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In vitro Effects of Two Extracts and Two Pure Alkaloid Preparations of *Uncaria tomentosa* on Peripheral Blood Mononuclear Cells

Abstract

In the traditional Peruvian medicine, hot aqueous extracts of *Uncaria tomentosa* have been used for the treatment of a wide range of health problems, particularly digestive complaints and arthritis. Some of the beneficial effects observed in patients suggest an immunomodulatory capacity of *Uncaria tomentosa* extracts. In this study, the effects of two extracts and two mixtures of tetracyclic and pentacyclic oxindole alkaloids of *Uncaria tomentosa* were investigated in freshly isolated human peripheral blood mononuclear cells (PBMC) stimulated with the mitogens phytohaemagglutinin (PHA) and concanavalin A (Con A) *in vitro*. Neopterin production and tryptophan degradation were monitored in culture supernatants to determine the effects of the test substances on immunobiochemical pathways induced by interferon-γ. Compared to unstimulated cells PHA and Con A increased the production of neopterin and degradation of tryptophan ($p < 0.01$). HCl and ethanol extracts and mixtures of alkaloids of *Uncaria tomentosa* inhibited both effects in a dose-dependent manner, the lowest effective concentrations of the extracts were 500 – 1000 μg/mL and of the alkaloid mixtures 100 – 175 μg/mL ($p < 0.05$ and < 0.01). With the highest concentrations of extracts and mixtures complete suppression of mitogen-induced neopterin production and tryptophan degradation was observed. These data demonstrate that *Uncaria tomentosa* extracts and mixtures of alkaloids modulate the immunobiochemical pathways induced by interferon-γ. The findings imply a potential application of the extracts as immunoregulators and would be in line with observations in patients using these extracts.

Key words

Uncaria tomentosa · Rubiaceae · immunomodulation · neopterin · tryptophan · indoleamine (2,3)-dioxygenase

Abbreviations

Con A:	concanavalin A
EDTA:	ethylenediaminetetraacetic acid, Titriplex III
IDO:	indoleamine (2,3)-dioxygenase
IFN-γ:	interferon-γ
kyn:	kynurenine
MTT:	3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide
PBMC:	peripheral blood mononuclear cells
PHA:	phytohaemagglutinin
POA:	pentacyclic oxindole alkaloids
ROS:	reactive oxygen species
TOA:	tetracyclic oxindole alkaloids
trp:	tryptophan

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Introduction

Uncaria tomentosa (Willd.) DC. is a giant vine of the Rubiaceae family, Cinchonoidae subfamily, and is indigenous to the Amazon rainforest and other tropical areas of South and Central America, including Peru, Colombia, Ecuador, Guyana, Trinidad, Venezuela, Suriname, Costa Rica, Guatemala and Panama. Traditionally, the bark of *Uncaria tomentosa* and the often confounded *Uncaria guianensis* were prepared as a decoction (extraction with hot water). Scientific and commercial interest in *Uncaria tomentosa* was elicited by reports of miraculous cures of diseases like arthritis, cancer, asthma, stomach ulcers, inflammation of the urinary tract, abscesses, in convalescence (as a tonic), in the treatment of viral diseases, menstrual disorders and disorders of wound healing [1].

Attempts to extract potentially therapeutic components from this plant led to the discovery of 2 chemotypes of *Uncaria tomentosa* with a different pattern of tetracyclic (TOA) or pentacyclic oxindole alkaloids (POA) [2]. Quinovic acid glycosides, sterols, epicatechin, and other ubiquitous components were found in both chemotypes. Some studies revealed immunomodulatory effects of POA. Besides enhancing phagocytosis, as reported for other plant-derived immune modulators [3], POA were shown to inhibit the proliferation of activated lymphocytes while stimulating the proliferation of resting or weakly activated lymphocytes. These effects were antagonistically inhibited by TOA [4].

