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Anti-inflammatory activity of two different extracts of *Uncaria tomentosa* (Rubiaceae)

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Abstract

We assessed in vivo the anti-inflammatory activity of two Cat's claw bark extracts, by comparing a spray-dried hydroalcoholic extract against an aqueous freeze-dried extract, to determine which extract was more effective. We used the carrageenan-induced paw edema model in mice. In addition, to assess the molecular mechanism of action, we determined the inhibition of NF- κ B through the Electrophoretic Mobility Shift Assay (EMSA) and the effects on cyclooxygenase-1 and -2. Results showed that the anti-inflammatory activity was significantly higher using the hydroalcoholic compared with the aqueous extract ($P < 0.05$). The extracts also showed little inhibitory activity on cyclooxygenase-1 and -2. It cannot be excluded that the slight inhibitory activity on DNA binding of NF- κ B is due to cytotoxic effects. © 2002 Elsevier Science Ireland Ltd. All rights reserved.

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1. Introduction

Uncaria tomentosa (Willd.) DC. (Rubiaceae), commonly known as 'Cat's claw' or 'uña de gato', is a vine that grows in the Amazon rainforest. In Peru, its bark has been traditionally used for the treatment of many ailments, such as viral infections, cancer, gastric illnesses (gastric ulcers), arthritis and other inflammatory disorders (Reinhard, 1997). Cat's claw bark contains a series of secondary metabolites, such as oxindole alkaloids (OAs) and polyphenols (flavonoids, proanthocyanidins, tannins) and small concentrations of other secondary metabolites, such as quinovic acid glycosides, polyhydroxylated triterpenes and saponins (Wagner et al., 1985; Aquino et al., 1989, 1990; Laus et al., 1997).

Preparations of Cat's claw are often used because of its anti-inflammatory activity which is related to more

than one secondary metabolite acting in synergy (Reinhard, 1997). Recently, its cytoprotective effect against oxidative agents such as peroxynitrite has been reported (Sandoval-Chacon et al., 1998). In the same study, it was reported that an aqueous bark extract inhibited lipopolysaccharide-induced inducible NO synthase gene expression and the activation of the transcription factor NF- κ B. Synthesis of TNF- α was also inhibited and it has been discussed that the primary mechanism for Cat's claw anti-inflammatory actions may be due to immunomodulation via suppression of TNF- α synthesis (Sandoval et al., 2000).

In traditional medicine, Cat's claw products have become increasingly popular in recent years. However, depending on the extraction procedure the preparations often differ in their anti-inflammatory activity. Therefore, it is very important to optimize the extraction process to obtain a Cat's claw product with a high anti-inflammatory activity. Here we studied an aqueous alcoholic spray-dried extract for its anti-inflammatory activity using the carrageenan-induced mice paw oe-

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dema model (Sugishita et al., 1981) in comparison with a freeze-dried aqueous extract. In order to elucidate the molecular mechanism of the anti-inflammatory activity, the extracts were studied for their ability to inhibit the activation of the transcription factor NF- κ B and the enzymes of the arachidonic acid pathway, cyclooxygenase-1 and 2. NF- κ B is a pivotal regulator of the human immune response (Baeuerle and Henkel, 1994); it regulates the transcription of various proinflammatory mediators, e.g. cytokines, such as IL-1, IL-2, IL-6, IL-8 and TNF- α or genes encoding cyclooxygenase-2 (Barnes and Karin, 1997). Cyclooxygenase-1 and -2 are enzymes involved in the arachidonic pathway and maintain inflammatory processes (Vane and Botting, 1995).

2. Methodology

2.1. Extracts

Two bark extracts, which basically differed in the extraction method, were used in this study.

- A) Extract A: Uncitolina[®] EA5: *U. tomentosa* hydro-alcoholic, (80% of ethanol) spray-dried extract (drug extract ratio 8:1) provided by, and trademark of, Naturalfa, Química Suiza S.A., containing 5.61% of total oxindole alkaloids (TOA).
- B) Extract B: Aqueous freeze-dried *U. tomentosa* extract, commercially obtained. It contained a total of 0.26% oxindole alkaloids.

