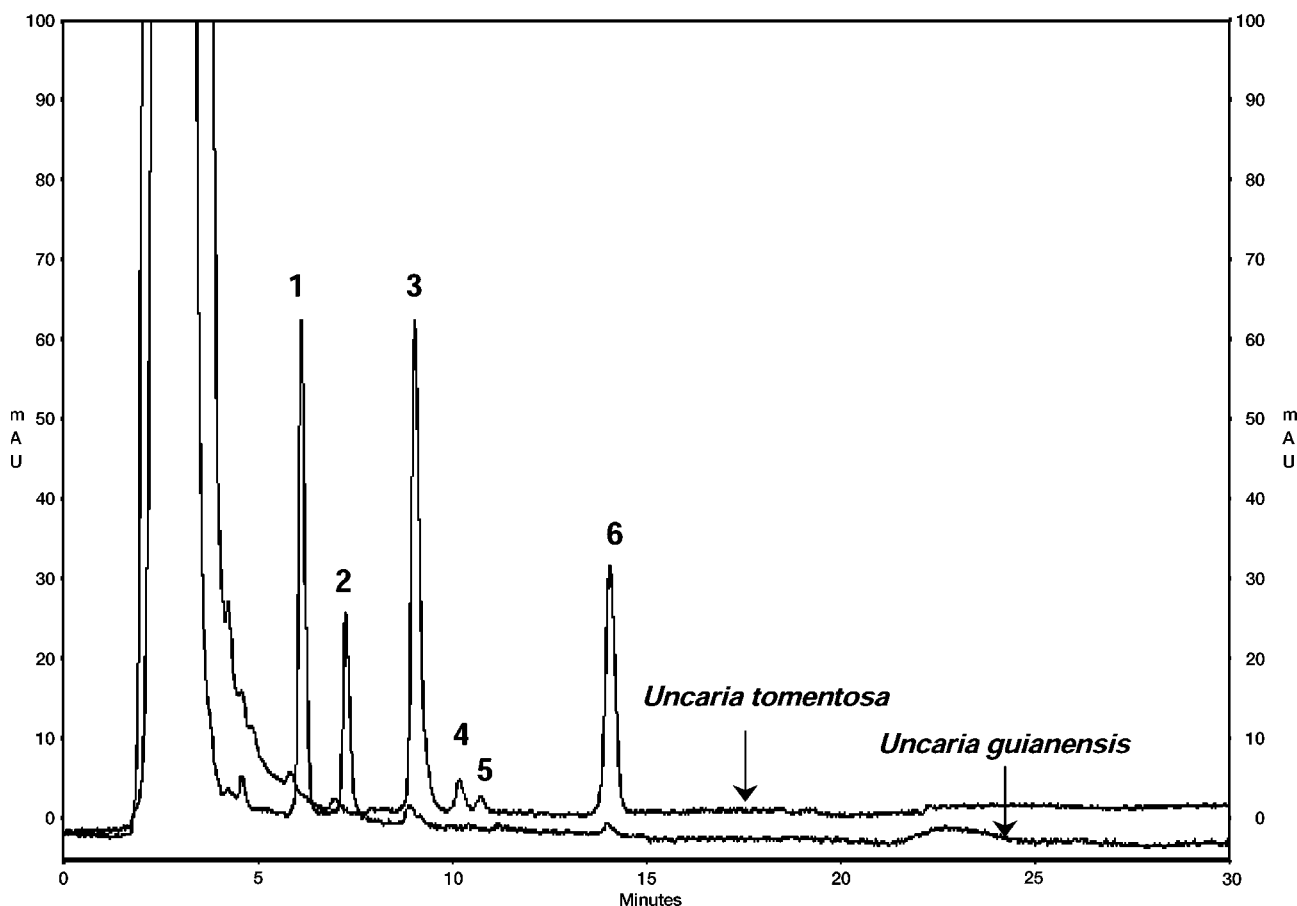
- **TNF $\alpha$  mRNA RT-PCR:** We used the Titan™ One tube RT-PCR system (Boehringer Mannheim, Indianapolis, IN) to perform the *in situ* RT-PCR reaction. The oligonucleotide primers (Life Technologies, Grand Island, NY) specific for rat TNF $\alpha$  (X66539) 295 bp were as follows: forward 5' TAC TGA ACT TCG GGG TGA TCG GTC C 3' (a 25-mer at position 149) and reverse 5' CAG CCT TGT CCC TTG AAG AGA ACC 3' (a 24-mer at position 443). The RT-PCR reaction and

amplification were carried out as reported previously (Zhang et al., 1998; Miller et al., 2000).

- *Immunohistochemical detection of digoxigenin:* We used a DIG nucleic acid detection kit (Boehringer Mannheim, Indianapolis, IN) to detect the digoxigenin tagged *in situ* RT-PCR amplified products. The slides were processed as reported previously, and following the manufacturer's recommendations (Miller et al., 2000).

