

CAT'S CLAW INHIBITS TNF α PRODUCTION AND SCAVENGES FREE RADICALS: ROLE IN CYTOPROTECTION

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Abstract—Cat's claw (*Uncaria tomentosa*) is a medicinal plant from the Amazon River basin that is widely used for inflammatory disorders and was previously described as an inhibitor of NF- κ B. Cat's claw was prepared as a decoction (water extraction) of micropulverized bark with and without concentration by freeze-drying. Murine macrophages (RAW 264.7 cells) were used in cytotoxicity assays (trypan blue exclusion) in response to the free radical 1,1-diphenyl-2-picrylhydrazyl (DPPH, 0.3 μ M) and ultraviolet light (UV) light. TNF α production was induced by lipopolysaccharide (LPS 0.5 μ g/ml). Cat's claw was an effective scavenger of DPPH; the EC₅₀ value for freeze-dried concentrates was significantly less than micropulverized (18 vs. 150 μ g/ml, $p < .05$). Cat's claw (10 μ g/ml freeze-dried) was fully protective against DPPH and UV irradiation-induced cytotoxicity. LPS increased TNF α media levels from 3 to 97 ng/ml. Cat's claw suppressed TNF α production by approximately 65–85% ($p < .01$) but at concentrations considerably lower than its antioxidant activity: freeze-dried EC₅₀ = 1.2 ng/ml, micropulverized EC₅₀ = 28 ng/ml. In conclusion, cat's claw is an effective antioxidant, but perhaps more importantly a remarkably potent inhibitor of TNF α production. The primary mechanism for cat's claw anti-inflammatory actions appears to be immunomodulation via suppression of TNF α synthesis. © 2000 Elsevier Science Inc.

Keywords—UV irradiation, Oxidant, *Uncaria tomentosa*, Inflammation, TNF α , Free radicals, NF- κ B, Cytoprotection

INTRODUCTION

Complimentary approaches to health care are of increasing interest to the public and health care providers; numerous alternatives are being evaluated for potential benefits [1,2]. Botanicals, or herbal medicines, represent a major component of this revolution. However, at issue with the increased utilization of medicinal plants is a lack of information as to applications, mechanisms of action, and appropriate doses and formulations. Recently, we have attempted to fill this informational gap, focusing on medicinal plants from South America, the last frontier of botanicals.

One of the most widely used and respected medicinal plants in South America is cat's claw or Uña de gato (*Uncaria tomentosa*). Cat's claw is a vine from the

Amazon River basin, which has a steep heritage of use in various states of inflammation [3,4]. Traditionally the bark of cat's claw is prepared as a decoction (extraction with hot water). Several pharmacognosy studies have attempted to isolate active chemical constituents from its bark, with quinovic acid glycosides [5–7], sterols [8], and oxindole or pentacyclic alkaloids being proposed [9–14]. Other studies indicated that cat's claw was effective in a model of inflammation, carrageenan-induced rat paw edema, but activity resided in a number of fractions and chemical components, not just quinovic acid glycosides [15]. Indeed, much of the cat's claw sold in Western countries is standardized for oxindole alkaloid content. However, oxindole alkaloids have been found to promote phagocytosis, leading many to claim that cat's claw is an immunostimulant [16]. This concept is confusing if not misleading, as inflammation is not normally approached therapeutically by agents that promote phagocytosis or immune activation. In this regard we have sought to find alternative explanations.

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Recently, we reported that cat's claw was cytoprotective against peroxynitrite, prevented the side effects of nonsteroidal anti-inflammatory drugs on the intestine and was an effective inhibitor of inducible nitric oxide synthase gene expression by suppressing the activation of the transcription factor NF- κ B [17]. These actions are more consistent with an ability to treat chronic inflammation. The purpose of this study was to extend this concept to other NF- κ B regulated genes implicated in inflammation as well as additional examples of oxidative injury. We chose TNF α as a prototypical NF- κ B dependent cytokine and because of its critical role in chronic inflammation [18]. Therapeutic benefits with TNF α antibodies in patients with arthritis or Crohn's disease have strengthened the therapeutic potential of targeting TNF α [19–22]. However, the costs of these agents are substantial (approximately \$2,000 per injection) and less expensive yet effective therapeutic strategies would be welcomed. This study was designed to explore the potential of cat's claw as an alternative anti-inflammatory therapy.

