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Uncaria tomentosa stimulates the proliferation of myeloid progenitor cells

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ABSTRACT

Ethnopharmacological relevance: The Asháninkas, indigenous people of Peru, use cat's claw (*Uncaria tomentosa*) to restore health. *Uncaria tomentosa* has antioxidant activity and works as an agent to repair DNA damage. It causes different effects on cell proliferation depending on the cell type involved; specifically, it can stimulate the proliferation of myeloid progenitors and cause apoptosis of neoplastic cells. Neutropenia is the most common collateral effect of chemotherapy. For patients undergoing cancer treatment, the administration of a drug that stimulates the proliferation of healthy hematopoietic tissue cells is very desirable.

It is important to assess the acute effects of *Uncaria tomentosa* on granulocyte-macrophage colony-forming cells (CFU-GM) and in the recovery of neutrophils after chemotherapy-induced neutropenia, by establishing the correlation with filgrastim (rhG-CSF) treatment to evaluate its possible use in clinical oncology.

Materials and methods: The *in vivo* assay was performed in ifosfamide-treated mice receiving oral doses of 5 and 15 mg of *Uncaria tomentosa* and intraperitoneal doses of 3 and 9 μg of filgrastim, respectively, for four days. Colony-forming cell (CFC) assays were performed with human hematopoietic stem/precursor cells (hHSPCs) obtained from umbilical cord blood (UCB).

Results: Bioassays showed that treatment with *Uncaria tomentosa* significantly increased the neutrophil count, and a potency of 85.2% was calculated in relation to filgrastim at the corresponding doses tested. An *in vitro* CFC assay showed an increase in CFU-GM size and mixed colonies (CFU-GEMM) size at the final concentrations of 100 and 200 μg extract/mL.

Conclusions: At the tested doses, *Uncaria tomentosa* had a positive effect on myeloid progenitor number and is promising for use with chemotherapy to minimize the adverse effects of this treatment. These results support the belief of the Asháninkas, who have classified *Uncaria tomentosa* as a 'powerful plant'.

1. Introduction

Uncaria tomentosa belongs to the Rubiaceae family and is commonly known as cat's claw. It is a woody vine native to the Amazon rainforest and other tropical areas of South and Central America. This plant has been used in traditional and cultural practices in South America for centuries, especially in Peru. References exist regarding indigenous groups such as the Asháninkas who use preparations of 'powerful plants' to restore health, and *Uncaria tomentosa* is one of these plants (Keplinger et al., 1999). Today, this plant is widely used in Peruvian traditional medicine as an anti-inflammatory, contraceptive, and cytostatic remedy (Aquino et al., 1991). It has been used in patients with rheumatic diseases

Abbreviations: BFU-E, burst forming units-erythroid; CFC assay, colony-forming cell assays; CFU-E, colony-forming units-erythroid; CFU-GEMM, colony-forming unit-granulocyte/erythrocyte/macrophage/megakaryocyte; CFU-GM, colony-forming unit-granulocyte/macrophage; CSFs, colony stimulating growth factors; GSH, glutathione; GM-CSF, granulocyte/macrophage colony-stimulating factor; hHSPCs, human hematopoietic stem/precursor cells; IL, interleukin; NPSH, non-protein thiols; NF-kappa B, nuclear factor kappa B; POAs, pentacyclic oxindole alkaloids; ROS, reactive oxygen species; SOD, superoxide dismutase; TOAs, tetracyclic oxindole alkaloids; TNF-α, tumor necrosis factor α; UCB, umbilical cord blood; WBC, white blood cell.

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(Mur et al., 2002) or cancer (Gonzales and Valerio, Jr., 2006) due its antioxidant (Pilarski et al., 2006) and anti-inflammatory properties (Allen-Hall et al., 2007). *In vitro* and *in vivo* studies using animal models have also demonstrated that it has DNA repair activity (Sheng et al., 2000a). Furthermore, micromolar concentrations of its purified pentacyclic oxindole alkaloids (POAs) inhibit the growth of neoplastic cells. For example, mitraphylline can induce apoptosis of human Ewing's sarcoma (MHH-ES-1) cells and breast cancer cells (MT-3 cells) (García Gimenez et al., 2010), pteropodine and uncarine F induce apoptosis of human lymphoblastic leukemia T cells (CCRF-CEM-C7H2) (Bacher et al., 2005), and mitraphylline induces apoptosis of human glioma (Gamgee cell) and neuroblastoma cells (SKN-BE) (Garcia Prado et al., 2007). Finally, these compounds stimulate the proliferation of myeloid precursors (Eberlin et al., 2005).