As quality control, we measured the oxindole alkaloids (OA) content in both extracts. Total OA concentrations were determined using high performance liquid chromatography (HPLC) techniques with a Phenomenex Luna column C18(2), (5 μ m, 250 mm \times 4.6 mm). The HPLC analysis was carried out using a Perkin-Elmer Series 200 (Figs. 1 and 2). The standard OAs mitraphylline, isomitraphylline, pteropodine, isopteropodine (Chromadex) were used for calibration as external standards, giving identical plots within statistical error. A linear relationship between peak area and concentration of mitraphylline, isomitraphylline, pteropodine and isopteropodine was established in the range 0.01–20 μ g/ml. The detection limits were in the range 1.0–5.0 ng/ml.

2.2. Anti-inflammatory effect: carrageenan-induced mouse paw oedema

2.2.1. Animals

Adequately nurtured female BALB/c mice of 30–50 days of age, weighing between 25 and 30 g were used. These animals were obtained from the National Health Institute (Lima, Peru).

2.2.2. Test on mice using Uncitolina[®] EA5 and a freeze-dried bark extract

Groups of eight mice were allotted to each experimental group. The two extracts used in the study were diluted in 1 ml of distilled water in the following concentrations; 500, 200, 100, and 50 mg/kg of the specimen's body weight.

Mice received their corresponding extract through a blunt-pointed metal gastric probe during 8 days, before any inflammations were induced. About 1 h after their last dose had been administered, they received a 0.05 ml s.c. injection of carrageenan (1% in physiological saline solution) in the right paw (Sugishita et al., 1981).

The control group (negative control) only received the vehicle (distilled water), while the positive control group received indomethacin as anti-inflammatory agent (7 mg/kg).

The paw inflammation was measured 4 h after the carrageenan injection, with the help of a micrometer. The inhibition percentage of the inflammatory reaction produced by the carrageenan was calculated in the acute phase 4 h after its administration.

2.3. Inhibition of NF- κ B: electrophoretic mobility shift assay

2.3.1. Cell culture

Jurkat T-cells were maintained in RPMI 1640 medium, 293 cells in Dulbecco's modified eagle medium. Both were supplemented with 10% fetal calf serum, 100 IU/ml penicillin and 100 μ g/ml streptomycin (all Gibco-BRL). Cycloheximide was purchased from Sigma (USA), and TNF- α was a kind gift of Professor Dr K. Decker, Freiburg, Germany.

2.3.2. NF- κ B electrophoretic mobility shift assays (EMSA)

Jurkat T cells were incubated with the extracts at various concentrations for 1 h. Subsequently, cells were stimulated for 1 h with 200 U/ml TNF- α , after which total cell extracts were prepared. These extracts were prepared using a high-salt detergent buffer (Totex: 20 mM Hepes, pH 7.9, 350 mM NaCl, 20% (v/v) glycerol, 1% (w/v) Nonidet P-40, 1 mM MgCl₂, 0.5 mM EDTA, 0.1 mM EGTA, 0.5 mM DTT, 0.1% phenylmethylsulfonyl fluoride, 1% aprotinin). Cells were harvested by centrifugation, washed once in ice-cold PBS (Sigma), and resuspended in 4 cell volumes of Totex buffer. The cell lysate was incubated on ice for 30 min and then centrifuged at 13 000 rpm at 4 °C for 5 min. The protein content of the supernatant was determined and equal amounts of protein (10–20 μ g) added to a reaction mixture containing 20 μ g of bovine serum albumin (Sigma), 2 μ g of poly(dI-dC) (Boehringer), 2 μ l of buffer D+ (20 mM Hepes, pH 7.9, 20% glycerol, 100 mM KCl, 0.5 mM EDTA, 0.25% Nonidet P-40, 2 mM DTT, 0.1%