MATERIALS AND METHODS

Chemicals

Methanol (HPLC grade) and 1,1-diphenyl-2-picrylhydrazyl (DPPH) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Unless indicated, all cellular reagents and culture medium were from Life Technologies (Grand Island, NY, USA).

Cell culture

RAW 264.7 cells were obtained from the American Type Culture Collection (Rockville, MD, USA). The cells were grown in DMEM (high glucose), 10% FCS and supplemented with 25 mM HEPES, pH 7.4; 4 mM L-glutamine; 40 μ g/ml penicillin; 90 μ g/ml streptomycin and 1.2 g/l NaHCO₃. Cell cultures were maintained in a humidified 5% CO₂ incubator at 37°C. At confluence cells were detached by scraping. Harvested cells were plated in 6-well tissue culture plates (1 \times 10⁶ cells/well) and allowed to grow to confluence over 24 h before use.

Preparation of cat's claw extracts

The bark of cat's claw, *Uncaria tomentosa*, was collected from the experimental plots of the Tulumayo Agricultural Station at Universidad Nacional Agraria de la Selva, Tingo Maria, Peru. Two forms of cat's claw extracts were prepared: (1) an aqueous decoction was made by boiling in water micropulverized cat's claw (mpCC) at 20 g/l for 30 min. Then it was filtered using a Whatman paper No. 4, and (2) a freeze-dried cat's claw

extract (fdCC) was prepared by boiling the bark of cat's claw (20 g/l) for 30 min then decanted and total solids separated by filtration with a Whatman No. 4 filter paper. The filtrate was then freeze-dried using a Freezemobile 6 concentrator (Virtis, Gardiner, NY, USA). For the cell culture experiments the cat's claw solutions of mpCC and fdCC were filtered at 0.2 μ m. Aliquots of the extracts were kept at –20°C until further use.

Evaluation of free radical-scavenging by cat's claw

The DPPH radical scavenging method previously reported [23] was modified as follows. The soluble solids content of the cat's claw extracts were standardized to give stock solutions containing 2 mg/ml water. An aliquot (25 μ l) of either mpCC or fdCC extracts was placed in a cuvette and a 6 \times 10^{–5} M methanolic solution of DPPH (final vol 1 ml) was added. The decrease in absorbance at 515 nm was determined continuously with data capturing at 10 s intervals with a Beckman Coulter DU-640 spectrophotometer (Beckman Instruments, Fullerton, CA, USA). The absorbance of the DPPH radical stock solution was measured for 60 min and during that time the absorbance was stable. All determinations (reactions) were performed in triplicate.

The DPPH scavenging capacity of cat's claw was determined by the following expression: SC = [(A_{S(10)} – A_{C(0)})/A_{C(0)}] * 100 where SC is a scavenging-capacity coefficient from 0 to 100%, A_{S(10)} is the absorbance at time = 10 min for the extract sample, and A_{C(0)} is the absorbance of the control (DPPH per se) at time = 0. Calculated antioxidant activity coefficients for cat's claw are shown in Table 1.

Free radical-induced cell death

To evaluate the in vitro cytotoxicity of DPPH, RAW 264.7 cells were covered with 2 ml Hanks' balanced salt solution (HBSS) containing DPPH (0.3, 1, 3, and 10 μ M) for 1 h at 37°C. After DPPH treatment, fresh media was replaced and cells were incubated overnight. In another set of experiments RAW 264.7 cells were treated simultaneously with HBSS containing freeze-dried cat's claw (fdCC, 10 μ g/ml) and DPPH (0.3 μ M) for 1 h. After treatment, fresh media was replaced and cells were incubated overnight. Cell death was quantified using trypan blue dye exclusion as previously reported [24].

UV irradiation

For UV irradiation experiments, RAW 264.7 cells were either pretreated for 2 h with freeze-dried cat's claw (fdCC, 1 or 10 μ g/ml) or without cat's claw. Irradiation

Table 1. Comparison of the Antioxidant and Anti-TNF α Activities of Cat's Claw¹

Assay	Freeze-dried cat's claw		Micropulverized cat's claw	
	EC ₅₀	Max inhibition, %	EC ₅₀	Max inhibition, %
Antioxidant (DPPH)	18 μ g/ml	88.5	150 μ g/ml	87.5
Anti-TNF α	1.2 ng/ml	86.0	28 ng/ml	84.0

