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Immunomodulating and antiviral activities of *Uncaria tomentosa* on human monocytes infected with Dengue Virus-2

Sonia Regina I.N. Reis [a], Ligia M.M. Valente [b], André L. Sampaio [c], Antonio C. Siani [d], Mariana Gandini [a], Elzinandes L. Azeredo [a], Luiz A. D'Avila [e], José L. Mazzei [e], Maria das Graças M. Henriques [c], Claire F. Kubelka [a,*]

[a] *Laboratório de Imunologia Viral, Instituto Oswaldo Cruz, Fundação Oswaldo Cruz, Brazil*
[b] *Departamento de Química Orgânica, Instituto de Química, Universidade Federal do Rio de Janeiro, Brazil*
[c] *Departamento de Farmacologia Aplicada, Instituto de Tecnologia em Fármacos, FIOCRUZ, Brazil*
[d] *Laboratório de Produtos Naturais, Instituto de Tecnologia em Fármacos, Fundação Oswaldo Cruz, Brazil*
[e] *Departamento de Processos Orgânicos, Escola de Química, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil*

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Abstract

Uncaria tomentosa (Willd.) DC., a large woody vine native to the Amazon and Central American rainforests has been used medicinally by indigenous peoples since ancient times and has scientifically proven immunomodulating, anti-inflammatory, cytotoxic and antioxidant activities. Several inflammatory mediators that are implicated in vascular permeability and shock are produced after Dengue Virus (DENV) infection by monocytes, the primary targets for virus replication. Here we assessed the immunoregulatory and antiviral activities from *U. tomentosa*-derived samples, which were tested in an *in vitro* DENV infection model. DENV-2 infected human monocytes were incubated with *U. tomentosa* hydro-alcoholic extract or either its pentacyclic oxindole alkaloid-enriched or non-alkaloid fractions. The antiviral activity was determined by viral antigen (DENV-Ag) detection in monocytes by flow cytometry. Our results demonstrated an *in vitro* inhibitory activity by both extract and alkaloidal fraction, reducing DENV-Ag+ cell rates in treated monocytes. A multiple microbead immunoassay was applied for cytokine determination (TNF-α, IFN-α, IL-6 and IL-10) in infected monocyte culture supernatants. The alkaloidal fraction induced a strong immunomodulation: TNF-α and IFN-α levels were significantly decreased and there was a tendency towards IL-10 modulation. We conclude that the alkaloidal

* Corresponding author. Laboratório de Imunologia Viral, Instituto Oswaldo Cruz, FIOCRUZ, Av. Brasil, 4365 Rio de Janeiro, Brazil, CEP 21040-360. Tel.: +55 21 25984354; fax: +55 21 25984373.
E-mail address: claire@ioc.fiocruz.br (C.F. Kubelka).

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fraction was the most effective in reducing monocyte infection rates and cytokine levels. The antiviral and immunomodulating *in vitro* effects from *U. tomentosa* pentacyclic oxindole alkaloids displayed novel properties regarding therapeutic procedures in Dengue Fever and might be further investigated as a promising candidate for clinical application.

1. Introduction

Traditional medicine within native South American inhabitants has commonly involved plants from the tropical rainforest [1]. Therefore, ethnobotany has been the primary source for target selection during scientific investigation and represents a rich trial potential for immunomodulating and antiviral products.

Uncaria tomentosa (Willd.) DC colloquially referred as cat's claw, is a large woody vine native to the Amazon and Central American rainforests. It has been adopted medicinally by indigenous people for at least 2000 years to treat several diseases such as gastritis, gastric ulcers, cancer, arthritis, asthma and inflammatory disorders [2–4]. The species displayed experimentally immunostimulant, cytotoxic, anti-inflammatory and antioxidant activities [5–7]. The discovery of these bioactivities turned the plant into a valuable natural product, leading to commercialization *in natura* or as phytopharmaceutical derivatives [8]. The species contains both oxindole and indole alkaloids, triterpenoid glycosides, sterols and flavonoids, which individually or synergistically contribute to their therapeutic properties [7].

Dengue fever is presently endemic in tropical and sub-tropical regions, mainly in Southeast Asia, Pacific Islands and the Americas [9,10]. Therefore, Brazil had dengue outbreaks with at least 1 million cases (2001–2002), and 800 thousand cases were reported from January, 2006 to July, 2007 [11]. In addition, severe disease forms have been occurring with ever increased frequency, accompanied by rising death rates [12]. This infectious disease has its pathogenesis related to immunological mechanisms that result in coagulation disorders and haemodynamic imbalance, mainly due to vascular permeability induction [13]. However as yet, neither vaccine nor specific treatment is available for dengue fever, and patients are currently treated only symptomatically.