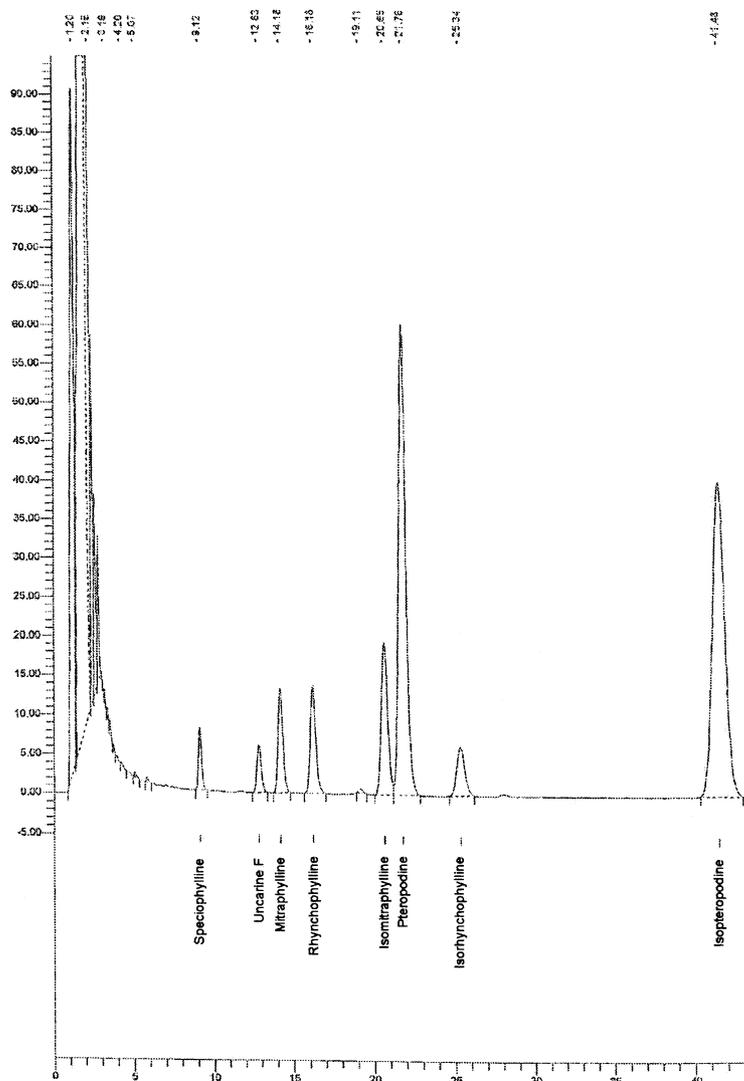


Fig. 1. HPLC analysis of OAs in Extract A.

phenylmethylsulfonyl fluoride), 4 μ l of Buffer F (20% Ficoll 400, 100 mM HEPES, 300 mM KCl, 10 mM DTT, 0.1% phenylmethylsulfonyl fluoride), and 100 000 cpm (Cerenkov) of a [32 P]-labeled oligonucleotide, made up to a final volume of 20 μ l with distilled water. Samples were incubated at room temperature for 25 min. NF- κ B oligonucleotide (Promega) was labeled using [γ - 32 P]ATP (3000 Ci/mmol; Amersham) and a T4 polynucleotide kinase (Promega). The electrophoresis was performed in a non-denaturing polyacrylamide gel (4%), in TBE buffer at pH 8.0, which was then developed using a Kodak XAR-5 film. Stimulation with TNF- α induced one novel DNA binding activity in Jurkat T cells, which was identified by competition assays as an NF- κ B p50/p65 heterodimer. Disappearance of this shift indicated that activation of NF- κ B is inhibited.

2.4. Cyclooxygenase-1 and -2 assays

The COX-1 assay was performed as described by Redl et al. (1994). In brief: 10 μ l of the sample solution were added to 190 μ l of 0.1 M Tris (USB)-HCl, 18 μ l of L-adrenaline-D-hydrogentartrate (Merck) and 10 μ l of hematine (Sigma). After addition of 0.2 units of COX-1 (Cayman) it was preincubated for 5 min, and 5 μ l arachidonic acid was added. The incubation at 37 $^{\circ}$ C was stopped after 20 min by 10 μ l formic acid 10%. The PGE $_2$ -concentration was measured with a PGE $_2$ -enzyme-immunoassay (R and D Systems).