Uncaria tomentosa-derived preparations are already used as complementary medication, without, however, sufficient clinical evidence of their safety and efficacy. Considerable scientific interest in the plant has been generated by the demonstration of its potent immunostimulatory activity [5], [6]. From such observations it appears reasonable that compounds in *Uncaria tomentosa* could play a role in modulating immune system functions, e.g., influencing the production and secretion of cytokines.

In vitro, monitoring biochemical effects such as neopterin formation or degradation of tryptophan is a convenient way to quantify the effects of cytokines and other immunomodulatory compounds on the T-cell/macrophage interaction [7], [8]. Neopterin, a metabolite of guanosine triphosphate, is produced in humans and primates by the enzyme GTP-cyclohydrolase in monocyte-derived macrophages and dendritic cells upon stimulation with interferon-γ [9], [10]. Therefore, increased neopterin concentrations represent an unspecific marker to detect the Th1-type (= cell-mediated) immune response [9]. In various cells including monocyte-derived macrophages and dendritic cells, the enzyme indoleamine 2,3-dioxygenase is induced by IFN-γ [11]. IDO converts tryptophan, an essential amino acid, to *N*-formylkynurenine, which deformylates to kynurenine [11]. The ratio of the concentration of the product of IDO, kynurenine, versus the substrate tryptophan (= kyn/trp) allows an estimate of IDO activity [12]. Kyn/trp is usually high during cellular immune activation [11]. Parallel induction of neopterin production and tryptophan degradation has already been shown in stimulated monocytic cells and peripheral blood mononuclear cells [7], [8], [13].

In this study, we investigated the influence of two *Uncaria tomentosa* extracts and of POA and TOA mixtures on PBMC by determination of neopterin formation and tryptophan degradation in stimulated and unstimulated PBMC of healthy donors.

Materials and Methods

Test substances

HCl and 96% ethanol (EtOH) extracts of *Uncaria tomentosa* were applied (Krallendorn® and Saventaro®, both from Immodal, Volders, Austria). The HCl extract contained 0.93% pteropodine, 0.55% speciophylline, 0.34% mitraphylline, 0.25% isopteropodine, 0.16% uncarine F and 0.05 isomitraphylline. The EtOH extract contained 0.73% isopteropodine, 0.28% pteropodine, 0.17 isomitraphylline, 0.13% mitraphylline, 0.05% uncarine F, 0.04% speciophylline, 0.015% rhynchophylline, 0.009% isorhynchophylline, 0.003% corynoxeine and 0.001% isocorynoxeine.

Further, POA hydrochlorides mixture containing 1% mitraphylline, 49% pteropodine and 50% isopteropodine, and TOA hydrochlorides mixture containing 1% isocorynoxeine, 4% corynoxeine, 39% isorhynchophylline and 56% rhynchophylline were examined (both mixtures were from Immodal). Considering the composition of the test substances it has to be kept in mind that isomers might interchange in the aqueous solutions used in these experiments. A concentration range of 500 – 4000 μg/mL Krallendorn® and Saventaro® and 50 – 500 μg/mL of POA and TAO hydrochlorides mixtures were applied to PBMC cultures.

Isolation of human PBMC

PBMC were isolated from whole blood from healthy voluntary donors after informed consent was obtained. Separation of blood cells was performed using density centrifugation (Lymphoprep, Nycomed Pharma AS, Oslo, Norway). After isolation, PBMC were washed three times in phosphate-buffered saline containing 0.2% 0.5 mM EDTA. Cells were maintained in RPMI 1640 supplemented with 10% heat-inactivated foetal calf serum (Biochrom, Berlin, Germany), 1% of 200 mM glutamate (Serva, Heidelberg, Germany) and 0.1% of gentamycine (50 mg/mL, Bio-Whittaker, Walkersville, MD) in a humidified atmosphere containing 5% CO_2 for 48 h. For each experiment, PBMC were freshly prepared. Four experiments with three parallels were performed of each experiment.