Dengue virus (DENV) targets are mainly mononuclear phagocytes such as monocytes and dendritic cells [14–17], which are activated after infection, producing pro-inflammatory mediators including TNF-α, Interleukin (IL)-6 and IL-8, among others. Cytokines are also known to be present in patients during dengue fever [18–22], likely playing a key role in endothelial cell activation and vascular permeability leading to the most severe clinical manifestations.

Some earlier investigations have been conducted in order to search for medicinal plant products with antiviral activities inhibiting viral replication [23–25]. Nevertheless, very little is known about plant potential against DENV. *U. tomentosa* has demonstrated immunoregulatory activities by inhibiting pro-inflammatory cytokines making it an interesting candidate for mitigating the intense inflammatory response during dengue fever.

In the present study our aim is to investigate new therapeutic agents for dengue and understand their mechanisms. We assessed the immunoregulatory and antiviral activities of a *U. tomentosa* crude hydro-ethanolic extract and two of its partioned fractions in DENV-infected monocytes, which were tested in an *in vitro* dengue infection model with primary human monocytes. The alkaloid-enriched fraction exhibited both antiviral properties by reducing infected cell rates and inhibiting cytokine activities diminishing TNF-α, IL-10 and IFN-α production levels.

2. Materials and methods

2.1. Plant material, extract and fractions and HPLC analysis

An EtOH:H_2O 1:1 extract previously obtained [26] from the stem barks of *U. tomentosa* wild specimens, collected in Cruzeiro do Sul, Acre, Brazil (donated by Biosapiens Co., Brazil—voucher data in Miranda and collaborators [27]) together with its derivative alkaloidal and non-alkaloidal fractions were assayed. These fractions were obtained by sonicating (10 min) the crude extract (94.2 g) with HCl 0.1 N (1 L), which was then partitioned with EtOAc (500 mL×9). The non-alkaloidal EtOAc fraction was treated with Na_2SO_4 and filtered, the solvent evaporated under low pressure at 37 °C to yield 10.4 g. The resulting aqueous fraction was treated with NH_4OH until pH 9–10 and extracted with EtOAc (500 mL×4). This alkaloid-rich EtOAc fraction was also dried as described above to yield 4.43 g. The fractions were submitted to TLC on pre-coated silica-gel plates $60F_{254}$ (Merck) with Hexane/EtOAc 5:95 as eluent. Spots were visualized by UV irradiation (254 nm) and Dragendorff reagent/10% aqueous sodium nitrite as described by Valente et al. [26]. The pentacyclic oxindole alkaloids (POA) present in the alkaloidal fraction were identified on the TLC plate by comparison to alkaloid standards previously isolated and characterized [26,28]. The additional characterization of the POA profile was carried out by reverse-phase HPLC system with a Lichrocart Lichrospher 5 μm, 125×4.6 mm i.d. column with a UV-detector at 245 nm under the same conditions described by Laus and Keplinger [29], system II. The chromatographic POA signals were identified by comparing their relative retention times (to pteropodine) to the relative retention time of the corresponding alkaloids described by those authors. The spectrometric identification was confirmed by subjecting the fraction to HPLC-DAD-MS, in the same chromatographic column. The mobile phase was 45:55 of MeCN/aqueous 30 mM NH_4OAc solution, pH 6.8–7.0, isocratic mode with a flow rate of 1.0 mL/min, at 60 °C. The ion $[M+1]^+$ at m/z 369 [30] and the corresponding UV spectra [31] were evaluated. The total alkaloid content was determined by HPLC under the indicated chromatographic conditions through external standardization relative to isopteropodine. Five independent points for the external calibration curve were established from triplicate injections of isopteropodine solution in MeOH. The curve presented linearity (R^2=0.9846) in the range of 4.75 to 76.0 μg/mL with standard deviation of 1.0 μg/mL, the minimum detectable concentration estimated as 1.0 μg/mL.

2.2. Cell viability assay

The effect of *U. tomentosa* extract and fractions on the C57Bl/6 peritoneal macrophage viability was determined by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay [32].

The yellow tetrazolium salt MTT is cleaved by the mitochondrial activity of viable cells, forming a purple formazan derivative. Cells (2.5×10^5/well) were incubated with *U. tomentosa* samples (0.1–500 μg/mL) for 48 h at 37 °C, dimethylsulfoxide then subsequently added to dissolve formazan crystals. Optical density was measured at 540 nm in a microplate reader (Molecular Devices).

2.3. Cell cultures, virus stock preparation and virus titration

An *Aedes albopictus* C6/36 cell clone was grown as monolayers at 28 °C on Leibovitz medium (L-15) supplemented with 200 mM glutamine, 1% non-essential amino acid solution, 0.5% tryptose phosphate broth, 100 U/penicillin, 10 μg/streptomycin and 5% fetal calf serum (FCS). The DENV serotype 2 strain 16681, provided by Dr. SB Halstead (Naval Medical Research Center, USA), was titrated by serial dilution cultures in microtiter plates and detected by immunofluorescence as previously described [33]. Virus titer was calculated as 50 percent tissue culture infectious dose ($TCID_{50}$) [34,35]. The inactivated virus was prepared by incubating the inoculum for 30 min at 56 °C. The virus stock used was at a concentration of 1.37×10^8 $TCID_{50}$/mL.