Neutropenia is the most common collateral effect of chemotherapy and may be a limiting factor in determining the frequency and dose of treatment. Patients with neutropenia are predisposed to infection due to an absence of granulocytes; the disruption of the integumentary, mucosal, and mucociliary barriers; and the inherent microbial flora shifts following severe illness and antimicrobial usage (Ozer et al., 2000). Colony stimulating growth factors (CSFs), especially for the myeloid lineage (G-CSF), are occasionally used to minimize the effects of neutropenia. They are part of the family of cytokines that regulate the proliferation, differentiation, and functional activation of myeloid hematopoietic cells. Filgrastim (recombinant human granulocyte colony-stimulating factor-rh-G-CSF) is a glycoprotein produced by recombinant DNA technology in *Escherichia coli*. Secondary administration of CSFs may allow chemotherapy dose maintenance, which is important because it improves overall survival, disease-free survival, quality of life, non-toxicity, and cost-effectiveness (Ozer et al., 2000).

For patients undergoing cancer treatment, the administration of a drug that stimulates the proliferation of healthy hematopoietic tissue cells is desirable because it could minimize the primary adverse effects of chemotherapy. *Uncaria tomentosa* has these characteristics, which make it a promising auxiliary to conventional treatments for cancer. Some studies have demonstrated the positive effect of *Uncaria tomentosa* on leukocyte counts over a period of eight weeks in healthy animals (Sheng et al., 2000a) and after 10 days of mild doxorubicin-induced neutropenia (Sheng et al., 2000b). It is important to observe its acute effect on myeloid tissue using a model of severe neutropenia and to assess whether the results obtained with the animal model can be extrapolated to humans (Gertsch, 2009). These observations are necessary to determine possible therapeutic and toxic doses (Keplinger et al., 1999). This study was conducted to investigate the effect of *Uncaria tomentosa* on myeloid progenitors. The objectives of this study were as follows: (1) to establish the dose-dependent relationship between *Uncaria tomentosa* and a reference drug (Filgrastim) on myeloid cell proliferation after ifosfamide-induced neutropenia; (2) to evaluate the interference of *Uncaria tomentosa* with reactive oxygen species (ROS) production using animal models; and (3) to assess the relationship of *Uncaria tomentosa* with cell proliferation. The result was confirmed using colony-forming cell (CFC) assays with human hematopoietic stem/precursor cells (hHSPCs).

2. Materials and methods

2.1. Drugs

Filgrastim (Leucin®, Bergamo, São Paulo, Brazil), ifosfamide (Glenmark, São Paulo, Brazil), and ascorbic acid (Redoxon®, Bayer, Brazil) were obtained. The *Uncaria tomentosa* extract was prepared by ultra-turrax extraction (Biotron-Kinematica AG) of ground bark (Centroflora) with 70% ethanol (Dipalcool). The fluid was centrifuged (Centrifuge Suzuki), concentrated in a heating tank (MCA-ALW) to remove the alcohol, and spray-dried (Kohls) using silicon dioxide (Evonik) and microcrystalline cellulose 102 (Blanver) as excipients.

2.2. Analysis of dry Uncaria tomentosa extract

The following reagents were used: acetonitrile (JTBaker), triethylamine (Fluka), acetic acid (JTBaker), polyamide (Fluka), ethanol (Vetec), and ultrapure water. Sample extraction was performed using a Unique® ultrasound, model USC 5000A, 40 kHz. Chromatographic analyses were performed on the Agilent 1100 HPLC system and a Zorbax® XDB C-18 column (150 mm × 4.6 mm, 3.5 μm Agilent) at 15 °C. Samples (80 mg) were diluted in 60% ethanol (10 mL) and subjected to sonication (20 min at 30 °C). Next, 2 mL of sample were passed through a column containing 200 mg of polyamide, and the eluate was injected into an HPLC system. Separation was achieved using a gradient elution of water (0.2% acetic acid) adjusted to pH 6.9 with triethylamine (A) and acetonitrile (B) at a flow rate of 0.8 mL/min. The composition of the mobile phase was 65% of A and 35% of B in the 0–18 min interval; a linear gradient approaching 50% of A and 50% of B in 18–29 min; 50% of A and 50% of B in 29–31 min; a linear gradient approaching 65% of A and 35% of B in 31–32 min; and 65% of A and 35% of B in 32–38 min. Each run was followed by an equilibration period of 6 min. Detection was performed at 245 nm, and the concentration of pentacyclic oxindole alkaloids (POA) were calculated with reference to external calibration curves of mitraphylline (Bertol, 2010).