The COX-2 assay was performed as described by Reininger and Bauer (1998). In brief: 10 μ l of the sample-solution were added to 190 μ l of 0.1 M Tris (USB)-HCl, 18 μ l of L-adrenaline-D-hydrogentartrate

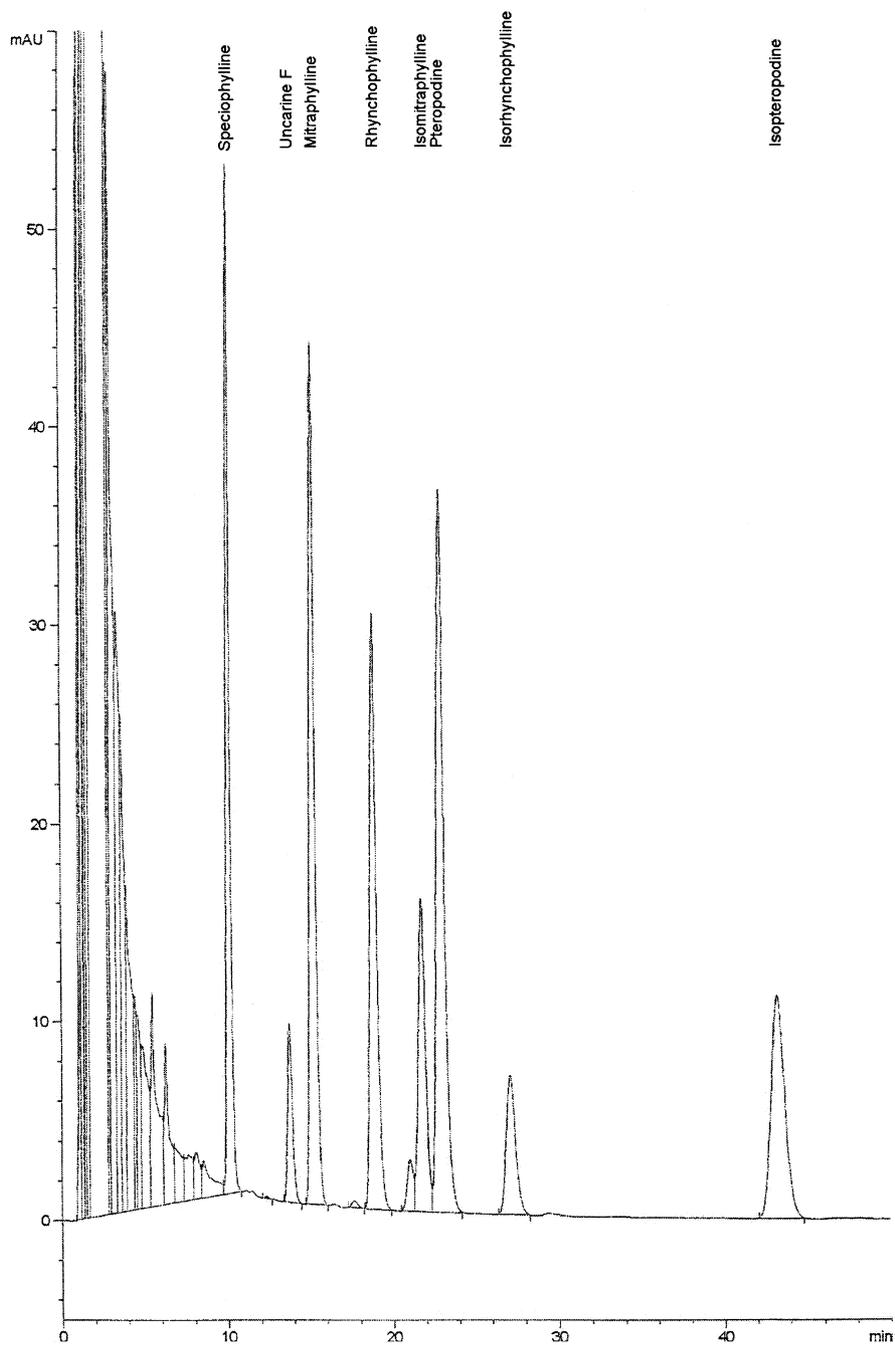


Fig. 2. HPLC analysis of OAs in Extract B.