2.4. Preparation of human peripheral blood mononuclear leukocytes

Human peripheral blood was provided by the Hospital Universitário Clementino Fraga, UFRJ. Human peripheral blood mononuclear leukocytes (PBMLs) were isolated from buffy coat cells from healthy donors through density gradient centrifugation (350 g, 30 min in Ficoll-Paque Plus Amersham Biosciences Corp, Piscataway, USA) according to standard procedures. Cells were suspended in RPMI 1640 supplemented with 200 mM glutamine, 100U/mL penicillin, 10 μg/streptomycin and afterwards incubated at 37 °C in a humid atmosphere with 5% CO_2 and allowed to adhere to uncoated polystyrene flasks 150 cm^3 during 90 min for monocyte enrichment. Non-adherent cells were removed by washing, adherent cells were detached by mechanical cell harvesting with cell scrapers in cold medium, resuspended and cultured in supplemented RPMI plus 10% FBS.

2.5. Monocyte infection and treatment with *U. tomentosa*

Enriched monocytes were suspended in supplemented RPMI 1640 medium plus 10% FCS and seeded at 2×10^6 cells/mL on 96- or 24-well plates. After an overnight incubation, infection was effected with a diluted inoculum (30 or 300 μL) in cell culture medium containing $1{,}37\times10^8$ $TCID_{50}$/mL. After a 2 h-incubation period for adsorption, the cell culture supernatant was replaced with a 2% FCS medium and incubated with *U. tomentosa* crude hydro-ethanolic extract or either alkaloid-rich or non-alkaloid fractions at different concentrations (100, 10, 1 or 0,1 μg/mL) and subsequently incubated at 37 °C with 5% CO_2. Dexamethasone 0,05 mM was adopted as a reference inhibitor. After 48 h, supernatants were collected and stocked at −20 °C for cytokine measurement and cells recovered for viral antigen determination, cell viability determined in culture by Trypan blue exclusion. Well content with cell control, inactivated and infectious DENV was assayed.

2.6. Viral antigen determination in monocytes by flow cytometry

Monocytes were recovered by scraping with a plastic microtip in a cold cell culture medium, set at 1×10^6 cells/microtube, then centrifuged (350 g, 10 min) and washed once with 1 phosphate-buffered saline pH 7.4 containing 1% bovine serum albumin and 0.1% NaN_3 (PBS/BSA). Afterwards, cells were fixed with a solution containing 2% paraformaldehyde PBS/BSA at 4 °C for 20 min and permeabilized with a solution containing 0.15% saponin in PBS/BSA. Permeabilized cells were then blocked with 5% inactivated plasma in PBS/BSA at 4 °C for 30 min and incubated with mouse anti-Dengue Complex monoclonal antibody (Chemicon) at 4 °C for 60 min. Cells were then washed and incubated for 30 min at 4 °C with anti-mouse IgG labeled with phycoerythrin (DAKO). After incubation, cells were washed with PBS/BSA, resuspended in 2% paraformaldehyde, and kept at 4 °C until cellacquisition (5000 events for gated monocytes) by FACS® Calibur flow cytometer (Beckon & Dickinson) and analyzed with FlowJo Software (TreeStar Inc.). An isotype-matched antibody was adopted as a staining negative control.

2.7. Cytokine detection in cell culture supernatant by multiplex microbead immunoassay

A multiplex biometric immunoassay, containing fluorescent dyed microspheres conjugated with a monoclonal antibody specific for a target protein, was employed for cytokine measurement according to the manufacturer's instructions (Upstate). Cytokines measured were: IL- 1β, IL-6, CXCL8 (IL-8), IL-10, IL- 12 (p70), IL-15, IFN-α and TNF-α. Briefly, cell culture supernatant (50 μl) was incubated overnight with antibody-coupled beads, complexes being washed and afterwards incubated with biotinylated detection antibody and then streptavidin–phycoerythrin prior to assessing cytokine concentration titers. A broad range, 1.95–8000 pg/mL concentrated human recombinant cytokines, provided by the vendor, was used to establish standard curves, maximizing assay sensitivity and dynamic range. Cytokine levels were determined with a multiplex array reader from Luminex® Instrumentation System (Bio-Plex Workstation from Bio-Rad Laboratories). The analyte concentration was calculated with software provided by the manufacturer (Bio-Plex Manager Software). It provided a regression analysis to derive the equation for cytokine concentration prediction in plasma samples.