2.3. Laboratory animals

Male 7- to 8-week-old BALB/c mice from the Central Animal House of the Federal University of Santa Maria (UFSM) were housed in air-conditioned controlled conditions (room temperature 22 ± 2 °C and relative humidity of 65%; artificial illumination, 12 h per day) and were used when they weighed between 19 and 24 g. They were given food and water *ad libitum*. All animal procedures were approved by the Animal Ethics Committee of the *Universidade Federal de Santa Maria*.

2.4. Biological assay

The bioassay was performed as previously described (Dalmora et al., 2006). The animals were allocated to *Uncaria tomentosa*, filgrastim reference standard, and control groups in a fully randomized method and were identified by color code for the assay. Each group consisted of 6 mice per treatment group. Standard and test samples were diluted to the concentrations of 6 and 18 μg/mL and 10 and 30 mg/mL, respectively, with phosphate buffered saline containing 0.1% bovine serum albumin. A single dose of 200 mg of ifosfamide/0.5 mL per mouse was injected intraperitoneally into each animal on day 0. Multiple intraperitoneal injections of 0.5 mL of filgrastim and oral doses of *Uncaria tomentosa* were given to the ifosfamide-treated mice from day 1 to day 4. Six hours after the last injection/administration, peripheral blood was collected from the orbital venous sinus. Blood films were prepared on glass slides and stained using the May–Grünwald–Giemsa method, in which the neutrophils were counted and expressed as a percentage of the total number of white cells.

Another bioassay was performed as previous described, with animals allocated into two groups: ascorbic acid and control. Ascorbic acid was diluted to the concentrations of 11 mg/mL according Jagetia et al. (2003). Multiple oral doses of 0.5 mL of ascorbic acid were given to the ifosfamide-treated mice from day 1 to day 4.

2.5. Hematological analysis

The white blood cell (WBC) count was performed in a Neubauer chamber (optical, 100×) in duplicate, and the blood films were stained with May–Grünwald–Giemsa for light microscopy observation. The hemoglobin concentration was determined using Drabkin's solution at a spectrophotometric absorbance of 540 nm. The samples were analyzed by two different individuals.

2.6. Antioxidants

Non-protein thiols in the plasma were assayed and evaluated with the Ellman's method (1959). Values are expressed in mmol/mL of protein. The determination of catalase activity in blood was carried out in accordance with the modified method of Nelson and Kiesow (1972). This assay involves the changes at an absorbance of 240 nm for 2 min due to the catalase-dependent decomposition of hydrogen peroxide (H_2O_2). The enzyme activity was calculated using the molar extinction coefficient (0.0432 cm^{-1} μmol^{-1}). The results were expressed in picomoles/mg protein. Superoxide dismutase (SOD) activity measurements were based on the inhibition of the superoxide radical reaction with adrenaline as described by Mc Cord and Fridovich (1969). In this method, SOD present in the sample competes with the detection system for superoxide radicals. The units of SOD are defined by the amount of enzyme that inhibits 50% of adrenaline oxidation. The oxidation of adrenaline leads to a colored product, adrenochrome, and is then detected by spectrophotometry. SOD activity is determined by measuring the speed of adrenochrome formation, observed at 480 nm, in a reaction medium containing glycine-NaOH (50 mM, pH 10) and adrenaline (1 mM).

2.7. Allometry calculation

To determine the mammalian metabolic rate, we used the following formula: (MR) = $aM_b{}^b$, with $a = 3.98$, $b = 0.686$, and M_b = body mass in grams (White and Seymour, 2005).

2.8. Colony-forming cell (CFC) assays

CFC assays were performed with hHSPCs obtained from umbilical cord blood (UCB). UCB was collected with full term deliveries ($n = 2$), and written informed consent was given by all mothers. All procedures were performed sequentially, immediately after collection and in aseptic conditions. The sample was diluted 1:3 in PBS, and the mononuclear cells were separated with density gradient solution polysucrose and diatrizoate sodium (Histopaque-1077, Sigma–Aldrich) following the manufacturer's instructions. Absolute and differential counting was performed (equipment Sysmex Xs1000i) in the cells obtained at the interface of mononuclear cells, and their viability was assessed by the trypan blue assay (Sigma–Aldrich). A positive selection was performed later using immunomagnetic separation for $CD34^+$ progenitors on the Dynal® CD34 Progenitor Cell Selection system (Invitrogen™ Dynal®) according to the manufacturer's instructions. $CD34^+$ cells were obtained from hemocytometer counting and assessed for viability using blue trypan before the CFC assays were carried out.