- *Detection of apoptosis by TUNEL:* Apoptosis was evaluated in paraffin embedded stomach sections of rats treated with indomethacin or pretreated with cat's claw as described above. We used the *in situ* direct immunoperoxidase detection of digoxigenin-labeled genomic DNA to assess the degree of apoptosis. The labeling target was the multitude of new 3'-OH DNA ends generated by DNA fragmentation and typically localized in morphologically-identifiable nucleic and apoptotic bodies. In deparaffinized tissue sections, Proteinase K (20 µg/ml) was applied for 15 min to digest

protein. Endogenous peroxidase was quenched using 2% H<sub>2</sub>O<sub>2</sub> in PBS for 5 min, rinsed and blotted. The equilibration buffer was then applied, along with the coverslip, for 10–15 sec. The coverslip was removed, excess fluid tapped off, and the section edges blotted. The section was then exposed to working-strength terminal deoxynucleotidyl transferase (TdT, 54 µl) and plastic coverslip applied. Incubation was for 1 h, at which time the coverslip was removed, stop/wash buffer was added, and incubated at 37 °C for 30 min. Agitation was performed every 10 min during this last step. Next the section was washed in PBS followed by addition of anti-digoxigenin antibody carrying a conjugated reporter enzyme construct (peroxidase). The section was then coverslipped and incubated at room temperature for 30 min. The coverslip was then removed, the section washed three times in PBS, and the substrate/chromogen mixture (H<sub>2</sub>O<sub>2</sub>/DAB) added for color development (3–6 min). The section was then



**Fig. 1.** HPLC chromatogram of freeze-dried cat's claw: *Uncaria tomentosa* and *Uncaria guianensis*. Calibration curves were obtained from standard solutions containing the alkaloids Mitrephylline (2) and Uncarine E (6). A linear relationship between peak size and concentration was observed. For quantification of the compounds (1, 3, 4 and 5), the peak height and area under the curve were used. Chromatographic conditions are described in Materials and Methods. Note: the flavanols discussed in Table 2 elute in the large polar peak shown in this chromatogram.

washed and counterstained (hematoxylin), and finally dehydrated in xylene and mounted.

• **Statistical Analysis:** Each experiment was performed at least three times and results are presented as the mean  $\pm$  SE. Statistical analyses were performed using the t-test and one-way ANOVA. Post-hoc comparison of means was done using the Least Significant Difference test and the unpaired t-test. A probability value of  $< 0.05$  was considered significant.

## Results

### Oxindole alkaloid content

The presence of total alkaloids (Oxindole and pentacyclic) in freeze-dried samples of the two species of cat's claw varied noticeably (Figure 1). *Uncaria tomentosa* contained higher ( $P < 0.001$ ) concentrations of Mitraphylline and Uncarine E than *Uncaria guianensis* (Table 1). The results also indicated that *Uncaria tomentosa* had 35 times more total alkaloids than *Uncaria guianensis*. This is the first report that demonstrates a significant difference in alkaloid content in freeze-dried samples obtained from the two species of cat's claw.

### Flavanol concentration

The content of flavanols in freeze-dried samples of cat's claw (UT and UG) was determined using reversed-phase HPLC. As shown in Table 2, four important flavanols were detected: catechin, epigallocatechin, epicatechin and epigallocatechin. With the exception of epigallocatechin, *Uncaria tomentosa* had higher ( $P < 0.01$ ) concentrations of flavanols than *Uncaria guianensis*. This is the first evidence of the difference in the content of flavanols that exist between the two species, and their presence in a freeze-dried form of cat's claw.

### Antioxidant activity

Experiments to assess the capacity to scavenge stable free radicals, DPPH and ABTS, were conducted using freeze-dried cat's claw (UT and UG). The inhibition of DPPH was correlated directly with cat's claw concentration. Despite lower concentrations of alkaloids and flavanols, *Uncaria guianensis* showed a greater DPPH scavenging capacity than *Uncaria tomentosa* ( $P < 0.01$ ). This difference was reflected as a lower  $IC_{50}$  value although maximal responses were comparable between species (Table 3). With respect to ABTS-radicals scavenging, the results indicated that both species showed similar tendencies to inhibit this free radical.

**Table 1.** Content of Oxindole alkaloids in freeze-dried cat's claw (*Uncaria tomentosa* and *Uncaria guianensis*)<sup>1</sup>.

Peak	Alkaloid	<i>Uncaria tomentosa</i>	<i>Uncaria guianensis</i>
		mg/g	
1	Speciophylline	1.60 $\pm$ 0.09*	0.13 $\pm$ 0.007
2	Mitraphylline	0.88 $\pm$ 0.04*	0.08 $\pm$ 0.008
3	Uncarine F	2.02 $\pm$ 0.07	ND
4	Pteropodine	0.15 $\pm$ 0.02	ND
5	Isomitraphylline	1.67 $\pm$ 0.11*	0.025 $\pm$ 0.004
6	Uncarine E	2.72 $\pm$ 0.14*	0.022 $\pm$ 0.003
Total		9.04 $\pm$ 0.36*	0.26 $\pm$ 0.026

<sup>1</sup> Separation of Oxindole alkaloids was carried out using a reverse HPLC system. The chromatographic conditions are described in Materials and Methods. Total Oxindole alkaloids were determined from the six peaks. Values represent mean  $\pm$  SE of three injections. \* Values within the same row differ ( $P < 0.001$ ). ND, not detected.

