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Uncaria tomentosa Aqueous-ethanol Extract Triggers an Immunomodulation toward a Th2 Cytokine Profile

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***Uncaria tomentosa* (Willd.) DC (Rubiaceae) is a large woody vine that is native to the Amazon and Central American rainforests and is used widely in traditional medicine for its immunomodulatory and antiinflammatory activities. The present work used *in vivo* immunotoxic and *in vitro* immunomodulatory experiments to investigate the effects of a pentacyclic oxindole alkaloid extract from *U. tomentosa* bark on lymphocyte phenotype, Th1/Th2 cytokine production, cellular proliferation and cytotoxicity. For the *in vivo* immunotoxicity testing, BALB/c male mice were treated once a day with 125, 500 or 1250 mg/kg of *U. tomentosa* extract for 28 days. For the *in vitro* protocol, lymphocytes were cultured with 10–500 μg/mg of the extract for 48 h. The extract increased the cellularity of splenic white pulp and the thymic medulla and increased the number of T helper lymphocytes and B lymphocytes. Also, a large stimulatory effect on lymphocyte viability was observed. However, mitogen-induced T lymphocyte proliferation was significantly inhibited at higher concentrations of *U. tomentosa* extract. Furthermore, an immunological polarization toward a Th2 cytokine profile was observed. These results suggest that the *U. tomentosa* aqueous-ethanol extract was not immunotoxic to mice and was able to modulate distinct patterns of the immune system in a dose-dependent manner. Copyright © 2011 John Wiley & Sons, Ltd.**

Keywords: Uncaria tomentosa; immune system; mice; immunotoxicity; cytokines; immunophenotype.

INTRODUCTION

Uncaria tomentosa (Willd.) DC (Rubiaceae), known as cat's claw, is a large woody vine that is found in the Amazon rainforest and the surrounding tropical areas (Obregón-Vilches, 1997). This species has shown immunostimulant, cytotoxic, antiinflammatory and antioxidant activities, and it is commercialized in its natural form or as a phytopharmaceutical derivative (Valente, 2006). Its chemical profile includes the presence of indole and oxindole alkaloids in the whole plant. Other components such as proanthocyanidins, sterols, triterpenoids and carboxyl-alkyl esters have been isolated from the bark (Sandoval *et al.*, 2000; Akesson *et al.*, 2005; Gonçalves *et al.*, 2005; Heitzman *et al.*, 2005). Individually or synergistically, these compounds contribute to the therapeutic properties of the species.

Several extracts from *U. tomentosa* bark with different constituents have exhibited interesting characteristics in terms of immunomodulatory and antiinflammatory activities. Aqueous extracts with low alkaloid contents have been shown to exhibit antiinflammatory activities and antioxidant, immunostimulant, antitumor and DNA-repairing properties. Carboxyl-alkyl esters (quinic acid derivatives) and/or polyphenolics may be responsible for these effects (Sandoval *et al.*, 2000; Gonçalves *et al.*, 2005; Akesson *et al.*, 2005). In contrast, a comparative study between the aqueous-ethanol and aqueous extracts from the bark revealed that the former, which contains a high alkaloid content, was more active as an antiinflammatory agent (Aguilar *et al.*, 2002). Furthermore, a chloroform: methanol 9:1 (v/v) and an acetone extract presented antiinflammatory activities that were correlated to quinovic acid glycosides and sterols, respectively (Senatore *et al.*, 1989; Aquino *et al.*, 1991).

The effects of these extracts on the transcription factors of cells and their immunological parameters have been described, but the effects vary according to the experimental conditions. Previous studies have reported that several *U. tomentosa* bark extracts can inhibit TNF-α, iNOS and NF-κB expression and can also increase the synthesis of IL-1β and IL-6, the phagocytosis index and the absolute number of leukocytes (Sandoval *et al.*, 2000; Akesson *et al.*, 2003; Allen-Hall *et al.*, 2010; Lemaire *et al.*, 1999; Wagner *et al.*, 1985; Wurm *et al.*, 1998). This immunomodulatory