2.8. Statistical analyses

Data were first tested for normality with the GraphPad Prism version 4.02 for Windows, GraphPad™ Software (http://www.graphpad.com) in order to determine the significance of differences in DENV-Ag+ cell rates under various culture treatment conditions. Data values which passed the normality test of Kolmogorow-Smirnov were evaluated for significance with the paired *t*-test (two-tailed). However, the cytokine detection data values obtained by the multiplex bead assay did not pass the normality test and were therefore analyzed by Friedman and the Dunn's Multiple Comparison Test. Altered parameters were considered significant when $P<0.05$.

3. Results

3.1. DENV infection model utilizing human primary monocytes for testing the *U. tomentosa* effect on cell infection rates and cytokine production

Human monocyte-enriched PBMLs infected with DENV-2 (strain 16681) were cultured *in vitro*. Preliminary experiments indicated that our DENV stock induced infection rates peaking on the second day after infection from three different PBML donors (data not shown). Target cells are CD14+ cells (data not shown), which constitute approximately 95% of the cells in the monocyte region from the FCS *vs*. SSC dotplots (cell size *vs*. granularity) in FACS analysis as described before [14]. We searched for DENV antigen (Ag) + cells by flow cytometry analysis in infected monocytes from 15 PBML donors and observed that only the infectious virus was present on the second day after infection and no DENV-Ag was detected when the inoculum was heat-inactivated, indicating that virus replication might have occurred (Fig. 1).

After DENV monocyte infection, cell culture supernatant was collected daily and tested for the presence of 8 different cytokines. In both PBML donors tested on the second day after infection TNF-α, IL-6, IL-10 and IFN-α were elevated when compared to control supernatants from cells incubated either with cell culture medium or with heat-inactivated DENV (Fig. 2). These cytokines were still present in the successive days at various detection levels. Other cytokines (IL-1β, IL-8, IL-12 and IL-15) were also tested but did not display altered levels during the 7 days the monocytes were cultured with DENV. The time point, 2 days after infection, was established to evaluate the *U. tomentosa* effect for both antiviral and immunomodulating activities.

3.2. *U. tomentosa* antiviral effect detected by FACS on DENV-Ag+ monocytes

Applying previously described methods [26], *U. tomentosa* derived samples were obtained from stem barks as in the Materials and methods. DENV-infected monocyte cultures from five different PBMC donors were treated with the *U. tomentosa* crude hydro-ethanolic extract or either alkaloidal or non-alkaloidal fractions (Fig. 3), which were tested simultaneously in the same PBML donors and compared to dexamethasone treatment after 2 days in culture. The crude hydro-ethanolic extract significantly decreased DENV-Ag detection in monocytes at the concentration of 10 μg/mL (Fig. 3A), whereas the alkaloidal fraction revealed the most effective inhibitory activity at 1 μg/mL (Fig. 3B). No effect was observed with the non-alkaloidal fraction (Fig. 3C) and dexamethasone inhibited virus detection at similar cell rates the those of to *U. tomentosa* (Fig. 3A, B and C).

3.3. *U. tomentosa* immunomodulating effect on the cytokine levels produced in DENV-infected monocyte supernatant

The *U. tomentosa* immunomodulating activity was assayed on DENV-infected monocytes cultured for 2 days by testing the hydro-ethanolic extract and both the alkaloidal and the non-alkaloidal fractions. TNF-α, IL-6, IL-10 and IFN-α were measured in cell supernatants from five different PBML donors. No significant alterations were detected with the hydro-ethanolic extract or non-alkaloidal fraction treatment (data not shown). However, the alkaloidal fraction inhibited both TNF-α and IFN-α at concentrations of 100 μg/mL (Fig. 4A and D) and similar cytokine levels resulted with the dexamethasone treatment.

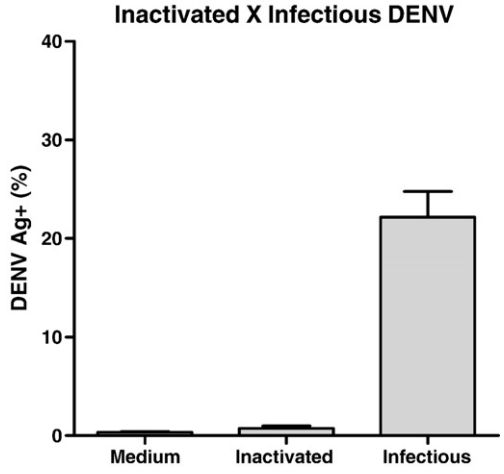

Figure 1 Dengue virus antigen (DENV-Ag) detection in monocytes by flow cytometry analysis. Human monocytes were obtained from healthy PBML donor buffy coats and infected cell culture medium, heat-inactivated or infectious DENV-2 (strain 16681). DENV-Ag + cells after 2 day-infection were detected by flow cytometry analysis. Histograms represent average ± standard error from 15 PBML donnors.