¹Cat's claw, freeze-dried and micropulverized, was used for these experiments. The antioxidant activity was assayed by in vitro inhibition of the free radical DPPH, and quantified spectrophotometrically at 515 nm. The anti-TNF α activity was determined using the Quantikine M mouse TNF α immunoassay as described in Materials and Methods.

was carried out with a UVC light source (TUV30V/G30T8 lamp, Philips, Holland) emitting a radiation of 253.7 nm. After 1 h exposure to UV light, the media was replaced and cells were incubated at 37°C overnight. Cell viability was assessed by trypan blue dye exclusion. The number of surviving cells is presented relative to the number initially plated (designated 100%). Stained and unstained cells were counted in three different fields of a hemocytometer. Apoptosis (DNA fragmentation) was assessed using a cell death detection ELISA kit (Boehringer Mannheim, Indianapolis, IN, USA) as previously described [24].

Nitrite measurements

Experiments were conducted to evaluate whether nitric oxide production is enhanced under UV irradiation or DPPH exposure. RAW 264.7 cells were either UV irradiated (1 h) or pretreated with fdCC (10 μ g/ml) for 2 h and then irradiated with UV light (1 h). After the UV irradiation, fresh media was replaced and cells were incubated overnight at 37°C. Nitric oxide release was determined spectrophotometrically by the accumulation of nitrite in the medium (phenol red free) with the Griess reagent as previously described [17]. For the DPPH experiments cells were either treated with 2 ml HBSS containing DPPH (0.3 μ M) or simultaneously given fdCC (10 μ g/ml) and DPPH (0.3 μ M) for 1 h. After treatment, the medium was replaced and cells were incubated overnight. Nitrite release into the medium was determined as outlined above.

Measurement of TNF α . The ability of cat's claw to inhibit TNF α synthesis/release in vitro was determined by stimulating TNF α production in RAW 264.7 cells after administration of lipopolysaccharide (LPS). Cells were either pretreated with cat's claw for 2 h and/or treated with LPS 0.5 μ g/ml for 1 h then the media replaced and cells were incubated at 37°C overnight. Culture medium was collected for determination of TNF α levels using the Quantikine M mouse TNF α Immunoassay kit (R & D Systems Inc., Minneapolis, MN,

USA). Samples were processed for ELISA determinations following the manufacturer's recommendations.

Statistical analysis. Each experiment was performed at least three times and results are presented as the mean \pm SEM. Statistical analyses were performed using *t*-test and one-way ANOVA. Post hoc comparison of means was done by Least Significant Difference test and unpaired *t*-test. A probability value of $< .05$ was considered significant.

RESULTS

Scavenging of free radicals by cat's claw extracts

The inhibition of the free radical DPPH by the soluble solids in the two cat's claw extracts is shown in Fig. 1. The results indicate that both forms of cat's claw, freeze-

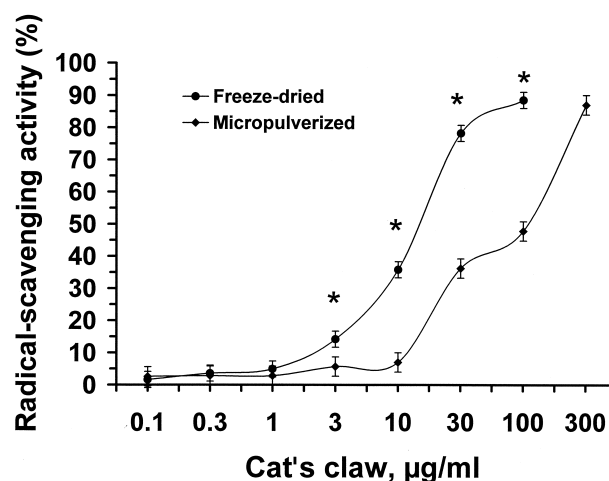


Fig. 1. Antioxidant activity of cat's claw assessed by the DPPH radical method. Cat's claw, freeze-dried and micropulverized, was used for these experiments. The DPPH scavenging capacity of cat's claw was assessed spectrophotometrically at 515 nm. In vitro reactions were carried out for 10 min and the degree of DPPH inhibition is expressed as absorbance depletion in percentage compared to the stable absorbance of DPPH (60 μ M) at 10 min. Values are mean \pm SEM of three experiments with three samples each. *Significant inhibition ($p < .001$) compared to the same concentration of micropulverized cat's claw.