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activity also includes effects on mononuclear cell proliferation. Alkaloid-rich extracts and ethanol extracts from *U. tomentosa* have been shown to inhibit the proliferation of human mononuclear cells induced by the mitogens phytohemagglutinin (PHA) and concanavalin A (Con A) (Winkler *et al.*, 2004). A study that used a commercial aqueous extract also revealed an inhibition of T and B lymphocyte proliferation without interfering with the production of IL-2 (Akesson *et al.*, 2003). Although the antiproliferative effects are dominant, the same standardized commercial aqueous extract increased the proliferation of PHA-stimulated murine lymphocytes after *in vivo* treatment (Sheng *et al.*, 2000). These conflicting activities add more complexity to this subject. More recently, Reis *et al.* (2008) reported that the alkaloid fraction from *U. tomentosa* had an immunomodulatory activity, characterized by a strong inhibition of TNF-α and IFN-γ production by human monocytes that were infected with type-2 Dengue virus.

Previous studies have reported a low incidence of systemic toxicity during *U. tomentosa* treatment. The LD_{50} (median lethal dose) for the aqueous extract was reported to be higher than 8 mg/kg body weight (Sheng *et al.*, 2000). This study also demonstrated that a commercial aqueous extract did not cause general signs of toxicity; however, the experimental conditions were limited to female Wistar-Furth rats and a low-dose regimen. Further, Wistar rats that were orally treated with 1000 mg/kg of an *U. tomentosa* preparation containing 7.5 mg of total oxindole alkaloids/g presented lymphocytosis and neutropenia in addition to an increase in kidney weight. However, these results seem to be restricted to an aqueous extract, and the authors did not test for a dose–response relationship (Svendsen and Skydsgaard, 1986 – unpublished data).

Despite the potential immunomodulatory effects of *U. tomentosa*, a lack of research exists regarding its immunotoxic potential. In addition, several controversial immunological effects need to be assessed. The main objective of the present work was to evaluate whether a 28-day oral treatment with an aqueous-ethanol extract from *U. tomentosa* bark would modulate the immune system without triggering a concomitant immunotoxic effect. Additional *in vitro* experiments were used to analyse this extract's effect on lymphocyte proliferation and Th1 and Th2 cytokine profiles.

MATERIAL AND METHODS

Plant material and preparation of the extract. An aqueous-ethanol 1:1 extract that was previously obtained (Valente *et al.*, 2006) from the stem bark of a wild specimen of *U. tomentosa*, collected in Cruzeiro do Sul, Acre, Brazil, and donated by Biosapiens Co., Brazil [voucher data in Miranda *et al.* (2003)], was used for the assays. The pentacyclic oxindole alkaloid (POA) profile and content of this extract was determined using high-performance liquid chromatography (HPLC) as described previously (Reis *et al.*, 2008; Pereira *et al.*, 2008). Briefly, the crude hydroalcohol extract was treated with 0.1 N HCl and then partitioned with EtOAc. The resulting aqueous fraction was treated with NH_4OH until a pH of 9–10 was reached, and then the fraction was extracted with EtOAc. This alkaloid rich EtOAc fraction was dried and filtered, and the solvent evaporated under low pressure. A MeOH solution of this alkaloid fraction was injected into a reverse-phase HPLC system using a Lichrocart Lichrospher 5 μm, 125 × 4.6 mm i.d. column and a UV-detector at 245 nm under the same conditions described previously (Laus and Keplinger, 1994). The identification of the POA signals was made by comparing their relative retention times with those of pteropodine. The total alkaloid content was determined using HPLC-UV with the same chromatographic column and a mixture of 45% of MeCN and 55% of an aqueous 30 mM NH_4OAc solution as the mobile phase. The pH of the mobile phase was adjusted to between 6.8 and 7.0, and the analysis was run in isocratic mode with a flow rate of 1.0 mL/min at 60 °C and detection at 245 nm. Five independent points for the external calibration curve were established using triplicate injections of an isopteropodine solution in MeOH. The curve presented linearity ($R^2 = 0.9846$) in the range of 4.75 to 76.0 μg/mL, with a standard deviation of 1.0 μg/mL. The minimum detectable concentration was estimated to be 1.0 μg/mL.

