



## Anti-inflammatory activity of Mitraphylline isolated from *Uncaria tomentosa* bark

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### ABSTRACT

**Ethnopharmacological relevance:** *Uncaria tomentosa* (Willd. ex Roem. & Schult.) DC. (Rubiaceae) is widely used by populations living in South America to treat many ailments associated with inflammatory disorders. Mitraphylline was shown to be the major pentacyclic oxindolic alkaloid present in the bark chloroformic extract of this plant. Its activity against cytokines involved in inflammation process was tested in a murine model *in vivo*.

**Materials and methods:** Mice received mitraphylline once a day for 3 days at 30 mg/kg/day by oral route. Then, they were subjected to bacterial lipopolysaccharide (LPS) endotoxin (15 mg/kg) and the LPS-induced production of 16 different cytokines was determined by Elisa multiplex. Control group received dexamethasone orally at 2 mg/kg/day. Toxicity on K565 cells and murine peritoneal macrophages, *in vitro*, at doses up to 100  $\mu$ M was monitored by XTT-colorimetric assay.

**Results and conclusions:** For the first time mitraphylline was tested *in vivo* against a large range of cytokines that play a crucial role in inflammation. Mitraphylline inhibited around 50% of the release of interleukins 1 $\alpha$ , 1 $\beta$ , 17, and TNF- $\alpha$ . This activity was similar to dexamethasone. It also reduced almost 40% of the production of interleukin 4 (IL-4) while the corticoid did not. Lastly it did not show any toxicity on K565 cells nor murine macrophages at doses up to 100  $\mu$ M.

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### 1. Introduction

*Uncaria tomentosa* (Willd. ex Roem. & Schult.) DC. (Rubiaceae) is a woody vine growing up to 30 m, distributed from the Amazonian to Central American rain forests. It is popularly known as “Uña de Gato” (Cat’s claw) because of its claw-shaped thorns. In traditional medicine, people use it in the form of a decoction taken orally. Three bark strips of approximately 11 cm  $\times$  3 cm are placed in 1 l of cold water and boiled gently for at least 15–20 min to obtain a strong, dark dyed extract. Three cups (ca. 150 ml) of this decoction are drunk for the treatment of various diseases related to inflammatory processes (Aguilar et al., 2002; Bourdy com. pers.). *U. tomentosa* is one of the most commonly used plants

in Latin America for the treatment of a wide-array of symptoms (Obregón, 1997; Keplinger et al., 1999; Williams, 2001; Heitzman et al., 2005). For example, we have previously shown that in Bolivia, the Tacanas administer *U. tomentosa* bark concentrated decoction for the treatment of rheumatism, irregular menstruation, and ailments of digestive tract, liver, and kidney (Bourdy et al., 2000). The traditionally reported medicinal properties of *U. tomentosa* have been validated by numerous experimental studies demonstrating its antiviral, antioxidant, antiproliferative, immunostimulant, antimicrobial, and anti-inflammatory activities (Aquino et al., 1989; Senatore et al., 1989; Desmarchelier et al., 1997; Sheng et al., 1998, 2000, 2001; Wurm et al., 1998; Lemaire et al., 1999; Lamm et al., 2001; Riva et al., 2001; Aguilar et al., 2002; Sandoval et al., 2002; Akesson et al., 2003; Deharo et al., 2004; Winkler et al., 2004; Goncalves et al., 2005; Pilarski et al., 2006; Allen-Hall et al., 2007; Hardin, 2007).

The anti-inflammatory activity of traditional extracts made from *U. tomentosa* bark is well documented (Erowele and Kalejaiye, 2009). A water extract of micro-pulverised bark of *U. tomentosa* was able to inhibit the expression of tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ), which is known to stimulate the acute

**Abbreviations:** IL, Interleukin; LPS, Lipopolysaccharide; THP-1, monocytic cells (ATCC TIB-202); TNF, tumor necrosis factor; XTT, sodium 3'-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro) benzene sulfonic acid hydrate.