Stimulation of PBMC

Isolated PBMC were plated at a density of 1.5×10^6 cells/mL in supplemented RPMI 1640, preincubated for 30 minutes with or without *Uncaria tomentosa* extracts and stimulated or not with phytohaemagglutinin (PHA, Sigma, Vienna, Austria) and concanavalin A (Con A, Sigma) for 48 h. The mitogens PHA and Con A induced neopterin production and tryptophan degradation in a dose-dependent way (not shown); 10 μg/mL PHA and 10 μg/mL Con A were used in our experiments. All test substances were dissolved in supplemented RPMI medium (see above), filtered at 0.2 μm and frozen at –20 °C until used.

Viability of cells after stimulation was controlled by the colorimetric 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test (Roche Diagnostics GmbH, Munich, Germany) and by the trypan blue exclusion method.

Measurement of neopterin production and tryptophan degradation

After incubation for 48 h, supernatants were harvested and neopterin concentrations were determined by ELISA (BRAHMS, Berlin, Germany) according to the manufacturer's instructions with a detection limit of 2 nmol/L. Tryptophan and kynurenine concentrations were measured by high pressure liquid chromatography using 3-nitro-l-tyrosine as internal standard [14]. The kyn/trp ratio was calculated and expressed as μmol kynurenine/mmol tryptophan.

Results

Unstimulated cells

In supernatants of unstimulated PBMC an average neopterin concentration of 9.0 ± 1.4 nmol/L was detected after an incubation period of 48 h. Addition of *Uncaria tomentosa* extracts suppressed neopterin production in a dose-dependent way (Fig. **1A**, white bars). The down-regulation of neopterin production differed significantly between HCl and EtOH extracts: EtOH extracts were effective already at lower concentrations, whereas HCl extracts had a significant effect only at the highest concentration of 4000 μg/mL (Fig. **1A**, white bars). The necessary concentrations of POA and TOA mixtures to diminish neopterin production were 50 – 150 μg/mL, TOA appeared to be effective at somewhat lower concentrations than POA (Fig. **2A**, white bars).

The average concentrations of tryptophan and kynurenine in unstimulated PBMC were mean ± S.E.M.: 20.0 ± 1.5 μmol/L and 3.7 ± 0.6 μmol/L, respectively. The Kyn/trp was 201.5 ± 52.1 μmol/mmol. In unstimulated PBMC, the addition of the HCl and EtOH extracts increased tryptophan up to 24.6 ± 0.2 μmol/L and 24.8 ± 0.5 μmol/L, respectively. Mixtures of POA and TOA hydrochlorides increased tryptophan concentrations even slightly more (details not shown). At the same time, all four test substances decreased kynurenine and kyn/trp in a dose-dependent manner (Figs. **1B** and **2B**, white bars).

Mitogen-stimulated cells

PHA and Con A increased neopterin concentrations to 26.1 ± 2.2 and 24.6 ± 0.8 nmol/L, respectively (all $p < 0.01$ compared to unstimulated cells). Both *Uncaria tomentosa* extracts diminished neopterin concentrations significantly with different concentration-dependencies (Fig. **1A**, dotted and filled bars): the lowest effective concentration of HCl and EtOH extracts of