CFC assays were performed using semi-solid Complete MethoCult® H4434 (methylcellulose, HSCF, HGM-CSFhIL-3, hEPO StemCells Technologies) according to the manufacturer's instructions for human hematopoietic CFC assays (human colony-forming cell assays using MethoCult®, Technical Manual, version 3.0, October 2004). The *Uncaria tomentosa* extract was added to the culture medium at a final concentration of 100 and 200 μg/mL before the addition of $CD34^+$ cells. The medium was vortexed, and 7.5×10^2 $CD34^+$ cells were added per 1.1 mL of medium. Then, 1.1 mL of medium (7.5×10^2 cells) was transferred to each 35-mm plate. Cultures were incubated at 37 °C under 5% CO_2 in air and humidity higher than 95% for 15 days. Subsequently, they were visualized under a microscope with phase contrast. The tests were performed in two independent experiments and in duplicate for controls and tests.

The trial was approved by the Human Research Ethics Committee of the Universidade *Federal de Santa Catarina*, Protocol number 311/2008, in November 2008.

2.9. Statistical analysis

Statistical analyses of the bioassay data were carried out according to Finney by parallel line methods (2×2) using a PLA 2.0 Program (Stegmann Systemberatung, Rodgau, Germany) to calculate the potency and confidence intervals ($P = 0.05$). Analysis of variance was performed for each assay, and the assumption of regression and parallelism of the log dose–log response lines were tested ($P = 0.05$). The data for hematological analysis were analyzed with the EpiInfo program, version 3.5.1, from the CDC/USA. The data were evaluated by analysis of variance and *t*-test and are expressed as the mean ± SD.

3. Results

3.1. Pentacyclic oxindole alkaloids from Uncaria tomentosa extract

The HPLC analysis of dry *Uncaria tomentosa* extract has a content of 2.57% POA. The concentrations of each POA were as follows: speciophylline – 0.26%; uncarine F – 0.07%; mitraphylline – 0.80%; isomitraphylline – 0.40%; uncarine C – 0.46%; and uncarine E – 0.58%. The chromatographic profile in Fig. 1 does not include the tetracyclic oxindole alkaloids (TOA), rhynchophylline and isorhynchophylline.

3.2. Hematological evaluations

The experimental mice receiving filgrastim and *Uncaria tomentosa* showed significant recovery of their neutrophil counts compared to the control group after ifosfamide-induced neutropenia. Additionally, the recovery (increase) of neutrophils was four and thirteen times higher for the two doses of *Uncaria tomentosa* tested, respectively. The minimum neutrophil count for the dose of 15 mg per mouse was 593.7 cells/μL, whereas the control group showed 0.0 cells/μL, with the highest value of 211.5 cells/μL. Thus, the dose of 15 mg of *Uncaria tomentosa* showed positive qualitative effects near the normal range, while the leukocytes of the control group were almost entirely lymphocytes (91.7%), as shown in Table 1 and Fig. 2. Additionally, the values obtained for the control group showed the utility of the experimental model of ifosfamide-induced leukopenia.It is essential to establish dose dependency with a well-established drug in clinical use in studies with herbal remedies because they are extracts instead of purified, active compounds. Therefore, a bioassay was performed to compare a biopharmaceutical sample of 3 and 9 μg doses of filgrastim and 5 and 15 mg of *Uncaria tomentosa*. The validity of the assay was shown by the analysis of variance, and the results were statistically calculated, giving a potency of 85.20%.

Ascorbic acid was used as a positive standard, in order to evaluate antioxidant activity. The use of ascorbic acid at dose of 5.5 mg per mouse (250 mg/kg) resulted in leukocytes count increase (4493.7/μL, SD 1545.9, $P = 0.02$) when related to control (2255.0/μL, SD 724.6). But, different from the *Uncaria tomentosa* or filgrastim treatment, the major increase occurred in lymphocyte

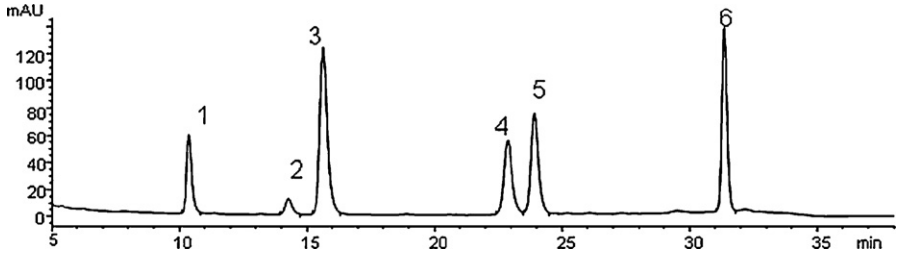

Fig. 1. HPLC-fingerprint analysis of dry extract from *Uncaria tomentosa*. Speciophylline (1), uncarine F (2), mitraphylline (3), isomitraphylline (4), uncarine C (5) and uncarine E (6).