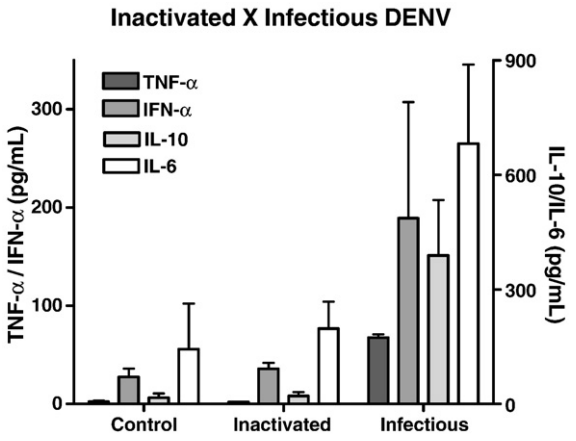

Figure 2 Cytokine production by monocytes after infection with DENV-2. Human monocytes were infected with DENV-2 virus during 2 days and supernatants were collected. Multiplex Cytokine Analysis Technologies (Luminex™) was used for cytokine detection (TNF-α, IFN-α, IL-10 and IL-6) in cultured monocyte supernatants. Histograms represent average ± standard error from 2 PBML donors.

IL-6, which is strikingly induced after monocyte infection, apparently was not inhibited by *U. tomentosa* or dexamethasone treatments (Fig. 4B). IL-10 was also hampered significantly by dexamethasone (Fig. 4C), but there was no significant alteration with the *U. tomentosa* treatment, although a strong tendency for IL-10 inhibition was observed in cultures treated with the alkaloidal fraction: IL-10 levels after DENV infection were 572 ± 219 pg/mL, lowered to 244 ± 60 pg/mL after treatment and presented ≥ 18% abrogation in all five PBML donors.

3.4. Pentacyclic oxindole alkaloid profile and content from the alkaloid fraction

The total alkaloid content in the crude extract obtained from wild Brazilian *U. tomentosa* was calculated as 29.1 mg/g (±1%) in agreement with commercially acceptable plant materials. The most effective activity from *U. tomentosa* emerged in the alkaloid fraction, the HPLC analysis of this bioactive alkaloid fraction exhibiting six characteristic pentacyclic oxindole alkaloid biomarkers with quite similar profiles (Fig. 5 and Table 1) to those already described for Peruvian plants [29,36].

4. Discussion

Anti-inflammatory and anti-allergic properties from *U. tomentosa* extracts have been earlier reported, among them cytokine modulation such as TNF-α downregulation and paw edema inhibition in mouse models [1,37,38]. We describe here for the first time a medicinal plant derived