Animals. Six week old pathogen-free BALB/c male mice were purchased from the Multidisciplinary Center of Biological Investigations (CEMIB, Campinas, São Paulo, Brazil) and housed in rooms with controlled environmental conditions (temperature 22 ± 2 °C, relative humidity 55 ± 20%, a 12/12 h light-dark cycle and 4 daily exhaustion periods). All animals received Nuvilab CR-1 commercial food (Nuvital, Brazil) and filtered water *ad libitum* and were submitted to a 2-week acclimation period before the experiment. Experimental protocols were approved by the Committee for Ethics in Animal Experimentation of the UNESP Medical School, São Paulo, Brazil (protocol number 635).

Experimental design. BALB/c mice were randomized into groups of ten animals each. Treatment with *U. tomentosa* extract or its dilution vehicle (alcohol 10%) was given orally for 28 consecutive days. The following groups were used for the *in vivo* evaluations: G1 contained animals treated with the dilution vehicle (alcohol 10%), and G2, G3 and G4 contained animals treated with *U. tomentosa* aqueous-ethanol extract at 125, 500 and 1250 mg/kg of body weight, respectively. Body weights, water consumption and food consumption were evaluated once a week during the experimental period. After 28 days, the animals were euthanized in a CO_2 chamber. Blood and tissue samples were collected for hematological analysis, histopathology and immunophenotyping of splenic lymphocytes.

To evaluate the *in vitro* immunological effects of the *U. tomentosa* extract, *s*plenocytes were stimulated with the extract (10–500 μg/mL) for 48 h. Cellular viability and proliferation were assessed using a 3-(4,5-dimethylthiazol-2-yl)-2,5-dyphenyltetrazolium bromide (MTT) assay (Mosmann, 1983). The production of Th1 and Th2 cytokines was evaluated using a sensitive cytometric bead array (CBA) in splenocyte culture supernatant.

Tissue processing and histological analysis. The liver, kidneys, spleen, thymus, mesenteric lymph node and femur were removed and fixed in 10% buffered formalin (phosphate buffer pH = 7.2) (Carlo Erba Reagents, Italy) for 48 h. The spleen was divided into

two portions: one for histology and the other for splenic single cell suspension. The paraffin blocks were cut into 5 μm thick sections and stained with hematoxylin-eosin (HE) (Sigma-Aldrich, USA). The histophatological analysis was conducted following previously published criteria by Society of Toxicologic Pathology (Haley *et al.*, 2005).

Hematological analysis. Blood samples were collected by cardiac puncture and blood smears were immediately prepared on glass slides. The samples were stained by Leishman (Merck Darmstadt, Germany), and the relative counting of granulocytes and mononuclear cells (200 cells/slide) was evaluated using a conventional light microscope at 40× magnification.

Splenic single cell suspensions. The spleens were pushed against a 50 μm sterile nylon mesh (BD, New Jersey, USA) in Petri dishes containing RPMI-1640 culture medium (Cultilab, Campinas, Brazil). The erythrocytes were lysed using an isotonic solution of ammonium chloride, potassium bicarbonate and ethylenediamine tetraacetic acid (pH 7.2) (Sigma-Aldrich, USA). Cell suspensions were washed twice (200 × **g**/10 min), and the pellet was resuspended in RPMI-1640 culture medium that was supplemented with 10% inactivated bovine fetal serum (Cultilab, Campinas, Brazil). Cellular viability was evaluated using trypan blue (Hyclone, Thermo Scientific, USA) and was considered adequate for values greater than 90%. Cell suspensions were adjusted to 5×10^6 cells/mL and cryopreserved in RPMI-1640 medium plus 10% dimethyl sulfoxide (Carlo Erba Reagents, Italy) for phenotypic analysis of the lymphocyte subpopulations by flow cytometry.

Lymphocyte immunophenotyping. Cell aliquots were thawed in a temperature-controlled bath (37 °C) and were immediately transferred to tubes that contained RPMI-1640 medium supplemented with 10% bovine fetal serum. After centrifugation (200 × g/10 min), the pellet was resuspended in 3 mL of an isotonic solution (Advia70, Bayer, Germany) and was washed twice. Cellular viability was evaluated using trypan blue, and these values were considered adequate when they were greater than 90%. The cellular concentration was then adjusted to 1×10^6 cells/mL, and aliquots (100 μL) were incubated in the dark for 30 min with the following antibodies: FITC rat anti-mouse CD4 (L3T4), PE-Cy5 rat anti-mouse CD8a (Ly-2) and PE rat anti-mouse CD45RA (BD Pharmingen, USA). Splenic lymphocyte subset counts were determined by FACS Calibur analysis (BD Pharmingen, USA) with gating on the total lymphocyte population. Lymphocytes were characterized by their appearance according to forward scatter (FC) and side scatter (SSC) analyses. For consistency between each flow cytometry analysis, the standard calibration bead (BD Pharmingen, USA) was used to set the forward scatter, side scatter and PMT voltage. For the experimental samples, a corresponding control was used to set gates or positive/negative cell populations.