Table 1
Recovery of leukocytes and hemoglobin values with *Uncaria tomentosa* (VO) or filgrastim (IP) four days after induction of leukopenia with ifosfamide (IP) in mice.

Group	Control	*Uncaria tomentosa*		Filgrastim	
Dose	X *n* = 6	5 mg VO *n* = 6	15 mg VO *n* = 6	3 μg IP *n* = 6	9 μg IP *n* = 5
Leucocytes (cell/μL)	2025.6^{A} (1267.6)	2230.0^{A} (1114.5)	2690.1^{A} (1261.1)	3395.1^{A} (2933.7)	4125^{A} (1933.5)
Neutrophils (cell/μL)	98.3^{C} (85.4)	464.4^{B} (255.1)	1366.5^{AB} (605.7)	1571.9^{A} (1408.9)	1979.5^{A} (1672.2)
Lymphocytes (cell/μL)	1919.5^{A} (1180.0)	1608.7^{A} (840.4)	1164.9^{B} (544.8)	1637.7^{A} (1440.4)	1849.8^{A} (358.1)
Monocytes (cell/μL)	27.2^{C} (20.4)	166.8^{AB} (180.2)	158.6^{B} (129.8)	185.4^{A} (136.0)	289.6^{A} (190.3)
Hemoglobin (g/dL)	12.7^{A} (1.9)	14.38^{A}a (1.8)	15.55^{A} (1.5)	13.87^{A} (1.6)	14.95^{A} (2.3)

Values expressed as means (SD). Values in the same row that do not share the same upper case superscript letters are significantly different among the treatments.

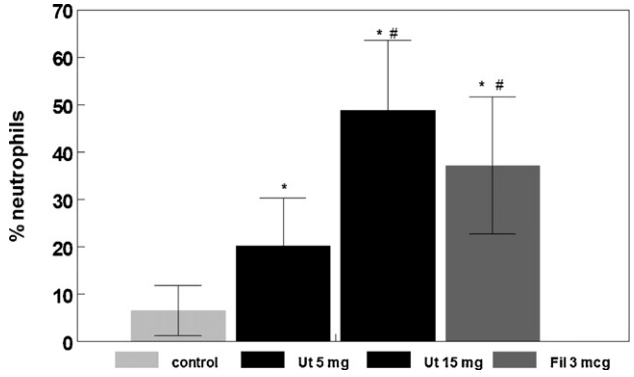

Fig. 2. Recovery of neutrophils (% values) with *Uncaria tomentosa* (VO) or filgrastim (IP) four days after induction of leukopenia with ifosfamide (IP) in mice. $^{*}P < 0.005$ in relation to the control group; $^{\#}P < 0.05$ between treatments (Ut 15 mg × Ut 5 mg; Fil 3 μg × Ut 5 mg); data were represented as means ± SD. Ut 5 mg = *Uncaria tomentosa* dry extract 10 mg/mL; dose 0.5 mL/day per animal; Ut 15 mg = *Uncaria tomentosa* dry extract 30 mg/mL; dose 0.5 mL/day per animal; Fil 3 μg = 6 μg/mL, dose 0.5 mL/day per animal.

count (3257.9/μL, SD 88.4 vs 1873.0/μL, SD 543.6 in the control group, $P = 0.01$).

3.3. Oxidative stress

There were no differences in levels of non-protein thiols or in the activities of antioxidant enzyme catalases or superoxide dismutase (SOD) among *Uncaria tomentosa*, filgrastin, and control groups (Table 2).

Table 2
Values of antioxidants in animals treated with *Uncaria tomentosa* or filgrastim, after ifosfamide-induced neutropenia.

	Control	*Uncaria tomentosa* 15 mg	Filgrastim 3 μg
NPSH [a] (μmol SH/mL)	0.73 (0.08)	0.75 (0.04)	0.73 (0.05)
SOD[b] (UI/mg of protein)	9.09 (0.11)	7.11 (1.92)	8.41 (1.66)
Catalase (nmol/mg of protein)	9.00 (1.64)	8.81 (0,90)	7.71 (1.64)

Values expressed as mean ± SD.
[a] Non-protein thiols.
[b] Superoxide dismutase.