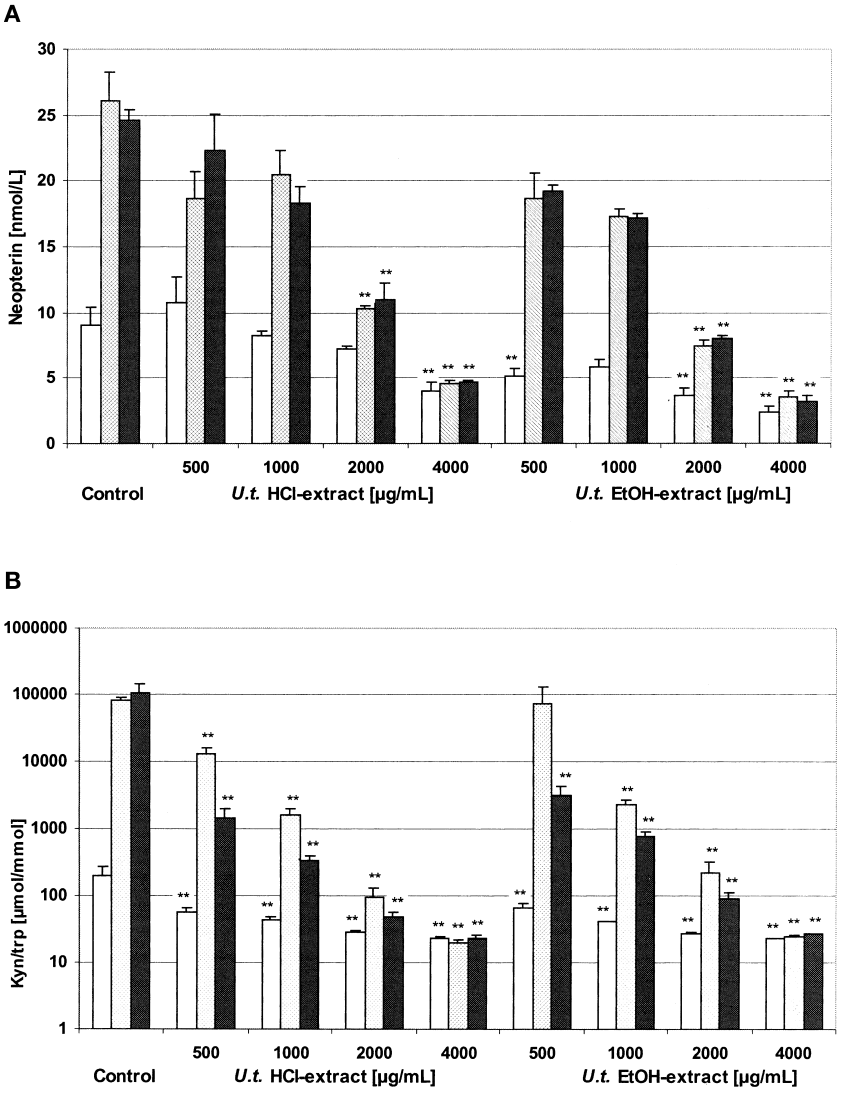

Fig. **1** Neopterin formation (**A**) and tryptophan degradation (**B**) in unstimulated peripheral blood mononuclear cells (white bars) and in cells stimulated with 10 μg/mL phytohaemagglutinin (PHA, dotted bars) and 10 μg/mL concanavalin A (Con A, filled bars) with or without coincubation with HCl and ethanol extracts of *Uncaria tomentosa* (*U.t.*) for 48 hours (* $p < 0.05$, ** $p < 0.01$ compared to the control).