Cytotoxicity and proliferation assays. Ten BALB/c mice were used for this experiment, and the single-cell suspensions were obtained as described above. The cellular concentration was adjusted to 5×10^6 cells/mL, and each sample was dispensed in triplicate in a 96-well flat-bottom tissue culture plate (Corning Costar, USA) and was treated with the *U. tomentosa* extract at concentrations of 10, 50, 100 and 500 μg/mL. Control samples were treated with 10% ethanol, which was the dilution vehicle. To evaluate lymphocyte proliferation, the cellular samples were treated with the same concentrations of the extract and were stimulated by mitogen Con A (Sigma-Aldrich, USA) at 10 μg/mL. Control wells lacking Con A were used as a reference for basal levels of lymphocyte proliferation. The plates were incubated for 48 h (37 °C, 5% CO_2). Then, MTT solution (Sigma Aldrich, USA) (5 mg/mL) was added to all the wells and incubated for 4 h (37 °C, 5% CO_2). The spent media were pipetted out, and DMSO (Carlo Erba reagents, Italy) was added to each well to dissolve the dark blue formazan crystals. The optical density (OD) values were measured using a microtiter plate reader at 570 nm.

Th1 and Th2 cytokines production. Splenocytes, obtained as described above, were dispensed in a 48-well flat-bottom tissue culture plate (Corning, Costar, USA) at 5×10^6 cells/mL. The samples were stimulated by mitogen Con A (10 μg/mL) and treated with 100 or 500 μg/mL of the extract. The plates were incubated for 48 h (37 °C, 5% CO_2), and the supernantants were collected and stored at −80 °C. Th1 and Th2 cytokines were quantified using a murine Th1/Th2 cytometric bead array (CBA) kit according to the manufacturer's instruction (BD Pharmingen, USA). The FACSCalibur flow cytometer (BD Pharmingen, USA) was calibrated using setup beads, and a total of 3000 events were acquired for each sample. Individual cytokine concentrations (pg/mL) were evaluated by their fluorescence intensities (FL-2 channel) and analysed based on the standard reference curve of CELLQUEST and CBA software (BD Pharmingen, USA).

Statistical analysis. The statistical analyses were performed using GraphPad Prism 5.0. The data were tested for normality (Kolmogorov-Smirnov) and homogeneity (Bartlett's), and if necessary, appropriated transformations were made. Body weights, water and food intake, organ weights, hematological parameters, immunophenotype, cytotoxicity and cytokine results were compared using an analysis of variance (ANOVA). When the results of the ANOVA were significant ($p < 0.05$), the comparison was followed by the Tukey test. For the heterogeneous cases, the Kruskal-Wallis test was applied.

RESULTS

Pentacyclic oxindole alkaloid profile and content

The previous HPLC analysis (Pereira *et al.*, 2008; Reis *et al.*, 2008) of this bioactive aqueous-ethanol extract of *U. tomentosa* indicated the presence of the six pentacyclic oxindole alkaloids that were considered to be chemical markers of the species. The total alkaloid content in the crude extract was calculated as 29.1 mg/g (±1%), which is in agreement with commercially acceptable plant materials (Fig. 1). The total pentacyclic alkaloid pool is usually the marker for assessing the pharmaceutical quality of the cat's claw extracts.

General parameters of toxicity and histopathology of the lymphohematopoietic organs, liver and kidneys

No treatment-related mortality of the animals occurred at any tested dose. The clinical analysis did not reveal abnormalities with respect to hair coat, eye color, touch response, strength or salivation. No significant differences were observed for body weight gain and water or food consumption compared with the control group. However, the relative weights of the liver (125 and 500 mg/kg), spleen (125, 500 and 1250 mg/kg) and kidneys (125 mg/kg) were affected by the extract (Table 1).