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phase reaction of the inflammation processes (Sandoval et al., 2000; Paul et al., 2006; Tincani et al., 2007). In addition, it has been demonstrated that an ethanolic extract of *U. tomentosa* bark was able to inhibit the liberation of TNF- $\alpha$  by lipopolysaccharide (LPS)-activated THP-1 monocytic cells (Allen-Hall et al., 2010).

From a chemical standpoint, *U. tomentosa* has also been extensively studied. The majority of alkaloids of *Uncaria* are of the indole and oxindole families (Laus, 2004). Mitraphylline is the most ubiquitous alkaloid being present in 20 of 34 *Uncaria* species (Heitzman et al., 2005). Nevertheless, its activity on immunomodulatory markers has not been studied so far. Thus, we decided to explore in a murine model the impact of mitrephylline on the production of 16 different cytokines involved in inflammation, using an ELISA multiplex system. We also measured its cytotoxicity on K565 cell line and murine macrophages.

## 2. Materials and methods

### 2.1. Plant material

Bark from *U. tomentosa* was collected in Pucallpa (Ucayali region), Eastern Peru. Extraction with solvents of increasing polarity was performed on dried milled material. A voucher (BM 1500) was deposited at the National Herbarium of the San Marcos University in Lima, Peru. Pr. Alban Castillo confirmed the botanical determination.

### 2.2. Bioguided fractionation studies

Fifty grams of *U. tomentosa* bark was extracted with chloroform (maceration, 5 days). Chloroform extract showed better anti-TNF- $\alpha$  activity than water extract (20% and 5% at 10  $\mu$ g/ml, respectively), then, the former was subjected to a bioguided fractionation process. Successive normal phase column chromatography, using mixtures of chloroform and ethyl-acetate at increasing polarities, led to the isolation of the pentacyclic oxindole alkaloid, the mitrephylline which was the major compound in the extract (Fig. 1).

HPLC was performed using a VWR-Hitachi Elite Lachrom with photodiode array detector. Samples were injected onto a Purospher®STAR RP-18e column (150  $\times$  4.6 mm; 5  $\mu$ m particle size) and eluted with 35% acetonitrile in phosphate buffer (pH 6.6) at a flow rate of 1.8 ml/min. The volume injected was 20  $\mu$ l and eluates were monitored at 245 nm. Authentic standards of oxindolic alkaloids were obtained from Chromadex (Laguna Hills, CA).  $^1\text{H}$  (300 MHz) and  $^{13}\text{C}$  (75 MHz) NMR experiments were run on a Bruker AC-300, using  $\text{CDCl}_3$  as a solvent for mitrephylline and oxindolic alkaloid standards.

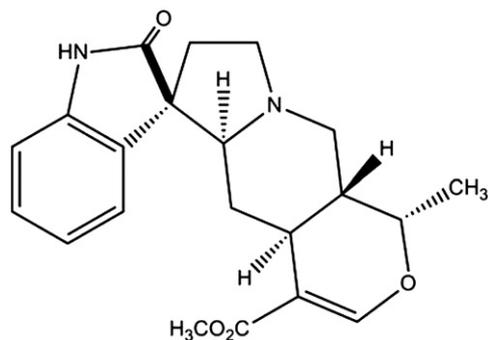


Fig. 1. Structure of mitrephylline.

### 2.3. Biological tests

#### 2.3.1. In vitro cytotoxicity assays

$20 \times 10^3$  cells K562 cells and  $5 \times 10^4$  cells murine peritoneal macrophages were incubated at 37  $^\circ\text{C}$  for 24 or 48 h in 96-wells microplates with increasing concentrations of mitrephylline (1–100  $\mu\text{M}$ ; total volume: 100  $\mu\text{l}$ ). Afterwards, 50  $\mu\text{l}$  of XTT were added to each well. Absorbance of the formazan dye produced by metabolically active cells was measured at 490 nm using a Muktiskan FC Microplate Photometer (Thermo Scientific). Each assay was performed in triplicate.