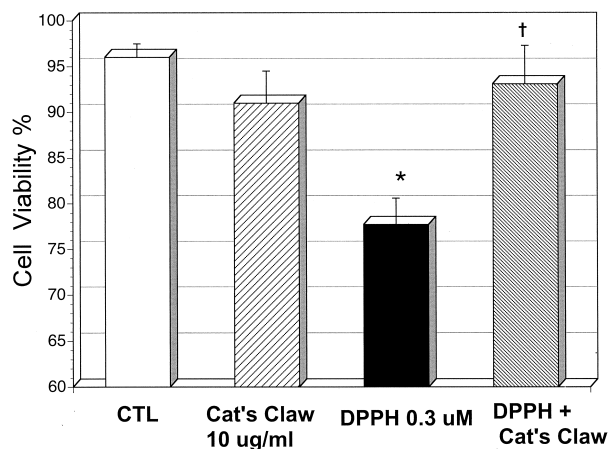


Fig. 2. Viability in RAW 264.7 cells after treatment with DPPH (0.3 μ M) and/or simultaneous administration of freeze-dried cat's claw extract (10 μ g/ml). Cells were plated at a density of 1×10^6 cells/well. Values are mean \pm SEM of three experiments, each with three samples. *Significant decrease ($p < .01$) compared to all other groups. † significant increase ($p < .01$) compared to DPPH.

dried and micropulverized, were effective at inhibiting DPPH in a concentration dependent manner (0.1–300 μ g/ml). Between the two forms of cat's claw, the freeze-dried was the most effective ($p < .01$) scavenger of DPPH (EC_{50} , 18 μ g/ml) compared to the micropulverized (EC_{50} , 150 μ g/ml).

Cell viability is influenced by DPPH

The cytotoxicity of the free radical DPPH was determined by exposing RAW 264.7 cells to DPPH (0.3, 1, 3, and 10 μ M) for 1 h and incubated for 16 h. Exposure to DPPH decreased ($p < .001$) cell viability at all concentrations tested. In another set of experiments, RAW 264.7 cells were treated with DPPH (0.3 μ M) and/or treated simultaneously with freeze-dried cat's claw (10 μ g/ml) and DPPH (0.3 μ M) and incubated for 16 h. The addition of cat's claw to an HBSS-containing DPPH prevented the loss in cell viability (Fig. 2). Comparable results were noted when a higher concentration of DPPH was used (1 μ M data not shown). Cytoprotection by cat's claw against DPPH was evident at concentrations of cat's claw that were able to sequester DPPH in vitro.

Cat's claw protects against UV-induced cell death

Experiments to assess the deleterious effect of UV irradiation for 1 h, with and without freeze-dried cat's claw pretreatment (10 μ g/ml), were conducted to delineate the protective contribution of cat's claw. Viability of RAW 264.7 cells was significantly ($p < .001$) compromised by the exposure to UV irradiation (Fig. 3). Cells pretreated with cat's claw were protected ($p < .01$)

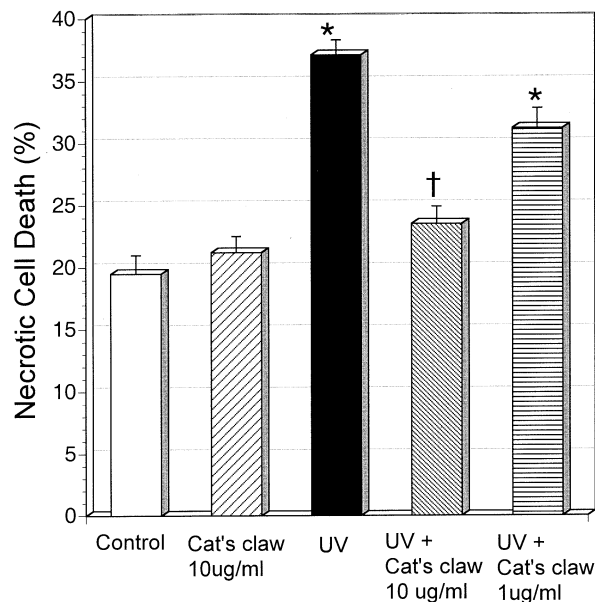


Fig. 3. Necrotic cell death of RAW 264.7 treated with UV light and/or freeze-dried cat's claw extract (10 μ g/ml). Cells were plated at a density of 1×10^6 cells/well. Bars represent the percentage of dead cells after 16 h incubation as described in Materials and Methods. Values are mean \pm SEM of three experiments, each with three samples. *Significant increase ($p < .001$) compared to all other groups. † significant protection ($p < .01$) compared to UV.

against cell death (necrosis) induced by UV irradiation. Results from the apoptosis experiments showed that RAW 264.7 cells irradiated with UV light showed a significant increase ($p < .01$) in cytosolic DNA fragments (apoptosis) compared to the control group. However, pretreatment with cat's claw did not show a significant reduction in apoptosis of RAW 264.7 cells compared to UV irradiation (Fig. 4).