The spleens from the animals treated with the highest dose of *U. tomentosa* extract (1250 mg/kg) exhibited an increase in marginal zone cellularity and an increase in the incidence of extra-medullary hematopoiesis. Furthermore, the cellularity of the thymic medulla was increased after 1250 mg/kg of the *U. tomentosa* extract. The bone marrow, lymph nodes, kidneys and liver did not exhibit any significant histological alterations.

Hematological parameters

Compared with the relative values that were observed in the control group, the animals treated with 1250 mg/kg of the *U. tomentosa* extract displayed a significant increase in the relative number of lymphocytes, which was associated with a decrease in granulocytes (Table 2).

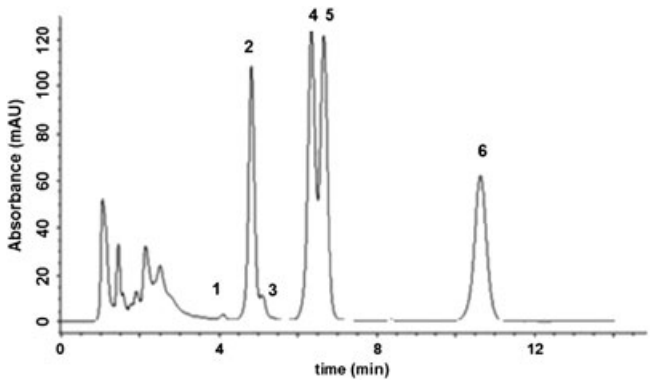

Figure 1. HPLC-UV pentacyclic oxindole alkaloid profile (Laus and Keplinger, 1994, conditions) of the studied *U. tomentosa* alkaloidal fraction. (1) speciophylline; (2) mitraphylline; (3) uncarine F; (4) pteropodine; (5) isomitraphylline; (6) isopteropodine. For extract quantification details see Reis *et al.* (2008).

Phenotypic analysis of splenic lymphocyte subpopulations

The phenotypic analysis of the lymphocyte subsets revealed a higher percentage of mature $CD4^+$ T lymphocytes in the groups that were treated with 125 mg/kg and 500 mg/kg of the *U. tomentosa* extract. These same doses caused a non-significant trend toward an increase in the percentage of immature $CD4^+$ $CD8^+$ T cells. In contrast, a significant increase in the percentage of $CD45RA^+$ B lymphocytes was present in the group that was treated with the highest dose of the extract (1250 mg/kg) when compared with the control group and the 125 and 500 mg/kg treated groups (Table 3).

Effect of *Uncaria tomentosa* on lymphocyte viability and proliferation

The *U. tomentosa* aqueous-ethanol extract did not cause any toxicity that affected the lymphocyte's survival. In fact, all the tested concentrations resulted in increased cellular viability compared with the values that were observed in the untreated control group (Fig. 2). Nevertheless, higher concentrations of the extract (50–500 μg/mL) caused an inhibition in T lymphocyte proliferation after mitogenic stimulus, resulting in proliferation indexes that were lower than those observed in the untreated control samples (Fig. 3).

Th1 and Th2 cytokine production

A standard curve with cytokine concentrations ranging from 0 to 5000 pg/mL was constructed for this assay, revealing a fitting-accuracy of 99.9% for each tested cytokine (data not shown). The *U. tomentosa* aqueous-ethanol extract increased the mitogen-induced production of IL-4 and IL-5 and caused a strong inhibition of IFN-γ at the highest tested concentration (500 μg/mL). In addition, both concentrations, 100 and 500 μg/mL, inhibited the production of IL-2. Despite the absence of changes in the TNF-α level at 500 μg/mL, the lowest tested concentration (100 μg/mL) led to a significant

Table 1. Body weight, body weight gain and organ relative weight in BALB/c male mice treated with *U. tomentosa* aqueous-ethanol extract for 28 days