The experimental mice receiving ascorbic acid showed mild reduction (33%) on non-protein thiols levels in relation to the control mice, four days after induction of leukopenia with ifosfamide. There were no differences in antioxidant enzymes (data not shown).

3.4. Allometry scale

To evaluate whether the dose tested in the animals is suitable to be used in humans, the allometric scale was used (White and Seymour, 2005). Allometry is the study of how a dependent variable (metabolic rate) varies in relation to an independent variable (body mass). The allometric comparison allows one to calculate dosages and frequencies of drug administration to individuals based on individual calorie needs. The *Mammalian basal metabolic rate* (MBR) for this animal study (mice) was 35.2 mL O_2 h^{-1}; for humans, it was 7545.6 mL O_2 h^{-1}. Thus, considering that the average weight of animals was 24 g, mice treated with 0.5 mL of solution containing 10 mg of dry *Uncaria tomentosa* extract received 208 mg/kg of *Uncaria tomentosa* that corresponded to a dose of 17.8 mg/kg or 1 g/day for a 60-kg human.

3.5. In vitro assay

CFC assays showed possible increases in cell proliferation after *Uncaria tomentosa* extract administration *in vitro*, evidenced by the overplating effect observed in these cultures. This effect did not occur in the control trials conducted simultaneously with the same cell samples. The overplating effect made it impossible to accurately count the number of colonies, making it undesirable for data collection; therefore, we opted for a semi-quantitative and qualitative assessment. Semi-quantitatively, the observed overplating effect was caused by an increase in the size of granulocyte-macrophage colony-forming cells (CFU-GM) and mixed colonies (CFU-GEMM), presenting considerable confluence/overlap (Figs. 3 and 4). Qualitatively, a reduction in the intensity of the reddish color given by the hemoglobin to the B-FUE colonies was observed. This reduction was more evident at the concentration of 200 μg extract/mL, which suggests lower hemoglobinization of these colonies in cultures with *Uncaria tomentosa*, as seen in Fig. 5. Images are representative of a semi-quantitative and general