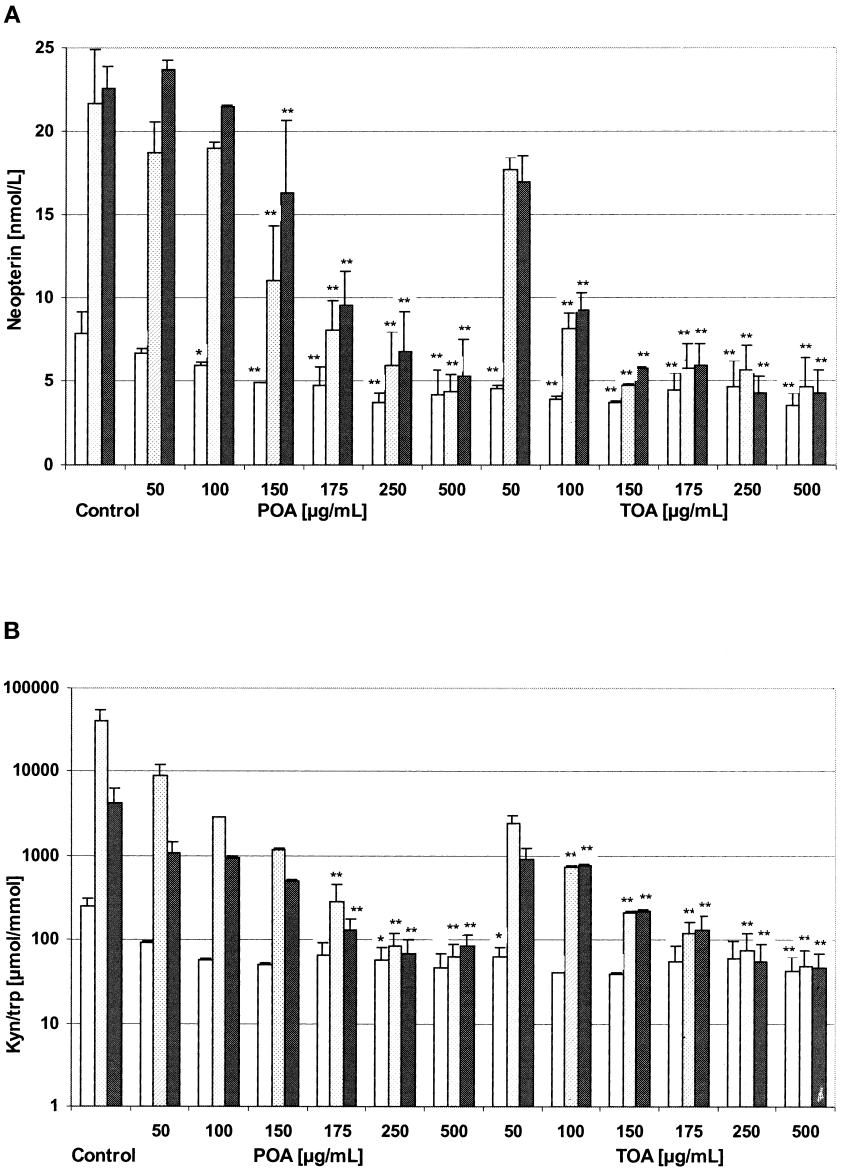

Fig. **2** Neopterin formation (**A**) and tryptophan degradation (**B**) in unstimulated peripheral blood mononuclear cells (white bars) and in cells stimulated with 10 µg/mL phytohaemagglutinin (PHA, dotted bars) and 10 µg/mL concanavalin A (Con A, filled bars) with or without coincubation with mixtures of pentacyclic oxindole alkaloids (POA) and tetracyclic oxindole alkaloids (TOA) of *Uncaria tomentosa* for 48 hours (* $p < 0.05$, ** $p < 0.01$ compared to the control).

Uncaria tomentosa was 500 µg/mL. POA and TOA mixtures were effective at 50 – 150 µg/mL and thus at much lower concentrations than the HCl and EtOH extracts and again POA appeared to be a little less effective than TOA (Fig. **2A**, dotted and filled bars).

Activation of IDO, as quantified by a decrease of tryptophan and a parallel increase of kynurenine concentrations and expressed as kyn/trp, was increased in mitogen-treated PBMC by approximately two orders of magnitude as compared with unstimulated cells (Fig. **2B**, dotted and filled bars). Stimulation of cells with PHA and Con A decreased tryptophan concentrations to 0.15 ± 0.02 and 0.17 ± 0.10 µmol/L, respectively. In parallel, kynurenine concentrations increased to 11.7 ± 0.79 µmol/L and 12.1 ± 1.57 µmol/L (all $p < 0.01$ compared to unstimulated cells). All test substances inhibited stimulation-induced tryptophan degradation and reduced kyn/trp ratio significantly (Figs. **1B** and **2B**, dotted and filled bars). The lowest effective concentrations were 500 µg/mL of HCl and EtOH extracts and 100 – 175 µg/mL of POA and TOA mixtures. At the highest doses used, kyn/trp reached levels beyond that of unstimulated cells (Fig. **2B**, dotted and filled bars) and tryptophan concentrations returned to the concentrations of unstimulated PBMC (23.7 ± 1.1 and 23.7 ± 0.6 µmol/L).

Cell proliferation and viability was not affected by the different test substances used.