(Merck), 10 μM Na_2EDTA , and 10 μM of hematine (Sigma). After addition of 0.2 units of COX-2 (Cayman) it was preincubated for 5 min. Then 5 μM arachidonic acid were added. The incubation at 37 $^\circ\text{C}$ was stopped after 20 min by 10 μl of formic acid 10%. The PGE_2 -concentration was measured with an PGE_2 -enzyme-immunoassay (R and D Systems).

2.5. Statistical analysis

The statistical significance between the two treated groups and the control group was calculated through the analysis of variance (ANOVA) for the in vivo assay using SPSS software. P values less than 0.05 ($P < 0.05$) were considered as indicative of significance. The

EMSA were carried out in duplicate. The results of the COX-1 and COX-2 assays are means of three experiments.

3. Results

3.1. Anti-inflammatory effect: in vivo model

The maximum paw inflammatory effect, caused by carrageenan (1%) injection, takes place 4 h after its administration. The extracts dose-dependently and significantly decreased the carrageenan-induced increase in paw volume as compared with control rats (Table 1). Hydroalcoholic extract (50 mg/kg) A, with 5.61% TOA, produced an anti-inflammatory effect similar to 7 mg/kg of the non-steroidal drug indomethacin. The aqueous freeze-dried extract B exhibited the same effect at the higher dose of 200 mg/kg.

3.2. Inhibition of NF-κB: in vitro model

In order to investigate whether the anti-inflammatory effect observed in the carrageenan-induced mouse paw oedema is caused by cytokine suppressing effects, the extracts were studied in the NF-κB EMSA.

As observed in Fig. 3 (lane 6), activation of NF-κB was nearly completely reduced by pretreatment of Jurkat cells with 500 µg/ml of the hydroalcoholic extract A, while the aqueous extract B only slightly prevented NF-κB DNA binding at this concentration.

3.3. Inhibition of cyclooxygenase-1 and 2

Inhibition of enzymes of the arachidonic acid pathway may also contribute to the anti-inflammatory effects. Therefore, both extracts were studied at a concentration of 50 µg/ml whether they inhibit cyclooxygenase-1 and -2. Interestingly, the hydroalcoholic Cat's claw extract A exhibited an inhibition of cyclooxygenase-1 and -2 of 7.8% and of 21.7%, respectively. In contrast, an inhibitory activity of 32.7% was measured

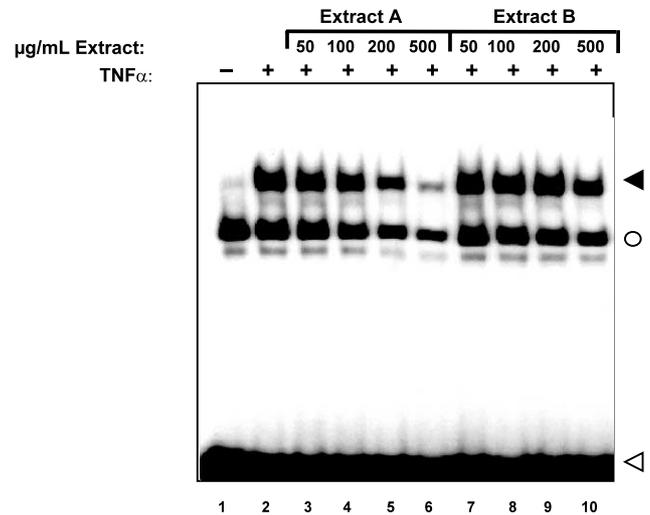


Fig. 3. The effect of the hydroalcoholic (A) and the aqueous (B) Cat's claw bark extract on NF-κB DNA binding. Lane 1 shows unstimulated Jurkat cells, in lane 2 cells were treated with 200 U/ml TNF-α alone. In the other lanes (3–10) cells were pretreated for 1 h with various concentrations of the respective bark extract and subsequently stimulated with TNF-α for 1 h. Equal amounts of protein from cell extracts were analyzed for NF-κB activity by EMSA. A filled arrowhead (▲) indicates the position of NF-κB DNA complexes. The open circle (○) denotes a non-specific activity binding to the probe and the open arrowhead (◁) shows unbound oligonucleotide.