	Control	UT 125 mg/kg	UT 500 mg/kg	UT 1250 mg/kg
Initial body weight	25.9 ± 1.6	25.4 ± 1.2	25.6 ± 1.5	25.7 ± 1.7
Final body weight	30.6 ± 1.5	30.4 ± 0.9	29.7 ± 2.6	29.9 ± 2.7
Body weight gain	4.7 ± 0.8	5.0 ± 1.6	4.2 ± 2.5	4.3 ± 2.6
Liver	4.51 ± 0.34	5.47 ± 0.49[b]	5.18 ± 0.56[a]	4.79 ± 0.43
Kidneys	0.79 ± 0.05	0.86 ± 0.07[b]	0.82 ± 0.06	0.75 ± 0.07
Adrenals	0.02 ± 0.01	0.02 ± 0.01	0.02 ± 0.01	0.02 ± 0.01
Spleen	0.30 ± 0.009	0.37 ± 0.05[b]	0.37 ± 0.02[a]	0.35 ± 0.05[a]
Thymus	0.23 ± 0.03	0.21 ± 0.06	0.19 ± 0.04	0.18 ± 0.04

Body weights are expressed as mean ± SD.

Body weight gain (Mean final body weight – Mean initial body weight).

Organ relative weights (organ weight/final body weight × 100).

Significantly different from control group, [a]$p < 0.05$ and [b]$p < 0.01$, respectively.

Table 2. Relative counting of mononuclear and polymorphonuclear cells in BALB/c male mice treated with *U. tomentosa* aqueous-ethanol extract for 28 days

	Control	UT 125 mg/kg	UT 500 mg/kg	UT 1250 mg/kg
Lymphocytes	82.0 ± 6.9	89.3 ± 5.3	88.9 ± 7.2	91.4 ± 3.7[a]
Granulocytes	16.1 ± 5.6	7.9 ± 4.6	10.2 ± 6.6	5.5 ± 2.1[b]
Monocytes	1.90 ± 1.0	2.6 ± 2.4	0.9 ± 1.3	3.3 ± 3.6

Percentage of mononuclear and polymorphonuclear cells, expressed as mean ± SD (200 cells/animal were counted).
Significantly different from control group, [a]$p < 0.05$ and [b]$p < 0.01$, respectively.

Table 3. Phenotypic analysis of splenic lymphocyte subsets in BALB/c male mice treated with *U. tomentosa* aqueous-ethanol extract for 28 days

	Control	UT 125 mg/kg	UT 500 mg/kg	UT 1250 mg/kg
$CD4^+$ T lymphocytes	21.3 ± 0.3	27.8 ± 3.4[a]	32.3 ± 3.0[a]	13.35 ± 1.57[a]
$CD8^+$ T lymphocytes	12.2 ± 1.3	14.3 ± 2.7	13.6 ± 1.4	7.00 ± 1.9[a]
$CD4^+CD8^+$ T lymphocytes	1.8 ± 0.5	3.14 ± 1.5	2.42 ± 1.7	1.16 ± 0.3
$CD45RA^+$ B lymphocytes	63.5 ± 1.6	54.5 ± 3.9	51.76 ± 0.8	80.1 ± 4.3[a]

Values are expressed as percentage of lymphocyte subsets (mean ± SD). 10000 cells/animal were counted.
Significantly different from control group, [a]$p < 0.05$.

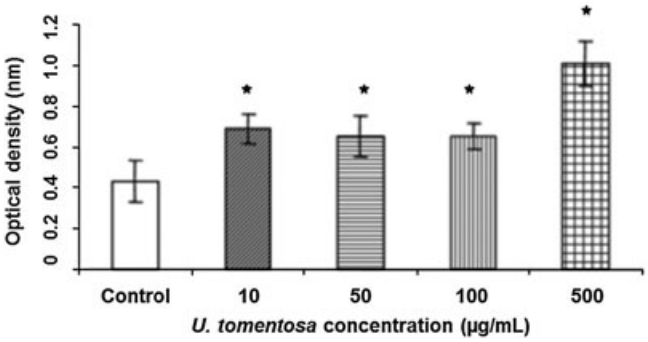

Figure 2. Evaluation of splenic lymphocyte viability by the MTT colorimetric assay after treatment with *U. tomentosa* aqueous-ethanol extract (10–500 µg/mL). Values are expressed as optical density (mean ± SD). *Significantly different from control, $p < 0.001$.