**Table 2.** Flavanol content of freeze-dried cat's claw determined by HPLC<sup>1</sup>.

Flavanol	<i>Uncaria tomentosa</i>	<i>Uncaria guianensis</i>
	mg/g	
Catechin	8.47 $\pm$ 0.1*	5.53 $\pm$ 0.07
Epigallocatechin	7.57 $\pm$ 0.2	9.51 $\pm$ 0.01
Epicatechin	4.22 $\pm$ 0.1*	2.47 $\pm$ 0.05
Epigallocatechin gallate	4.41 $\pm$ 0.2*	0.79 $\pm$ 0.07

<sup>1</sup>The aqueous extract of the two species of cat's claw (*Uncaria tomentosa*, *Uncaria guianensis*) was freeze-dried and used for these experiments. The flavanol content was determined using HPLC as described in Materials and Methods. Values represent mean  $\pm$  SE of three injections. \* Means within the same row differ ( $P < 0.01$ ).

**Table 3.** Free radical scavenging capacity of cat's claw determined by the DPPH assay<sup>1</sup>.

$\mu$ g/ml	DPPH Inhibition, %	
	<i>Uncaria tomentosa</i>	<i>Uncaria guianensis</i>
1	3.5 $\pm$ 1.0	9.1 $\pm$ 0.7
3	7.8 $\pm$ 3.5	16.6 $\pm$ 2.8
10	17.6 $\pm$ 1.3	44.8 $\pm$ 1.1*
30	63.8 $\pm$ 0.5	67.1 $\pm$ 2.3
100	85.5 $\pm$ 0.8	90.5 $\pm$ 1.7

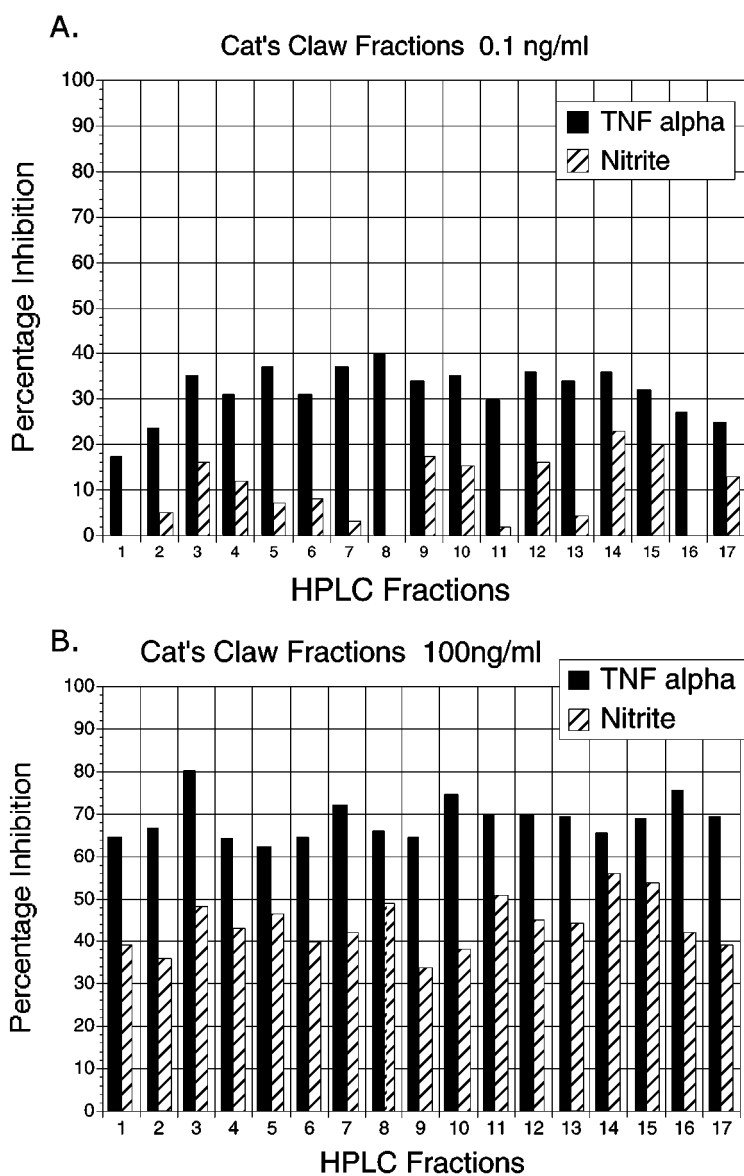
<sup>1</sup>Freeze-dried cat's claw, *Uncaria tomentosa* and *Uncaria guianensis*, was used for these experiments. The inhibition of the free radical DPPH was determined using a spectrophotometer (515 nm). Values represent mean  $\pm$  SE of three different reactions carried out for 5 min as described in Materials and Methods. \*Means within the same row differ ( $P < 0.01$ ).