Table 2
Inhibition of cyclooxygenase-1 and -2 by an hydroalcoholic (A) and an aqueous (B) Cat's claw extract at a concentration of 50 µg/ml (S.D. in brackets)

Extract	Cyclooxygenase-1	Cyclooxygenase-2
Hydroalcoholic (A)	7.8 (±10.4)	21.7 (±3.1)
Aqueous (B)	32.7 (±6.6)	12.2 (±6.5)

Table 1
Anti-inflammatory effect of an hydroalcoholic (A) and an aqueous (B) Cat's claw extract in mice's paw oedema after 4 h

Dose (mg/kg)	n	Anti-inflammatory activity (%)		
		Extract A	Extract B	Indomethacin
7	8			37.8
50	8	35.3	21.9	
100	8	37.3	23.8	
200	8	43.0	33.0	
500	8	51.7	26.7	

for cyclooxygenase-1 and of 12.2% for cyclooxygenase-2 (see Table 2) for the aqueous freeze-dried extract B.

4. Discussion and conclusions

The results show that both Cat's claw bark extracts have an anti-inflammatory action in various degrees. However, this activity was much more evident when the hydroalcoholic extract A was used. This difference may be due to the higher concentration of compounds present in this preparation compared with the aqueous extract B. As is well known, when an aqueous alcoholic mixture is used as an extraction system, it is possible to extract secondary metabolites of a relatively wide range of polarities. On the contrary, when water is used, only high polarity compounds are extracted. However, since both extracts have not been obtained from the same plant material, it is not absolutely clear, whether the different content of alkaloids is only due to the extraction procedure.

Further studies were undertaken to elucidate the molecular mechanism by which the extracts exert their anti-inflammatory activity. Only the hydroalcoholic extract A impaired NF- κ B DNA binding at a higher concentration (500 μ g/ml) using Jurkat T cells and TNF- α as stimulus. Therefore, it is unlikely that this transcription factor is the only responsible for Cat's claw anti-inflammatory effect. However, it cannot be excluded that the high amount of extract A used could be causing cytotoxic effects influencing the results on NF- κ B inhibition. On the other hand, it has to be kept in mind that an aqueous extract of Cat's claw, with unknown composition has already inhibited NF- κ B DNA binding in RAW cells (macrophages) stimulated with lipopolysaccharide at a concentration of 100 μ g/ml (Sandoval-Chacon et al., 1998).

Both extracts A and B showed moderate to weak activity against COX-1 and COX-2 in vitro. Both extracts differed in their activity against cyclooxygenase-1 and 2, with extract A being more effective against COX-2 and extract B towards COX-1. This effect, however, is not significant and further studies are necessary to clarify whether there is any selectivity towards these enzymes.

The question of which secondary metabolites cause the anti-inflammatory effect cannot be answered up to now. Evidence has demonstrated that some OAs of the pentacyclic type, which could be detected in both extracts by HPLC analysis in different concentrations, affect the cellular immune system. Several in vitro studies have shown that these pentacyclic OAs activate T and B-lymphocytes, increase the rate of phagocytosis of granulocytes and of reticulo-endothelial system cells (Wagner et al., 1985; Wurm et al., 1998). At the same

time, the proliferation of transformed and activated T and B-lymphocytes is inhibited (Keplinger et al., 1999).

Inflammatory disorders are characterized among other events, by the production of significant amounts of free radicals, nitrogen reactive species, as well as cytokines such as TNF- α , IL-1, IL-6 (Holtmann and Resch, 1995). The use of particular compounds of Cat's claw may modify the action or production of these (Sandoval-Chacon et al., 1998; Wurm et al., 1998; Lemaire et al., 1999; Sandoval et al., 2000). Since the inflammatory response is intimately associated to the immune process, benefits of Cat's claw in the immune system could also be expressed as an anti-inflammatory action.

This study demonstrates that both Cat's claw bark extracts have an anti-inflammatory activity. The effect of the tested hydroalcoholic extract A was stronger, suggesting that this action could be due to the presence of pentacyclic OAs acting alone or synergistically with other metabolites.

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