#### 2.4. In vivo assays

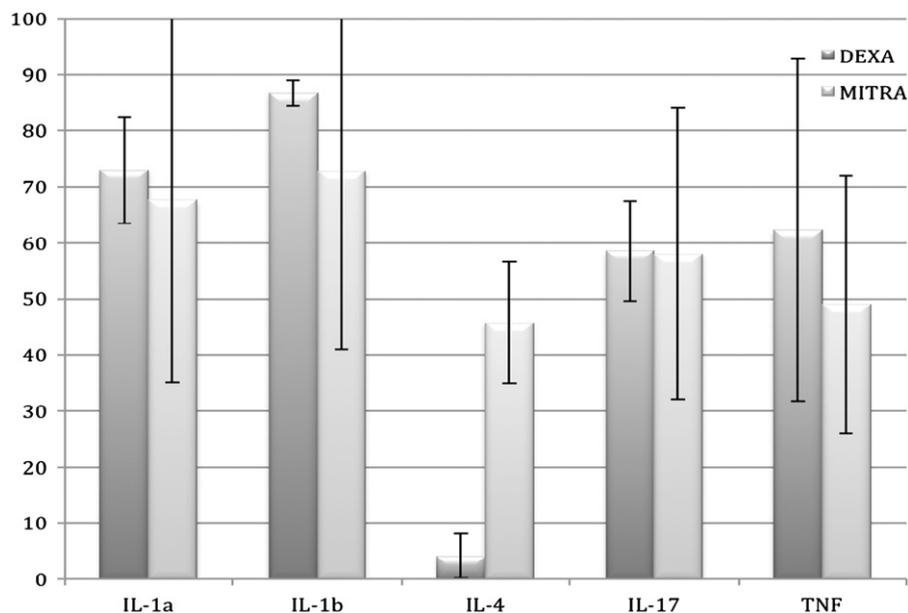
Eight-week-old Balb/c female mice were maintained on a 24 h light/dark cycle, with food and water *ad libitum*. All experimental animal procedures were conducted in accordance with the Guidelines of the National Legislation on Animal Care of the French statutory law (N $^\circ$ 2001-464). Batches of six mice were treated orally with 30 mg/kg/day of mitrephylline, for 3 days. The dose was defined according to preliminary test on mice to assess tolerability, leading to the highest cytokine signal. At that dose no apparent toxicity was observed. The control group received 0.9% saline solution, while dexamethasone (2 mg/kg/day, oral route) was used as reference. Two hours after the last dose, mice were injected intraperitoneally with saline-diluted LPS, which is an endotoxin of Gram-negative bacteria that causes massive release of cytokines. Two hours later, they were sacrificed and blood quickly collected. After coagulation, the serum was recovered and centrifuged for 10 min at 2500 rpm. Sixteen different mouse cytokines [Interleukin (IL)-1 $\alpha$ , IL-1 $\beta$ , IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12, MCP-1, INF- $\gamma$ , TNF- $\alpha$ , MIP-1, GM-CSF, and Rantes] were detected using the mouse cytokine Elisa multiplex Q-plex system, where distinct capture antibodies are spotted to each well of a 96-well plate in a defined array. A total of 30  $\mu\text{l}$  of supernatant was used following manufacturer's instructions (Quansys Biosciences, USA). Detection was performed using the Q-View<sup>TM</sup> Imager. The images were processed using Q-View<sup>TM</sup> Software. Sensitivity typically ranged between 30 pg/ml and less than 1 pg/ml. Statistical analysis was performed with GraphPad Prism 5.01 (GraphPad Software, San Diego).

## 3. Results and discussion

As previously described, our fractionation of a chloroformic extract of *U. tomentosa* revealed that mitrephylline was the preponderant alkaloid of chloroformic *Uncaria* extract (Laus, 2004). We evaluated the biological relevance of this molecule (Fig. 2) and showed that it was able to impair the liberation of TNF- $\alpha$  by 50% when administrated to mice orally at 30 mg/kg for 3 days. It also inhibited nearly 70% of the release of IL-1 $\alpha$  and -1 $\beta$ . This is comparable to dexamethasone, which is known to abolish LPS-induced IL-1 $\beta$  and TNF- $\alpha$  productions (Teeling et al., 2010). This result could have spectacular implications in the treatment of some auto-immune diseases, such as rheumatoid and osteoarthritis, given the crucial roles of IL-1 $\beta$  and TNF- $\alpha$  in their aggravation and progression (Dinarello, 2011).