**Table 4.** Comparison of the antioxidant and anti-TNF $\alpha$  activities of cat's claw<sup>1</sup>.

Assay	Cat's claw			
	<i>Uncaria tomentosa</i>		<i>Uncaria guianensis</i>	
	IC <sub>50</sub> value	Max Inhibition, %	IC <sub>50</sub> value	Max Inhibition, %
Antioxidant:				
DPPH	20.8 $\mu$ g/ml	85.5	12.6 $\mu$ g/ml*	90.5
ABTS-radicals	7.7 $\mu$ g/ml	82.1	4.8 $\mu$ g/ml*	82.6
Lipid peroxidation	148 $\mu$ g/ml	58.6	99 $\mu$ g/ml*	60.6
Anti-TNF $\alpha$	14.1 ng/ml	70.6	9.5 ng/ml*	75.5

<sup>1</sup>Freeze-dried cat's claw, *Uncaria tomentosa* and *Uncaria guianensis*, was used for these experiments. The antioxidant activity of cat's claw was assayed by inhibition of DPPH, ABTS-radicals, and lipid peroxidation. The anti-TNF $\alpha$  activity was determined using the Quantikine M mouse TNF $\alpha$  immunoassay. Details of the experiments are described in Materials and Methods.

\*Means for IC<sub>50</sub> value within the same row differ ( $P < 0.01$ ).



**Fig. 2.** Effect of cat's claw on LPS-mediated nitric oxide and TNF $\alpha$  production by macrophages (RAW 264.7). Freeze-dried cat's claw, *Uncaria tomentosa*, was used for these experiments. Cells were seeded at  $5 \times 10^4$  cells/well. Bars represent TNF $\alpha$  and nitrite release into the media from cells pre-treated with non-alkaloid HPLC fractions of cat's claw for 2 h then challenged with LPS (50 ng/ml) for 1 h, and incubated for 16 h as described in Materials and Methods. Each fraction was tested at two concentrations, 0.1 (Panel A) and 100 ng/ml (Panel B). Results are depicted as the mean of three samples from two experiments expressed as percentage inhibition from the LPS positive control ( $P < 0.01$ ).

The  $IC_{50}$  values for scavenging of DPPH, ABTS-radicals, and lipid peroxidation are shown in Table 4. The ability to scavenge DPPH was higher ( $P < 0.01$ ) for *Uncaria guianensis* than for *Uncaria tomentosa* (12.6  $\mu\text{g/ml}$  vs 20.8  $\mu\text{g/ml}$ ). Similar responses were observed for ABTS-radicals (4.8  $\mu\text{g/ml}$  vs 7.7  $\mu\text{g/ml}$ ), and protection against lipid peroxidation (99  $\mu\text{g/ml}$  vs 148  $\mu\text{g/ml}$ ). The higher concentrations of cat's claw required to quench lipid peroxides may reflect the water solubility of the active components. When cat's claw was evaluated for its ability to protect deoxyribose against hydroxyl radicals, it was found that both species protected ( $P < 0.01$ ) deoxyribose degradation in a dose-dependent manner. The results also indicated that *Uncaria guianensis* was more potent ( $P < 0.01$ ) than *Uncaria tomentosa*.

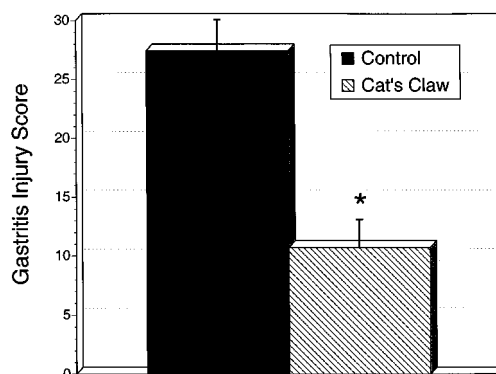
#### *In vitro* inhibition of TNF $\alpha$

The anti-inflammatory activity of freeze-dried cat's claw was assessed by its ability to inhibit TNF $\alpha$  production in RAW cells treated with LPS. As shown in Table 4, the  $IC_{50}$  values for the anti-TNF $\alpha$  activity of *Uncaria tomentosa* and *Uncaria guianensis* were 14.1 and 9.5 ng/ml, respectively. These results indicated that *Uncaria guianensis* was more potent ( $P < 0.01$ ) in decreasing TNF $\alpha$  production in macrophages (RAW 264.7) was compared to *Uncaria tomentosa*.

In separate experiments, the most polar HPLC peak (non-alkaloid peak) from *U. tomentosa* was fractionated further, with seventeen fractions collected for bioactivity determination. These fractions were administered at 0.1–100 ng/ml to RAW 264.7 cells for 2 h followed by a challenge with LPS (50 ng/ml) for 1 h. After 16 h incubation, TNF $\alpha$  and nitrite release into the media was measured. The results showed that TNF $\alpha$  and nitrite inhibition was concentration-dependent (Figure 2). The non-alkaloid fractions were more effective at decreasing TNF $\alpha$  release than nitrite, as noted before with the parent botanical (Sandoval et al., 2000). The fractions were administered at concentrations that were below (0.1 ng/ml) and above (100 ng/ml) the  $IC_{50}$  value for freeze-dried *U. tomentosa*. The inhibitory effects of these fractions on TNF $\alpha$  and nitrite production spanned the results observed with the whole freeze-dried extract.