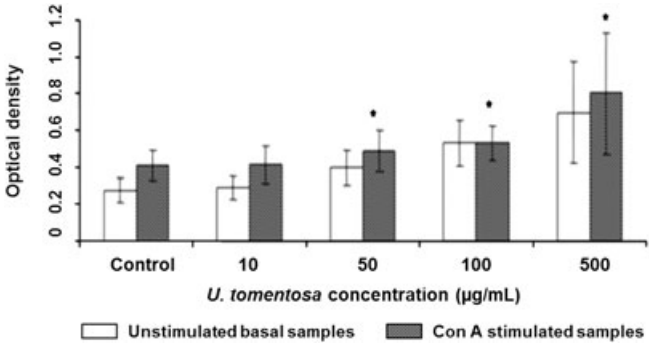

Figure 3. Proliferation of splenic lymphocytes treated with *U. tomentosa* aqueous-ethanol extract. Values are expressed as optical density (mean ± SD). The proliferation index was calculated following the formula: OD from Con A samples/OD from basal samples. *Indicates inhibition of the Con A-induced lymphocyte proliferation compared with the unstimulated basal samples, $p < 0.05$.

increase in the production of this pro-inflammatory cytokine by murine splenocytes (Fig. 4).

DISCUSSION

Uncaria tomentosa is a medicinal plant that possesses immunomodulatory and antiinflammatory properties. Previous studies correlated six pentacyclic oxindole alkaloids (POA) that are present in its leaves, bark and roots to the immunomodulatory property of this species (Winkler *et al.*, 2004). As a result of this finding, several phytopharmaceuticals that were based on the bark ethanol extract and the alkaloid-rich fraction from *U. tomentosa* have been standardized on the basis of their oxindole alkaloid content (Keplinger *et al.*, 1989; Keplinger and Keplinger, 1994). However, studies concerning the immunotoxicity and the immunomodulatory potential of such compounds still need to be conducted. As a result, this immunomodulating and antiviral *U. tomentosa* bark extract (aqueous-ethanol 1:1), which has been shown to be effective for Dengue fever (Reis *et al.*, 2008), was investigated in the present study to determine its immunotoxic potential. Our results demonstrated that this extract has a low systemic toxicity. These findings are consistent with a previous work that demonstrated that the LD_{50} for an aqueous extract was higher than 8 g/kg (Sheng *et al.*, 2000). Physiological alterations such as an increase in the relative weights of the liver, spleen and kidneys were observed; however, no treatment-related mortality was observed. Furthermore, there were no signs of structural changes or of an enzymatic induction that suggested a toxic effect. Additionally, the higher splenic relative weight could be attributed, at least partially, to the increased incidence of extra-medullary hematopoiesis and to the enlargement of the marginal zone. These findings are relevant because these changes have not been described in previous toxicity studies with *U. tomentosa* extracts and