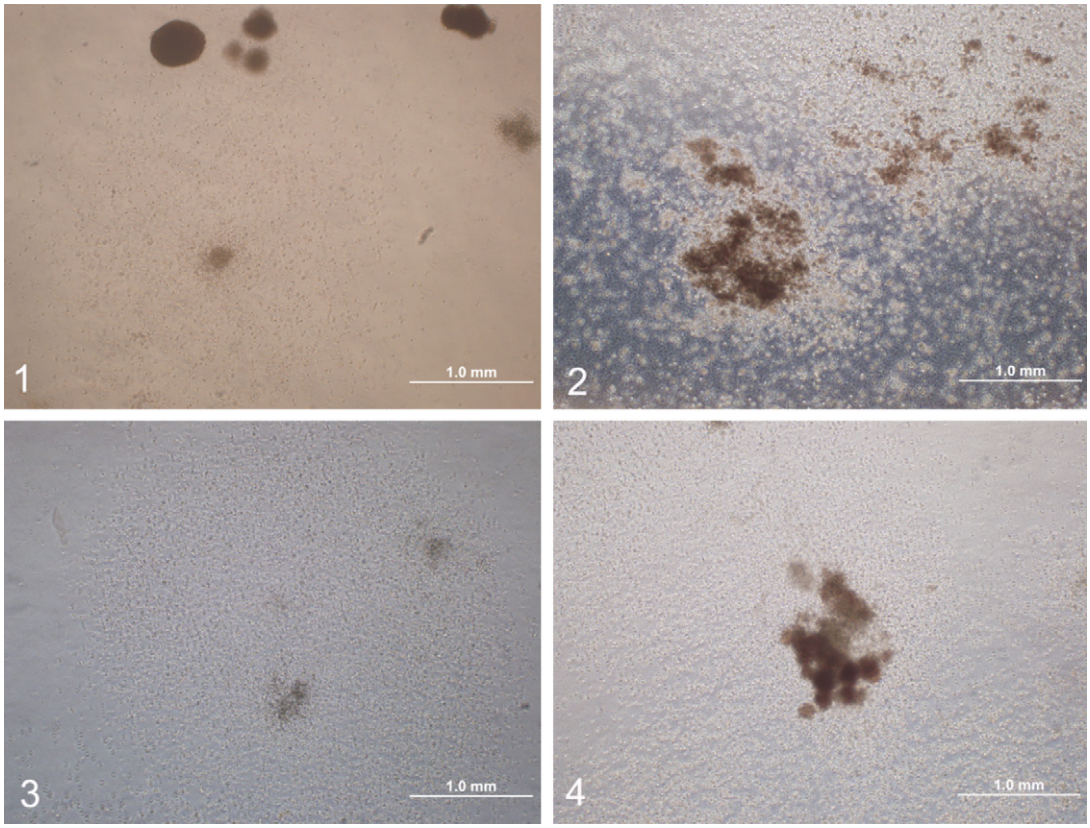

Fig. 3. CFC assays treated with extract of *Uncaria tomentosa*, final concentration of 100 μg of extract/mL medium (40×). Photo 1: CFC assay controls showing the absence of overplating (40×). Photo 2: demonstration of the overplating effect with the addition of *Uncaria tomentosa* extract. Photos 3 and 4: CFU-GM and CFU-GEMM, respectively, with high granulocytic proliferation, occupying nearly the entire microscopic field in extract-treated cell.

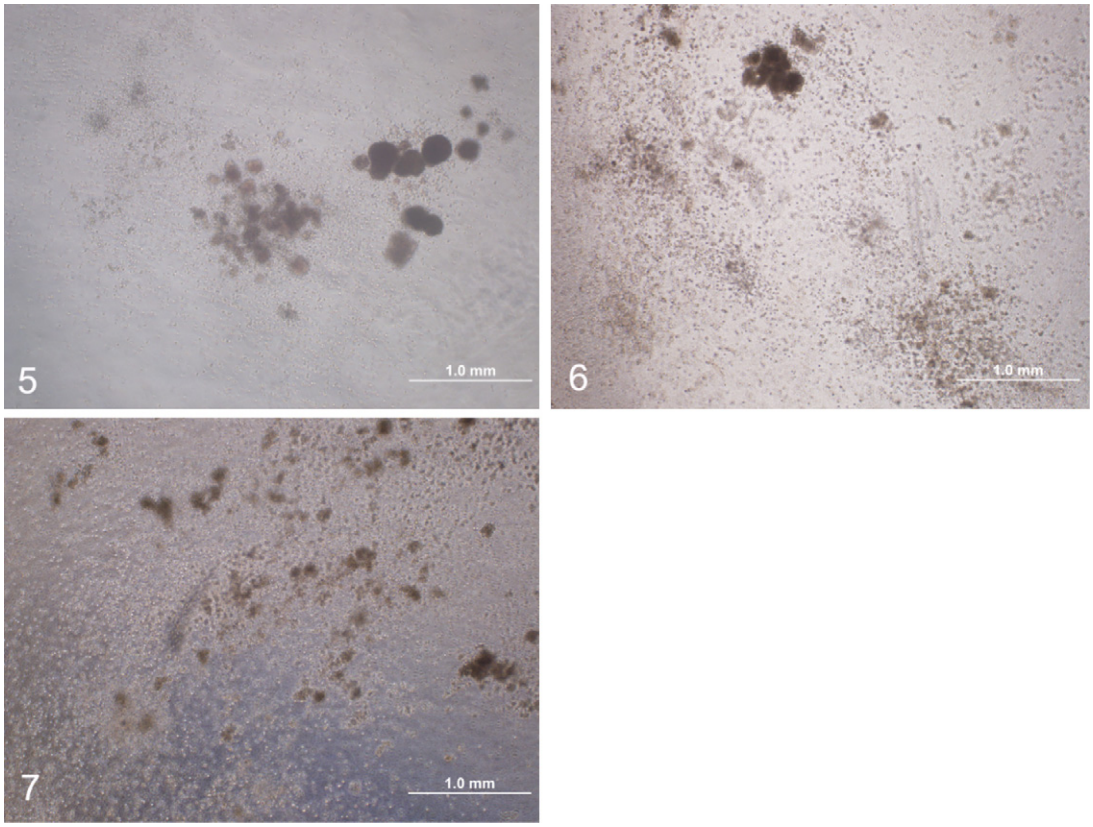

Fig. 4. CFC assays treated with dry extract of *Uncaria tomentosa*, final concentration of 200 μg/mL medium. Photo 5: CFC assay control showing the absence of overplating (40×). Photos 6 and 7: The overplating effect is demonstrated with addition of *Uncaria tomentosa* extract (40×).