#### *In vivo* anti-inflammatory activity of cat's claw

Oral administration of indomethacin (20 mg/kg body wt.) caused linear gastric lesions in the stomach of rats, characterized by hemorrhagic necrosis on the superficial epithelia. This model is thought to be representative of nonsteroidal anti-inflammatory drug (NSAID)-induced gastritis. In this study, we assessed the benefits



**Fig. 3.** Gastric injury in response to acute indomethacin administration. Micropulverized cat's claw, *Uncaria tomentosa*, (5 mg/ml) prepared as decoction, and administered to rats in the drinking water for three days prior to indomethacin (20 mg/kg body wt. p.o.), as described in Material and Methods. \*Significant protection ( $P < 0.01$ ) compared to indomethacin-induced gastritis.

of cat's claw (*Uncaria tomentosa*) given in the drinking water for 3 days prior to the administration of indomethacin. Cat's claw elicited a protective effect ( $P < 0.01$ ), and the degree of gastric mucosal injury was markedly attenuated (Figure 3).

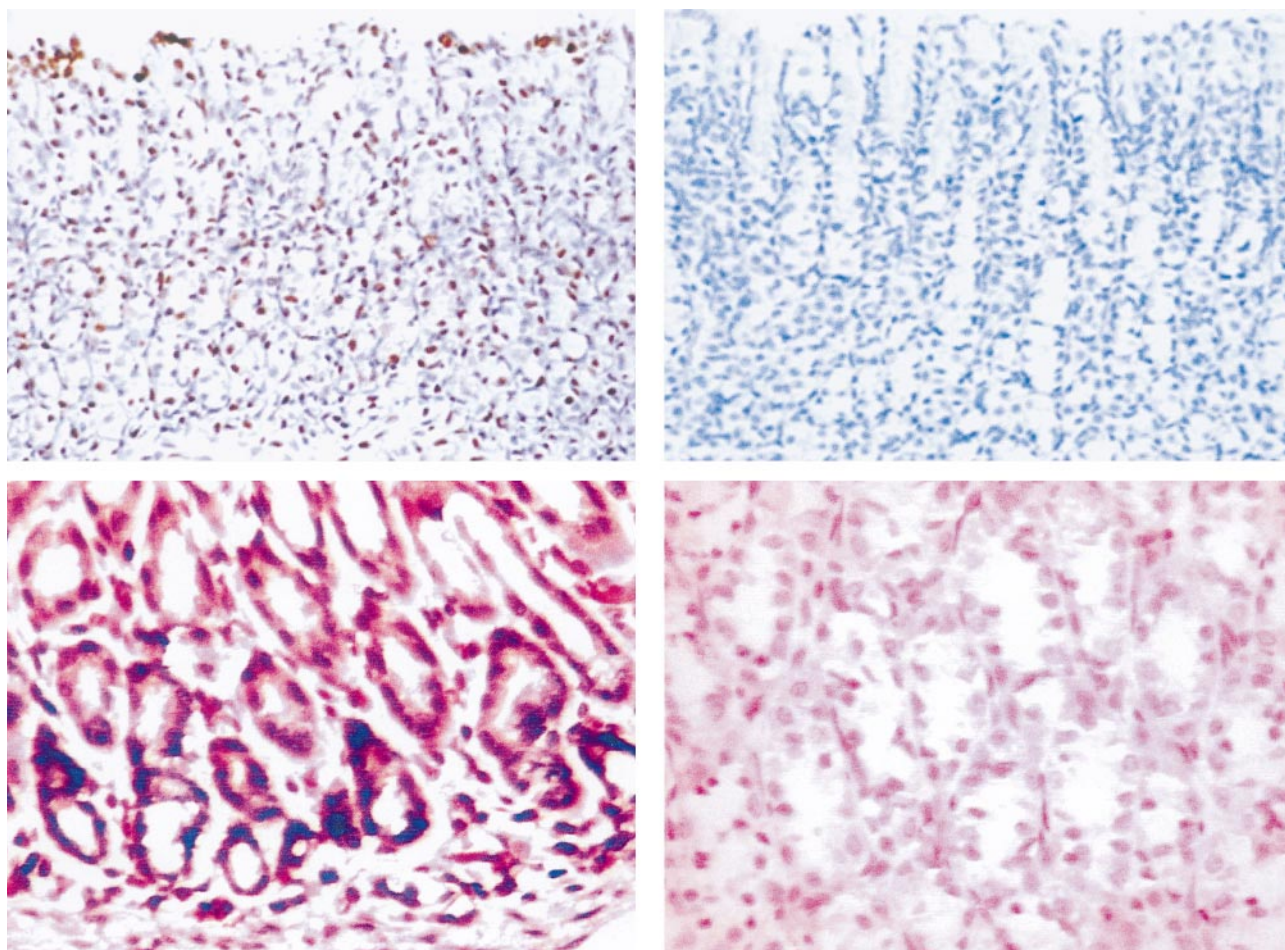
#### Stomach epithelial apoptosis

We investigated the cytoprotective effect of *Uncaria tomentosa* in decreasing indomethacin-induced apoptosis in stomach epithelial cells of rats. This response was assessed *in vivo* using the TUNEL immunohistochemical assay for double-strand DNA fragmentation, a hallmark of apoptosis. In intact, control rats, apoptosis was minimal and restricted to superficial epithelial cells. However, apoptosis was readily detected in epithelial cells of the gastric mucosa of rats treated with indomethacin (Figure 4). By contrast, *Uncaria tomentosa*, given as a pretreatment, prevented this response. Under this condition the gastric mucosa was similar in appearance to that of the control group.

#### TNF $\alpha$ mRNA expression

As the induction of TNF $\alpha$  gene expression has been implicated in NSAID toxicity, we evaluated the levels of TNF $\alpha$  mRNA in this model using *in situ* RT-PCR techniques reported previously (Wallace and Whittle, 1985), for inducible nitric oxide synthase (iNOS). In normal animals, TNF $\alpha$  gene expression was absent (data not shown), as expected. With indomethacin administration, a marked induction of TNF $\alpha$  mRNA in epithelial cells of the gastric mucosa was evident (Figure 5). Pretreat-





**Fig. 4.** Apoptosis of gastric mucosa assessed by the TUNEL histochemical technique. Micropulverized cat's claw, *Uncaria tomentosa* (5 mg/ml), prepared as a decoction, was administered to rats in the drinking water for three days prior to indomethacin (20 mg/kg body wt. p.o.), as described in Material and Methods. Representative sections counter stained with hematoxylin and with similar magnification ( $\times 200$ ). **A**, Stomach from an indomethacin-treated rat (i.e., drinking water). The degree of apoptotic cells is denoted by the dark brown color. **B**, Stomach from a rat receiving cat's claw for 3 days prior to administration of indomethacin.

**Fig. 5.