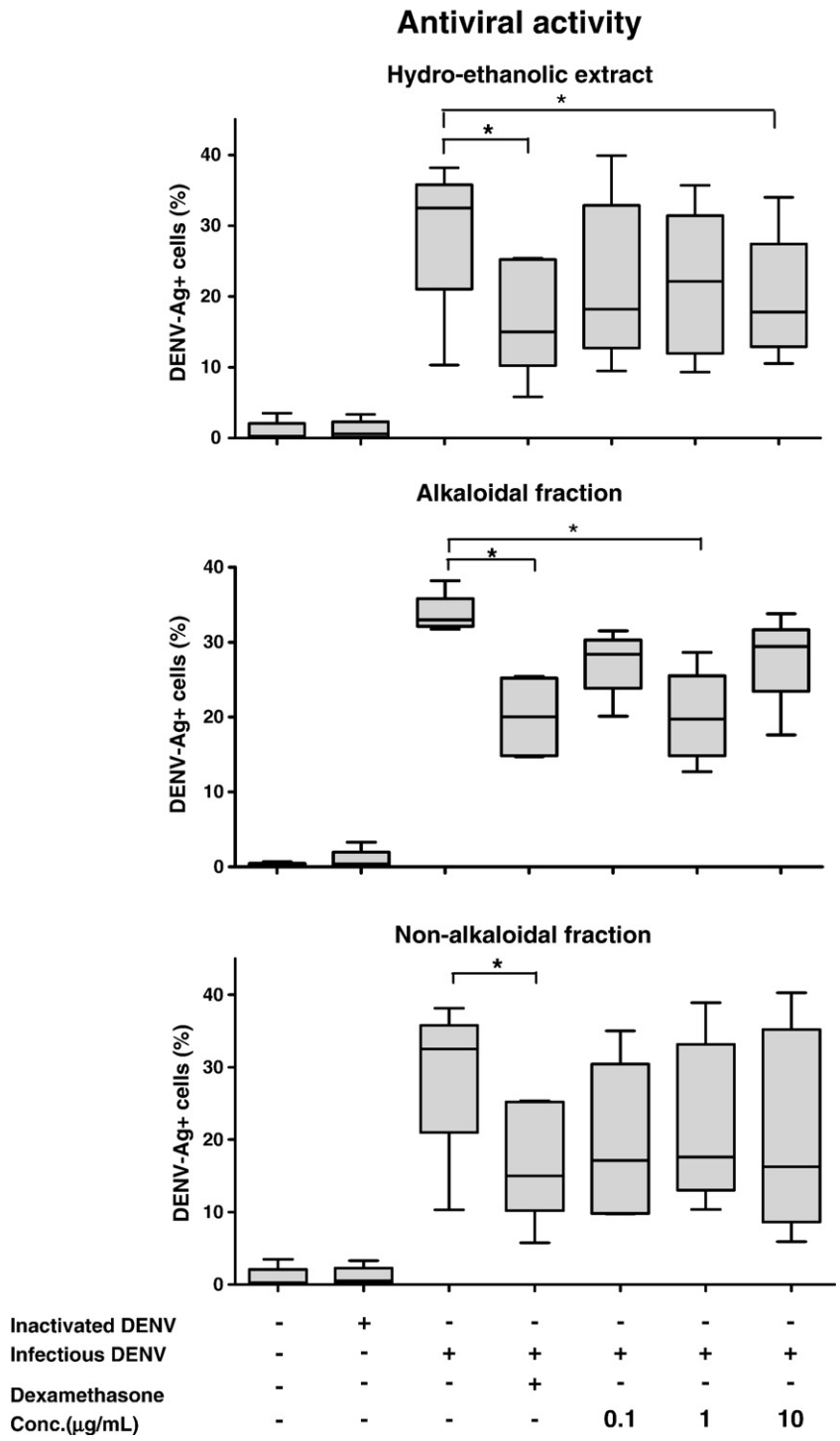

Figure 3 *U. tomentosa* effect on DENV-2 *in vitro* infection in monocytes. DENV-2 infected human monocytes were incubated with hydro-ethanolic extract, alkaloid-rich non-alkaloid fractions at concentrations of 0.1–10 μg/ml during 2 days. Inhibitory activity against DENV-2 was detected by viral antigen (DENV-Ag) reduced rates in treated monocytes after flow cytometry analysis. The box-and-whiskers graph represents data from 5 PBML donors. The box extends from the 25th to the 75th percentiles, the middle line being the median. The error bars, or whiskers, extend down to the lowest value and up to the highest. The paired *t*-test was used to evaluate differences between cytokine concentration produced by non-treated and *U. tomentosa* treated DENV-infected monocytes. * $P<0.05$.

product that has cytokine inhibitory activities *in vitro* in a dengue infection model, also reducing cell infection rates. In our studies we utilized human primary monocytes, the main target cells for DENV in patients [14], demonstrating an effective TNF-α, IL-10 and IFN-α inhibition and lower DENV-Ag cell detection.

No specific therapeutic agents exist for dengue, neither immunosuppressant nor antivirals, which have been clinically