Nitrite production

RAW 264.7 cells exposed to UV irradiation did not show an increase in nitric oxide production as assessed by nitrite release in to the media (average 22.4 nM). In another set of experiments with RAW 264.7 cells, the administration of the free radical DPPH (0.3 μ M) failed to increase nitrite production. These results indicate that cell death induced by DPPH or UV irradiation is unlikely to involve increased NO production secondary to expression of inducible nitric oxide synthase (iNOS).

TNF α production

In vitro experiments with RAW 264.7 cells were conducted to evaluate the protective effect of cat's claw against LPS-induced TNF α production. Results shown in Fig. 5 demonstrated that a pretreatment with cat's claw

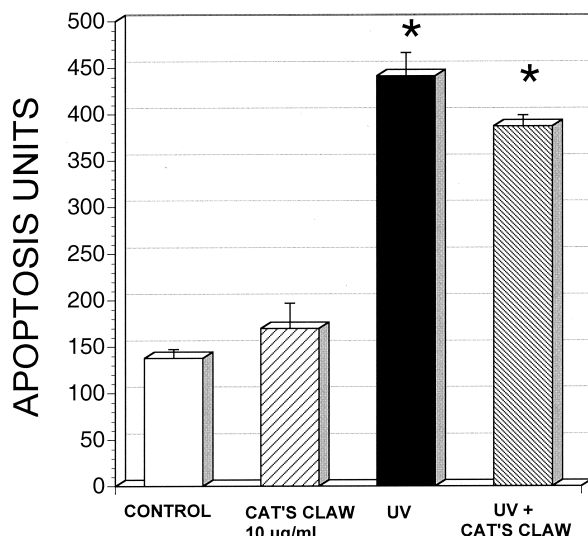


Fig. 4. Apoptosis in RAW 264.7 cells after treatment with UV light and/or freeze-dried cat's claw (10 $\mu\text{g}/\text{ml}$). Cells were seeded at a density of 1×10^6 cells/well. Bars represent enrichment of cytosolic DNA fragments of cells incubated for 16 h. Details of the experiments are described in Materials and Methods. Values are mean \pm SEM of two experiments, each with three samples. *Significant increase ($p < .001$) in apoptosis compared to control and cat's claw.

for 2 h exerted a significant reduction ($p < .001$) in TNF α synthesis in cells challenged with LPS. This response was evident over the entire dose range studied (0.001 to 3 $\mu\text{g}/\text{ml}$) for the freeze-dried cat's claw. A

similar protective response against LPS-induced TNF α production was also observed in cells pretreated with micropulverized cat's claw, although it was less effective (Fig. 5). This is the first demonstration of TNF α inhibition by cat's claw; an action registered at remarkably low concentrations especially for an aqueous extract. A comparison of the antioxidant activity assessed by DPPH and the efficacy to inhibit TNF α production by cat's claw is shown in Table 1. These results demonstrate that freeze-dried cat's claw is a more potent TNF α inhibitor and DPPH scavenger than micropulverized cat's claw. Additionally, the ability of cat's claw to block TNF α production is approximately 1.5×10^4 more potent than its antioxidant activity.

DISCUSSION

Cat's claw was an effective antioxidant, scavenging the free radical generator, DPPH in vitro, as well as preventing necrotic cell death in culture in response to DPPH and UV irradiation. In these states of oxidative stress, media nitrite levels were unchanged, indicating that macrophage cell death did not involve the induction of inducible nitric oxide synthase (iNOS) and increased NO production. The concentrations of cat's claw that afforded cytoprotection were consistent with direct free radical scavenging, with an EC_{50} of 18 $\mu\text{g}/\text{ml}$ (freeze-dried formulation). It was not surprising that the freeze-dried formulation of cat's claw was a more effective