** Expression of TNF $\alpha$  mRNA in gastric mucosa assessed by the *in situ* RT-PCR technique. Micropulverized cat's claw, *Uncaria tomentosa*, (5 mg/ml) was administered to rats in the drinking water for three days prior to indomethacin (20 mg/kg body wt. p.o.), as described in Material and Methods. Positive evidence of TNF $\alpha$  mRNA was noted in the gastric epithelia of indomethacin treated rats, as indicated by the dark blue color ( $\times 400$ ). **A**, Stomach from an indomethacin-treated rat (i.e., drinking water). **B**, Stomach from a rat receiving cat's claw for 3 days prior to administration of indomethacin. Rats pretreated with cat's claw failed to evoke the transcription of TNF $\alpha$  (panel B). Nuclear fast red was used as background stain.

ment with cat's claw, *Uncaria tomentosa*, for 3 days before indomethacin administration blocked the expression of TNF $\alpha$ . It was noticeable that the sites of TNF $\alpha$  expression appear to be coincident with apoptosis, both occurring in epithelial cells of the gastric mucosa.

## Discussion

The two species of cat's claw, *Uncaria tomentosa* and *Uncaria guianensis*, displayed excellent antioxidant

properties and a remarkably potent ability to suppress the production of TNF $\alpha$ . As reported previously, cat's claw (*Uncaria tomentosa*) has the ability to protect DNA against oxidants such as peroxynitrite (Sandoval-Chacón et al., 1998), and is also cytoprotective to macrophages (RAW 264.7) when they are exposed to ultraviolet irradiation and DPPH (Sandoval et al., 2000). This latter study also demonstrated that the antioxidant property of cat's claw was influenced by the form of processing, i.e., the freeze-dried cat's claw (*Uncaria tomentosa*) was superior to the micropulver-

ized in its ability to scavenge DPPH and inhibit TNF $\alpha$  production (Sandoval et al., 2000). In the present study, we sought to compare the two species of cat's claw (*Uncaria tomentosa* and *Uncaria guianensis*) with the purpose of determining whether there were differences in their antioxidant and anti-inflammatory properties. The present results indicate that *Uncaria guianensis* is more potent than *Uncaria tomentosa* in all assessments of scavenging free radicals, protecting deoxyribose, inhibiting lipid peroxidation, and suppressing of TNF $\alpha$  formation.

The antioxidant properties of cat's claw were determined in part by the cellular locus of the oxidative process. Higher concentrations were required to inhibit lipid peroxidation, but this is to be expected for a water-soluble inhibitor where it is less likely to have access to the lipid peroxides (Sandoval-Chacón et al., 1998; Sandoval et al., 2000). When the auto-oxidation of lipids is initiated, the primordial radicals can be produced either in the aqueous phase or in the lipid bilayer. Because the cat's claw used is water-soluble, two effects may occur. First, radicals from the water-soluble initiators are trapped easily before they can diffuse into the lipid phase. Second, the water-soluble inhibitors of cat's claw may not interact very well with radicals in the lipid phase and trap them.