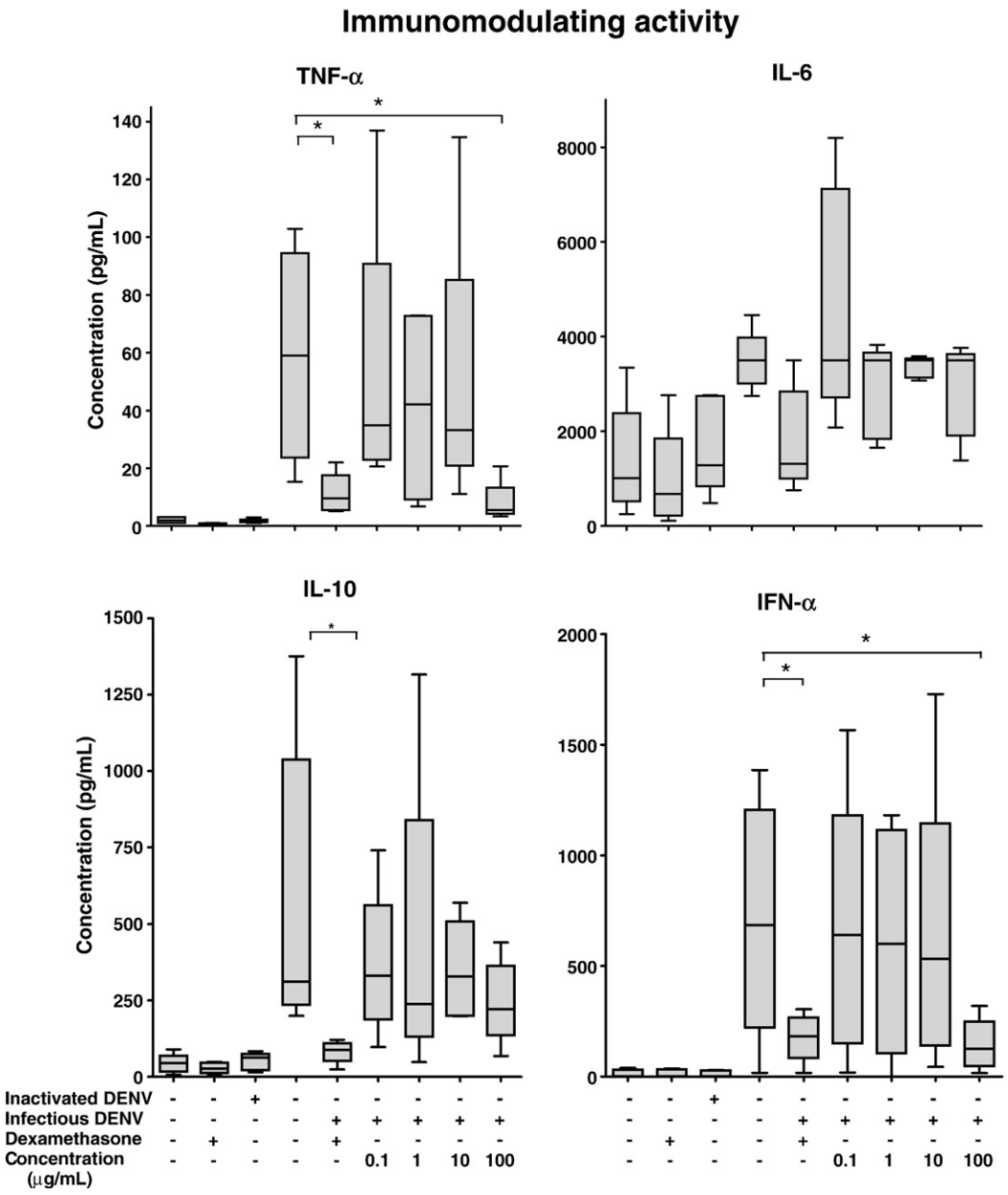

Figure 4 *U. tomentosa* effect on cytokine levels produced by DENV-2 infection in monocytes. DENV-2 infected human monocytes were incubated with alkaloid-rich fraction FA03/142 at concentrations of 0.1–100 μg/ during 2 days. The box-and-whiskers graph represents data from 5 PBML donors. The box extends from the 25th to the 75th percentiles, the middle line being the median. The error bars, or whiskers, extend down to the lowest value and up to the highest. The Friedman and Dunn's Multiple Comparison Tests were used to evaluate differences between cytokine concentration produced by non-treated and *U. tomentosa*-treated DENV-infected monocytes. * $P<0.05$.

tested and proven to have any impact. So far the main procedures during dengue fever are oral/parenteral hydration associated with analgesics. [39]

Clinical investigations support a key role for cytokines in dengue fever pathogenesis; TNF-α, IL-6 and IL-8, among others, being related with severity in various studies [19,21,22,40,41]. They may be associated with hemorrhagic manifestations [20], coagulation activation and fibrinolysis [42], perhaps being involved in vascular permeability, a key phenomenon for severity development during disease [13]. This immunologically mediated activation, more striking in patients with severe clinical manifestations, can also be found in lower degrees in patients with mild disease [43,44]. The identification of substances with immunomodulating properties, acting on inflammatory responses would be extremely appealing in treating the infection, since the disease is directly related to an exacerbated immunological response.

We showed herein that *U. tomentosa* pentacyclic oxindole alkaloids significantly reduce the DENV-Ag expression in monocytes as well as TNF-α, IFN-α and IL-10 production in culture after infection. We propose that this cytokine modulation, if achieved *in vivo*, may lead to milder clinical

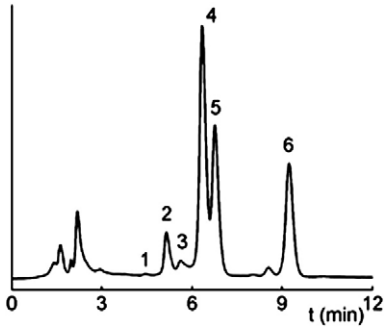

Figure 5 HPLC-UV pentacyclic oxindole alkaloid profile [Laus and Keplinger [29] conditions] of the studied *Uncaria tomentosa* alkaloidal fraction. (1) speciophylline, (2) mitraphylline, (3) uncarine F, (4) pteropodine, (5) isomitraphylline and (6) isopteropodine.

manifestations in patients. TNF-α, classically associated with dengue hemorrhagic fever, is known to activate endothelial cells, resulting in the adhesion molecule expression that facilitates blood cell extravasation. On the other hand, TNF-α downregulated molecules such as cathenin, involved in cell-to-cell junctions, therefore favor endothelial permeability and fluid extravasation, ultimately resulting in hypotension and hemoconcentration [45,46], IL-10, a modulator factor associated with dengue and sepsis severity [20,47,48], may be involved in inducing molecules such as suppressor-of-cytokine-signaling (SOCS) factors that downregulate IFNs and nitric oxide production, both known for their antiviral activities [14,25,49]. Inflammatory cytokines not controlled by this pathway may still be virus elicited contributing therefore to pathogenic mechanisms [49–51].