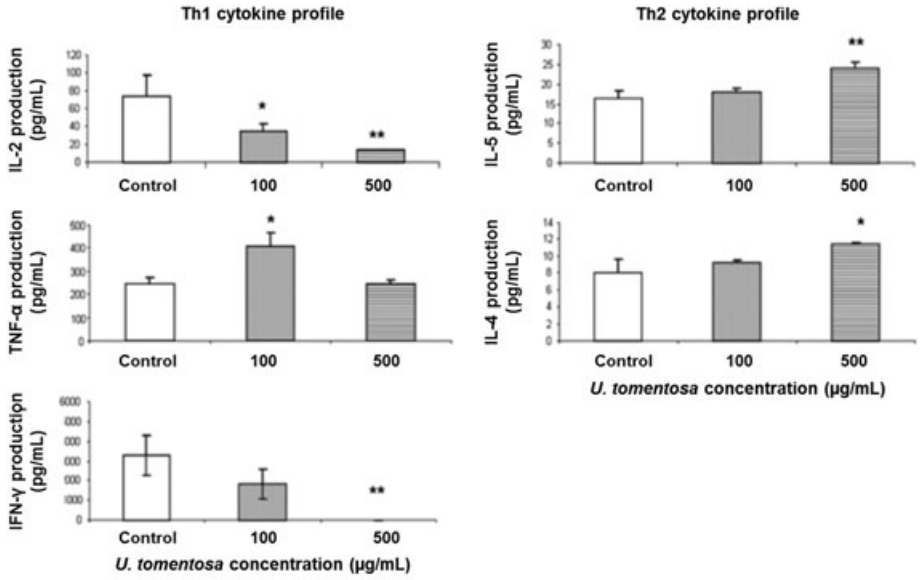

Figure 4. Mitogen-induced Th1 and Th2 cytokine production by murine splenocytes after *in vitro* treatment with 100 and 500 µg/mL of *U. tomentosa* aqueous-ethanol extract. Values are expressed as mean ± SD (pg/mL). Significantly different from control samples, $*p < 0.01$ and $**p < 0.001$, respectively.

there is a lack of reports that determine a dose–response relationship for this aqueous-ethanol extract following repeated dosing.

Concerning the immunomodulatory potential of this extract, treatment with different doses of *U. tomentosa* resulted in an increase in the relative number of lymphocytes, in a similar manner to the aqueous extract effects (Sheng *et al.*, 2000). In addition, using a flow cytometry analysis, it was found that this extract increased the number of lymphocytes carrying the CD4 (125 and 500 mg/kg) and CD45RA molecules (1250 mg/kg). This result suggests that this extract has the potential to modulate distinct patterns of the immune system in a dose-dependent manner. $CD4^+$ T cells are viewed as the major orchestrators of the immune system, which can be differentiated into distinct lineages such as Th1 and Th2 cells, depending upon their microenvironment (Zhu and Paul, 2010). Based on the *in vitro* data, a concentration-dependent immunostimulation was achieved, as observed by the increased number of viable lymphocytes, particularly at the highest extract concentration (500 µg/mL). Because *U. tomentosa* reduced the *in vitro* proliferation of lymphocytes that were stimulated by the T-cell mitogen Con A, the increased cellular viability may be related mainly to $CD45RA^+$ B cells. This result is in accordance with the *in vivo* increase of $CD45RA^+$ B lymphocytes at the highest dose (1250 mg/kg) and with the splenic histology, which revealed an enlargement of the splenic marginal zone, a region that is populated mainly by B cells. In addition, our results suggested that an alternative mechanism that was able to increase $CD4^+$ cells also promoted a trend toward an increased number of $CD4^+CD8^+$ T lymphocytes. These double-positive lymphocytes are considered to be partially immunocompetent cells that undergo post thymic maturation at the periphery, becoming functionally mature $CD4^+$ T cells (Jiménez *et al.*, 2002). Therefore, *U. tomentosa* may stimulate the transition of $CD4^+CD8^+$ lymphocytes to a mature $CD4^+$ helper phenotype. This possibility is partially supported by a clear increase in the cellularity of thymic medulla, as determined by a careful histological analysis. Literature reports have suggested that many of the immune-related effects of medicinal plants could be due to cytokine modulation (Spelman *et al.*, 2006). The dose-dependent increase in the number of $CD4^+$ T and $CD45RA^+$ B cells could suggest a Th2 polarization, i.e. this extract is determining a differentiation of Th0 cells toward Th2 cells. Our findings reinforce this possibility by showing that splenic cells stimulated *in vitro* with Con A and the highest extract concentration (500 µg/mL) produced significant levels of the Th2 cytokines IL-4 and IL-5. Under these same conditions, IL-2 and IFN-γ production, which are considered Th1 type cytokines, were clearly inhibited. This finding is in agreement with the classical literature that reports that IL-4 promotes the differentiation of Th2 cells *in vitro* through its action on the transcription factor STAT6 and the upregulation of GATA-binding protein (GATA3), which is the master regulator of Th2 differentiation (Zheng and Flavell, 1997). To ensure that these results were not related to alterations in cell viability, MTT assays were performed to measure cell proliferation, confirming that the extract was not cytotoxic to splenic lymphocytes at any of the tested concentrations.

From these findings, it was concluded that the aqueous-ethanol extract from *U. tomentosa* bark did not cause significant systemic toxicity or immunotoxicity. This extract was endowed with a clear immunomodulatory activity that affected lymphocyte subsets and promoted a cytokine polarization toward a Th2 profile, suggesting its potential to treat Th1 immunomediated disorders.

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Conflict of interest

The authors declare that there is no conflict.

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