The antioxidant defense system of the body consists of endogenous and exogenous antioxidants that work together at the molecular level to protect cell membranes, lipoproteins, and DNA from the damaging effects of free oxygen radicals (Gaté et al., 1999). Antioxidants play a significant role in the pathogenesis of a number of diseases. Epidemiological data strongly support the belief that high consumption of fruits and vegetables protect against degenerative diseases such as cancer (Kong et al., 2000), and heart disease (Law and Morris, 1998). Recent studies produced substantial evidence that reactive oxygen species (ROS) are intimately involved in the pathogenesis of inflammatory processes that can exacerbate tissue damage (Hensley et al., 2000). Besides the consumption of fruits and vegetables, consumers are using botanicals as source of natural antioxidants and anti-inflammatory agents to improve their health. In this regard, cat's claw (*Uncaria tomentosa* and *Uncaria guianensis*) possess properties that make this medicinal plant an excellent natural source of antioxidants (flavanols) and bioactive anti-inflammatory components with far greater efficacy than extracts obtained from *Pinus maritima* (Packer et al. 1999).

For both species of cat's claw (*Uncaria tomentosa* and *Uncaria guianensis*) the critical distinguishing action, based on potency, appears to reside in their ability to prevent the activation of redox-sensitive genes. In this study, suppression of TNF $\alpha$  formation was ob-

served in both forms of cat's claw at low concentrations, although *U. guianensis* was significantly more potent. TNF $\alpha$ -induced apoptosis is evident in several inflammatory conditions and disease states (Chandel et al., 2000), including NSAID gastritis and enteropathy (Fiorucci et al., 1998b). TNF $\alpha$  and other pro-inflammatory cytokines (IL-1, IL-6), chemokines, growth factors and enzymes (cyclooxygenase 2, inducible nitric oxide synthase) are quiescent genes under normal conditions; however, inflammation, immune activation and infection activate several transcription factors leading to transcription and translation. In terms of TNF $\alpha$  gene expression, emphasis has been placed on NF- $\kappa$ B as the main transcription factor in inflammation and tissue injury (Barnes and Karin, 1997, Jourdeuil et al., 1997). We have previously demonstrated that cat's claw is an inhibitor of transcriptionally-regulated gene expression, specifically through suppression of NF- $\kappa$ B activation (Sandoval-Chacón et al., 1998). It is possible that the antioxidant properties of cat's claw contribute to this response (Sandoval-Chacón et al., 1998; Sandoval et al., 2000; Desmarchelier et al., 1997) as NF- $\kappa$ B is an oxidant-sensitive transcription factor (Shreck et al., 1992).

Given the ethnomedical application of cat's claw for gastritis, we postulated that cat's claw may be useful in the treatment of gastritis through its actions on gene expression and cell death; the present results confirm this hypothesis. Cat's claw was indeed effective in preventing NSAID-induced gastric damage, as we have noted previously for NSAID enteropathy (Sandoval-Chacón et al., 1998). Associated with the protective effects of cat's claw is a suppression of TNF $\alpha$  expression by epithelial cells, and apoptosis of the epithelium. As TNF $\alpha$  can induce apoptosis in a variety of cell types (Fiorucci et al., 1998b), it is logical to assume a link between these two events, as proposed by other investigators (Fiorucci et al., 1998a). In the present study, rats received cat's claw in a manner consistent with the ethnomedical tradition, i.e., a decoction was prepared, filtered, and freeze-dried.

Oxindole alkaloids have been touted as the active ingredients in cat's claw based on an immunostimulatory action (Wagner et al., 1985; Keplinger et al., 1999; Lemaire et al., 1999; Reinhard, 1999). Several problems exist with this connection. First, few studies have demonstrated that alkaloids possess the same, but enriched, bioactivity as the parent botanical, cat's claw. While the oxindole alkaloids may indeed promote phagocytosis, there is a lack of evidence linking this action to the *in vivo* or *in vitro* actions of cat's claw. Lemaire et al. (1999), did provide evidence that a freeze-dried concentrate of cat's claw could elevate cytokine production (immune stimulation), but this effect was evident only at concentrations that far exceeded

the normal dosing of cat's claw (by several hundred-fold). Thus, this observation reflects a toxicity response, and not a therapeutic response.