IFN-α has been detected during dengue fever in patients, but its protective role is still controversial, since significantly elevated concentrations are associated with dengue hemorrhagic fever [52]. It is known that DENV may induce IFN type I production in infected cells, but viral proteins, such as non structural NS4B, can inhibit IFN signaling, impairing this antiviral pathway [53]. IFN-α in excess may induce autoimmune diseases, for example treatment in patients with hepatitis [54]. However, IFN-α downregulation may represent an effective immunomodulatory procedure not interfering with virus clearance, possibly induced by other molecules such as nitric oxide.

Antiviral compounds such as castanospermine significantly reduced DENV viremia in mice leading as well to a splenomegaly reduction, decreasing IL-6, TNF-α, IFN-γ and MCP-1 levels [55]. As these cytokines may be indicated as potential targets for immunomodulating agents in humans, we present for the first time successful data on immunomodulatory activities in human primary cells infected by DENV. Assuming viral load may be related with both severity [47] and immune response intensity, it still remains to be elucidated if effects observed here are a result of drug action in controlling virus life cycle replication or either stimulating antiviral host pathway or both in association.

U. tomentosa has been recently described to act on a monocyte-like human cell lineage THP-1 inhibiting LPS-induced TNF-α by a MAP kinase pathway blocking ERK1/2 and MEK1/2 phosphorylation [5]. It is very likely that its action on DENV-infected monocytes may be mediated by similar pathways. It has also been reported that oxindole alkaloids from *U. tomentosa* induce apoptosis that may favor the virus infected cell elimination [56].

Several studies from Sandoval and collaborators demonstrated the *U. tomentosa* immunomodulator effect by inhibiting LPS-induced proinflammatory cytokines, mainly TNF-α, iNOS gene expression, nitrite formation, cell death and activation of the transcription factor NF-κB [37,57–59]. The NF-κB activation was nearly totally reduced by *U. tomentosa* mediated impairment of NF-κB binding to DNA. It was shown that the hydro-ethanolic extract has significantly greater anti-inflammatory activity than the aqueous [37].

In vitro and *in vivo* assays have previously demonstrated that pentacyclic oxindole alkaloids are the main source of immunostimulant and cytotoxic activities of *U. tomentosa* alcoholic extracts [6,60,61], which justifies as their species chemical biomarker. However, the *U. tomentosa* anti-inflammatory activity is so far associated to other bioactive compound classes as well, suggesting a possible synergic action of alkaloids with other constituents [7,62,63], quinovic acid glycosides from *U. tomentosa*, hazing displayed *in vitro* antiviral properties [64]. The present work demonstrated that *U. tomentosa* pentacyclic oxindole alkaloid fraction was more effective than the hydro-ethanolic crude extract as an antiviral agent, since lower doses retain the anti-inflammatory effect.

U. tomentosa use in clinical procedures for inflammatory disorders, *e.g.* osteoarthritis, presented some efficacy in reducing pain and swollen joints [57,65,66]. These clinical manifestations are common during Dengue Fever leading us to speculate that they might also be decreased upon *U. tomentosa* treatment.

Cytokines operate both as cascades and as a network, regulating the production of other cytokines. Hence efficacious therapy most likely depends on more than one target interference [67]. Many plants have been so far studied and their immunomodulating effects have only been proven to affect few cytokines. Approaches such these herein presented with multiple microbead assays for testing several factors simultaneously may broaden the activity scope of herbal medicine.

The present study revealed that *U. tomentosa* pentacyclic oxindole alkaloids displayed novel antiviral and anti-inflammatory *in vitro* effects in a DENV-infected monocyte model. A successful immunomodulating drug for dengue fever would be a breakthrough for treatment, considering that such an approach has never been taken before, *U. tomentosa* being suggested as a promising candidate for dengue dever therapy.

Table 1 Relative retention times to pteropodine of the pentacyclic oxindole alkaloids (POA) present in the studied *Uncaria tomentosa* alkaloidal fraction in comparison to those found by Laus and Keplinger [29], system II

Identified alkaloids	Observed relative retention time[a]	Literature relative retention time[a]
Speciophylline (1)	0.66	0.69
Mitraphylline (2)	0.81	0.79
Uncarine F (3)	0.89	0.90
Pteropodine (4)	1.00	1.00
Isomitraphylline (5)	1.07	1.10
Isopteropodine (6)	1.46	1.52

[a] tR-POA/tR-pteropodine.

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