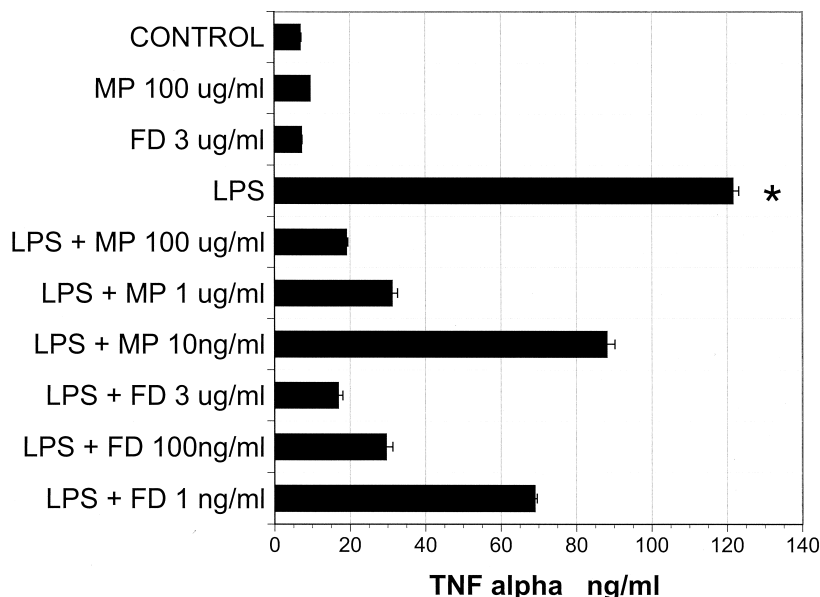


Fig. 5. Effect of cat's claw on LPS-mediated TNF α production by macrophages (RAW 264.7). Cells were seeded at 1×10^6 cells/well. Bars represent TNF α release into the media from cells treated with LPS (0.5 $\mu\text{g}/\text{ml}$) for 1 h or pretreated with freeze-dried (FD) or micropulverized (MP) cat's claw for 2 h then challenged with LPS for 1 h, and incubated for 16 h as described in Materials and Methods. All data represent means \pm SEM for triplicate determinations from three experiments. *Significant increase ($p < .001$) compared to control or cat's claw treated groups.

antioxidant and TNF α inhibitor than micropulverized. Both are water-extracted formulations, consistent with how cat's claw is prepared in the Amazon [4], but the freeze-drying process concentrates solutes from the decoction.

Other studies have addressed the antioxidant activity of cat's claw. In hydroperoxide-induced chemiluminescence [25] the investigators noted activity only at high concentrations of a cat's claw decoction (100–1000 $\mu\text{g/ml}$); concentrations that are unlikely to be achieved in vivo. Lower doses of methanol extracts were mildly effective in preventing TBARS and DNA-sugar damage induced by ferrous ammonium sulfate (1–100 $\mu\text{g/ml}$). However, collectively these results do not provide strong support for the concept that antioxidant activity is the primary means by which cat's claw is an effective anti-inflammatory agent.

We have previously described the induction of iNOS with ionizing radiation [26]. This cellular response does not appear to occur with UV irradiation, as media nitrite levels were unaltered. Cytoprotection against UV irradiation has been noted with a number of antioxidants, including vitamin E, vitamin C, and β -carotene [27,28]. In some cases, gene expression has been induced by UV irradiation consistent with an inflammatory response [29]. However, in this study cat's claw was effective in preventing cell death at doses consistent with an antioxidant action. Cat's claw may offer a natural approach for skin protection from UV irradiation, like other antioxidants, but additional research is necessary to determine the time course and extent of protection. Other investigators have demonstrated that extracts of cat's claw were negative in the AMES test and attenuated mutagenesis induced by 8-methoxy-psoralen and UVA irradiation [30]. They also noted that consumption of cat's claw tea by smokers reduced the mutagenicity of their urine in *S. typhimurium* (AMES test), further emphasizing the safety and antioxidant attributes of this botanical.

Apoptosis induced by UV irradiation was not reduced by cat's claw at the doses studied. Apoptosis in response to UV irradiation is likely to reflect a cellular response to damage. Cells that were previously destined to die by necrosis may have been partially salvaged by cat's claw, yet protection was insufficient to prevent apoptosis. It is possible that the number of apoptotic cells noted in the presence of UV irradiation and cat's claw may reflect a population of cells that in the absence of cat's claw would have died via necrosis. In any case, it is necrotic cell death that primarily extends an inflammatory process, and the actions of cat's claw are representative of an antioxidant and anti-inflammatory agent.