It is important to relate *in vitro* doses to those used *in vivo* or clinically, which is why the remarkably low concentrations of cat's claw required to prevent TNF $\alpha$  formation have substantial appeal in furthering our understanding of the mechanism of action of this medicinal plant. This is an immunomodulatory role, not an immune-stimulatory effect. Indeed, cat's claw is used in conditions where the immune response is excessive. We have recently noted that *U. guianensis* treatment was an effective stand-alone therapy for osteoarthritis (Piscoya et al., 2001) when administered at only 100 mg per day. Osteoarthritis and rheumatoid arthritis are conditions in which TNF $\alpha$  production is excessive (Martel-Pelletier et al., 1999; Charles et al., 1999). This trial, with a low dose of freeze-dried *U. guianensis*, emphasizes that mechanisms observed at low concentrations of cat's claw (inhibition of TNF $\alpha$  production secondary to NF- $\kappa$ B suppression) are those most likely to mediate the therapeutic response. Inhibition of NF- $\kappa$ B-dependent gene expression, particularly TNF $\alpha$ , is observed at the lowest concentrations of cat's claw reported to date (IC<sub>50</sub> value of approximately 10 ng/ml for a freeze-dried formulation). Consequently, weight must be given to this action when considering the therapeutic utility of *U. guianensis*.

Further considerations for concluding that the biological actions of cat's claw are not related to the alkaloid content are reflected in the observation that alkaloid content of these freeze-dried concentrates was almost negligible in *U. guianensis*, representing less than 0.03% of the solutes, compared to 0.9% for *U. tomentosa*. As the bioactivities observed with *U. guianensis* and *U. tomentosa* were qualitatively similar but with *U. guianensis* being more potent, it is very difficult to see these effects as the result of the alkaloid constituents. Furthermore, fractionation studies of *U. tomentosa* indicated that this biological activity (inhibition of LPS-induced macrophage production of TNF $\alpha$  and nitric oxide) could be found in multiple fractions obtained from the non-alkaloid polar HPLC peak evident in both subspecies. In terms of potency, these fractions were consistent with the bioactivity of the whole freeze-dried concentrate, suggesting that they were the source of *U. tomentosa*'s effects on TNF $\alpha$  and nitric oxide production. With numerous individual fractions sharing this bioactivity, structural identification of individual components was beyond the scope of this study. Nevertheless, it is consistent with the reports that a myriad of chemicals contribute to the therapeutic actions of cat's claw (Aquino et al., 1991, 1989; Cerri et al., 1988; Sheng et al., 1998; Kitajima et al., 2000).

Cat's claw has been implicated in diseases/disorders distinct from inflammation, although many of these indications are only supported by anecdotal evidence. Nevertheless, we propose that inhibition of redox-sensitive gene expression may well explain the breadth of these reports. In terms of cancer and benign prostatic hypertrophy, cell proliferation is linked to local production of growth factors; events that are regulated by several transcription factors, including NF- $\kappa$ B. Suppression of NF- $\kappa$ B has been linked to a reduction in proliferation *in vivo* (Miagkov et al., 1998). The only evidence linking cat's claw to direct cell death in cancer has been in an *in vitro* study using a hemopoietic line (HL-60, Sheng et al., 1998). Others, and we have shown that inhibition of NF- $\kappa$ B can promote apoptosis in these cells (Beg and Baltimore, 1996; Mannick et al., 1997). In addition, the clinical development program by Phytopharm, Plc for its botanical extract, P-54, which is an inhibitor of redox-sensitive transcription, has focused on both colon cancer and inflammatory bowel disease. This extract, P-54, is in Phase-II clinical trials for both of these conditions. This demonstrates that botanically derived inhibitors of NF- $\kappa$ B have potential utility for both inflammation and tumor proliferation. That the diverse therapeutic effects of cat's claw lie in suppression of redox-sensitive gene expression appears to be meritorious but not necessarily unique. In any case, this effect appears to be mediated by phytochemicals distinct from oxindole alkaloids.

If the importance of alkaloids (oxindole or pentacyclic) as contributors to the therapeutic actions of cat's claw can be challenged, what then is the outcome as to their utility as a means of standardizing the quality and biological actions of cat's claw? For example, if the alkaloid content of *U. guianensis* is meaningless, this subspecies is more potent than *U. tomentosa*, then it seems that we should address an alternative means of offering a standardized product. One possibility is that cat's claw should be standardized to a meaningful and relevant biological response, e.g., TNF $\alpha$  inhibition or free radical scavenging. For the most part, medicinal plants are not standardized to a biological assay, although the concept has particular relevance in cases like this where multiple components appear to contribute to the bioactivity. Cat's claw certainly fits this category. While we have determined that catechins are present in cat's claw, the amounts are low and are not useful for determination of authenticity. Perhaps the quinovic acid glycosides noted to be present in both species (Aquino et al., 1991; Yopez et al., 1991) may provide a better biochemical fingerprint. Whatever the approach chosen, manufacturers should be reminded that the goal is to offer a reliable and relevant means of quality assurance.

In conclusion, this study provides experimental validation for the ethnomedical and biological properties of cat's claw for inflammatory states, oxidative stress and gastritis. In terms of subspecies, *U. guianensis* and *U. tomentosa* have similar actions but in all determinations, *U. guianensis* was more potent. In this regard, *U. guianensis* may be the preferred subspecies for therapeutic use. Cat's claw appears to be a particularly potent and effective inhibitor of TNF $\alpha$  production, a cytokine associated with immune activation and inflammation. Suppression of redox-sensitive regulation of gene expression may well be the key to understanding the therapeutic potential of cat's claw.

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