Discussion

HCl and EtOH extracts of *Uncaria tomentosa* and pure preparations of POA and TOA were found to suppress the degradation of tryptophan and the production of neopterin in mitogen-stimulated PBMC. Even in unstimulated cells, the baseline degradation of tryptophan and production of neopterin were suppressed. The capacity of *Uncaria tomentosa* extracts and POA and TOA mixtures to suppress neopterin production and tryptophan degradation was similar to that obtained earlier when using, e. g., anti-inflammatory cytokines or atorvastatin [8], [13]. These data sug-

gest a suppressive effect of *Uncaria tomentosa* on the formation and release of IFN-γ in mitogen-stimulated PBMC, as the degradation of tryptophan and production of neopterin are both triggered by this particular cytokine [13]. The effects of test substances were dose-dependent and no toxicity was observed over the whole range of concentrations applied (highest concentrations tested were 4000 μg/mL Krallendorn® and Saventaro® and 175 μg/mL of POA and TAO hydrochlorides mixtures).

Mixtures of POA and TOA hydrochlorides achieved similar efficacy at up to 10-fold lower concentrations than the crude HCl and EtOH extracts. With POA and TOA mixtures, 100 – 150 μg/mL concentrations were sufficient to obtain similar results as compared with 500 – 1000 μg/mL HCl and EtOH extracts of *Uncaria tomentosa*. One can extrapolate that even the higher concentrations of HCl and EtOH extracts contained smaller quantities of POA and TOA as compared with the effective concentrations of the prepared mixtures of POA and TOA. Thus, our results suggest that the effects observed on PBMC could be referred only in part to the presence of POA and/or TOA, probably acting synergistically with other metabolites present in the HCl and EtOH extracts as described earlier by Aguilar et al. [15].

In our study, mixtures of POA and of TOA had similar influences on neopterin production and on tryptophan degradation. This finding contrasts earlier data showing a difference of POA and TOA, e.g., addition of TOA even reduced the effect of POA on Raji and Jurkat cells in a dose-dependent manner [16]. Thereby POA did not directly affect the proliferation, but rather induced endothelial cells to release a compound which influences the proliferation of lymphocytes. The secretion of this compound was induced by POA but not by TOA. Our experiments using PBMC were performed in the absence of endothelial cells. Thus, extracts may have distinct effects on different cell types. It appears possible that TOA and POA may achieve divergent effects only in endothelial cells.

At high concentrations of *Uncaria tomentosa* decoctions (100 – 1000 μg/mL) antioxidant activity has been demonstrated, extracts scavenged free radicals and inhibited TNF-α production [18]. Likewise, aqueous extracts of *Uncaria tomentosa* were found to interfere with intracellular signal transduction pathways involving NF-κB, suggesting the antioxidant capacity to be of importance [17], [18]. Anti-inflammatory activity of *Uncaria tomentosa* could also be involved in the suppression of stimulation-induced neopterin production and tryptophan degradation in PBMC which was observed in our experiments.

The increased production of oxidants and free radicals during inflammatory disorders has become widely recognised as an integral component of cell and tissue injury [19]. IFN-γ potently induces production and release of reactive oxygen species (ROS) in macrophages [20], and the enhanced neopterin production and tryptophan degradation in stimulated PBMC are as well due to IFN-γ activity. Thus, POA and TOA and other ingredients of *Uncaria tomentosa* may not only act as chemical antioxidants, by reducing effects mediated by IFN-γ, but they may also reduce the formation of ROS. Certainly the sometimes high concentrations effective in our *in vitro* system are unlikely to be reached in the circulation. On the other hand, administration of Krallendorn® already has shown some benefit, e.g., in 40 patients with rheumatoid arthritis [21].

In summary, our study demonstrates that extracts of *Uncaria tomentosa* interfere with immunopathogenetic pathways which involve the Th-1 type cytokine IFN-γ. Especially in clinical conditions with enhanced endogenous formation of IFN-γ and characterised by increased neopterin concentrations and elevated kyn/trp, e.g., autoimmune diseases, infections and coronary heart disease [9], [11], extracts of *Uncaria tomentosa* may be able to interfere with cytokine cascades and thus immunopathogenesis.

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