Although NF- κ B is an oxidant sensitive transcription factor [31,32], the ability of cat's claw to suppress LPS-induced TNF α release was noted at remarkably lower

concentrations than its ability to interact with DPPH or afford cytoprotection against UV radiation, DPPH or our previous findings with peroxynitrite [17]. Antioxidant activity was observed at 3–10 $\mu\text{g/ml}$ and anti-TNF α effects were observed at doses as low as 1.2–30 ng/ml. This is particularly impressive when one considers that this is not a pure chemical but rather a simple aqueous extraction process of the bark of a vine from the Amazon jungle. It is highly likely that the active principle from this mixture will have an EC₅₀ for TNF α inhibition in the low pM range, a result that would be ideal for a pharmaceutical, let alone a botanical that has centuries of clinical experience. Indeed cat's claw was approximately 1.5×10^4 more potent in suppressing TNF α synthesis than its antioxidant activity; a result that is highly suggestive that the anti-inflammatory actions of cat's claw in vivo may involve TNF α suppressive actions and related activities (NF- κ B inhibition). These actions are well within the dose range expected with current formulations. For example, recommended doses for commercial freeze-dried cat's claw are 100–300 mg/day. If cat's claw distributes evenly throughout the total body water without first pass metabolism, a concentration of 2–6 $\mu\text{g/ml}$ would be expected. This is within the range for activity as an antioxidant and well within the effective dose range to inhibit TNF α production.

Cat's claw does not however, fully suppress LPS-induced TNF α production. It results in a steady 65–85% inhibition over an extensive dose range. This incomplete response may reflect the agonist, LPS, used in this evaluation. LPS is an aggressive stimulant of transcription and activates multiple transcription factors, not just NF- κ B [33]. We have only evaluated the effects of cat's claw on transcription in terms of NF- κ B: other potential transcriptional factors remain to be assessed. Thus, cat's claw may afford only partial inhibition of TNF α production because other transcription factors remain operative with this agonist. While this is conjecture, a selective action is a potential attribute and not a drawback. Complete inhibition of TNF α and other inflammatory/immune mediators may result in a dangerous state of immune suppression, leaving the host vulnerable to invading microorganisms and tumor development. This fear is born out with anti-TNF α antibody therapy where the incidence lymphomas are increasing [20]. It is perhaps typical that a botanical can afford efficacy with reduced toxicity because its actions are limited in degree but not scope.

Besides the anti-inflammatory properties of cat's claw this herbal medicine has ethnomedical applications in cancer and hyperproliferative disorders like benign prostate hypertrophy [3,4]. Suppression of NF- κ B activity may offer an explanation for these anecdotes, as activation of NF- κ B is linked to cell proliferation evident in a

number of disorders and inflammatory states. In a recent study, a water extract of cat's (C-Med 100) suppressed proliferation and induced apoptosis in two human leukemic (K562 and HL60) and one EBV-transformed lymphoma cell line (Raji) [34]. While these responses were noted at high concentrations (100–400 $\mu\text{g/ml}$) of this extract it is possible that suppression of NF- κB activity was involved. Others and we have noted that cells of lymphoid origin have a constitutive NF- κB activity that acts to prevent apoptosis upon cell activation, and suppression of NF- κB can lead to apoptosis [35,36]. However, it is worthwhile emphasizing that the present study describes suppression of TNF α production by extracts in the ng/ml concentration range, and the antiproliferative responses require much higher concentrations ($> 100 \mu\text{g/ml}$). Another report has also demonstrated an increase in IL-1 and IL-6 with cat's claw [37], but these effects were registered at high doses of a freeze-dried concentrate (100–500 $\mu\text{g/ml}$), indicating that L-1 release was associated with cell death induced by toxic doses of cat's claw.

While the present report is an in vitro study, it is consistent with our previous in vivo finding [17]. Nevertheless, additional clinical studies are needed to confirm these mechanistic evaluations. Thus, these findings support the proposed anti-inflammatory actions of cat's claw. The ability of cat's claw to reduce TNF α production at low concentrations suggests that cat's claw may be an excellent adjunctive therapy in chronic inflammation and worthy of consideration in disorders characterized by an excessive Th1 response, and in particular, aberrant TNF α production.

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