Mitrephylline also reduced the production of IL-4 about 40% in our murine model, while dexamethasone was inactive. IL-4 is a pleiotropic cytokine produced by activated T cells, mast cells, and basophils. This interleukin is particularly involved in allergies and inflammation (Saggini et al., 2011). Impairing the production of these pro-inflammatory cytokines might offer new therapeutic tactics for the control of allergy and inflammation.

Mitrephylline also reduced IL-17 production by 50%. This interleukin is known to play a protective role in host defence



**Fig. 2.** Percentage of inhibition of IL 1a,b, 4, 17 and TNF in mice receiving DEXA: dexamethasone (2 mg/kg/day) and MITRA: mitraphylline (30 mg/kg/day). The percentage is related to control mice. *P*-value < 0.001 for IL4 only.

especially in bacterial infections. Nevertheless, excessive activation contributes to autoimmunity. Reports from pharmaceutical industries indicate that neutralizing members of this interleukin family have beneficial therapeutic impact in the treatment of some auto-immune diseases (Pappu et al., 2011). Lastly, mitraphylline displayed a focused immunological effect, as it did not show any *in vivo* impact on the levels of IL-2, IL-3, IL-5, IL-6, IL-9, IL-10, IL-12, MCP-1, INF- $\gamma$ , MIP-1, GM-CSF, or Rantes.

Mitraphylline did not show any toxicity against K562 cells nor murine macrophages *in vitro* at doses up to 100  $\mu$ M. This activity seems to be cell specific, as some authors showed that it had a cytotoxic effect on Human Ewing's sarcoma and breast cancer cell lines (Garcia Gimenez et al., 2010). It was also shown to be more cytotoxic than cyclophosphamide and vincristine on human glioma cell lines (Garcia Prado et al., 2007).

For the first time, we showed herein that mitraphylline, the major alkaloid of *U. tomentosa*, is able to modulate the immunological status of mice subjected to LPS, and is probably, at least partially, responsible for the anti-inflammatory activity of *Uncaria* bark extracts. Indeed, extracts have been shown to inhibit the activation of nuclear factor- $\kappa$ B (NF- $\kappa$ B), which regulates host immune and anti-inflammatory responses (Aguilar et al., 2002). It has also been demonstrated that extracts inhibit *in vitro* the expression of TNF- $\alpha$ , promote the liberation of lymphocyte-proliferation-regulating factor by human endothelial cells, and extends lymphocyte survival (Wurm et al., 1998; Akesson et al., 2003). According to certain authors, the anti-inflammatory activity of *Uncaria* extracts would not rely solely upon alkaloids (Sandoval et al., 2002). Nevertheless, alkaloids *per se* are at least partially responsible for the anti-inflammatory activity in traditional medicine. Indeed, a clinical trial carried out on patients affected with rheumatoid arthritis reduced the pain in 50% of patients treated with alkaloids-enriched extract of *U. tomentosa* (Mur et al., 2002).

#### 4. Conclusion

Mitraphylline could be used as a quality control marker for herbal medicines based on the *Uncaria* extracts. It could also be considered as a new lead compound for the development of

anti-inflammatory treatment. Further studies should be conducted to determine the concentration of mitraphylline in extracts and its exact role in the context of whole herbal remedies. In a recent review, Deharo and Ginsburg (2011) clearly pointed out that for many plants used in traditional medicine, the isolated active molecules alone could not explain the activity of the remedy because of their low concentration in the preparation. It would be interesting to search for other compounds, which contribute to the medicinal activity of *U. tomentosa* extracts in an effort to optimize the traditional preparation.

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