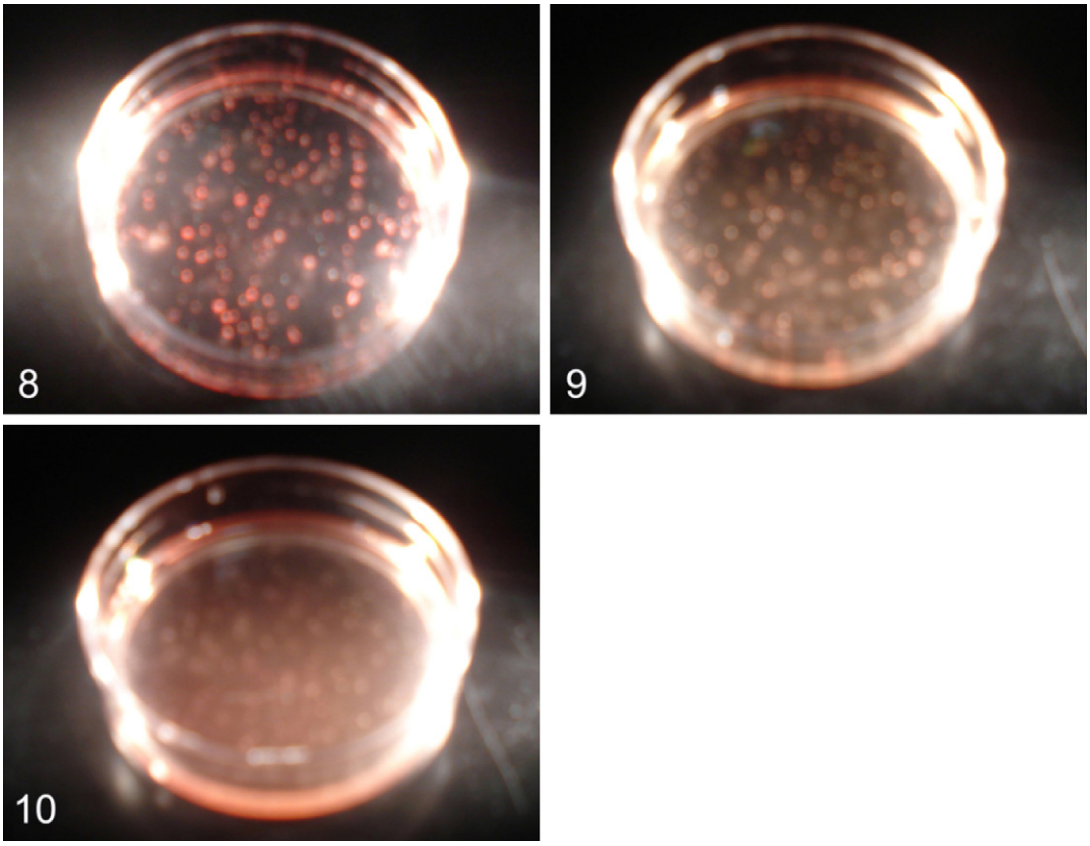

Fig. 5. Reduction in hemoglobin content in the CFU with addition of *Uncaria tomentosa* to the medium at final concentrations of 100 and 200 μg of dry extract/mL medium. Images of the plates of CFC assay control (Photo 8) and those treated with *Uncaria tomentosa* extract at final concentrations of 100 (Photo 9) and 200 (Photo 10) μg of extract/mL medium, respectively, showing the reduction of the reddish color afforded by hemoglobin (40×).

qualitative evaluation of observations in control and test culture plates.

4. Discussion

The alkaloid profile of the material used has great importance in evaluating the results obtained because POAs and tetracyclic oxindole alkaloids (TOAs) have different pharmacological properties. TOAs appear to have accentuated action in the cardiovascular and central nervous system (Shi et al., 2003); however, POAs are associated with antioxidant and anti-inflammatory action, as previously mentioned. Furthermore, TOAs are described to antagonize the pharmacological functions of POAs; therefore, inducing human endothelial cells to release a lymphocyte-proliferation-regulation factor (Wurm et al., 1998). Thus, the absence of TOAs in the sample allows its use for therapeutic and research purposes in accordance with U.S. Pharmacopeia (USP 32, 2009).

The use of ifosfamide in the present study resulted in severe neutropenia, as the control animals showed means of only 98 neutrophils/μL. Similar events are observed in clinical oncology and are a part of the focus in this study. This situation was reversed by *Uncaria tomentosa*, in which the group receiving 15 mg per animal showed 500 neutrophils/μL or higher. The administration/injection of *Uncaria tomentosa* and filgrastim during four consecutive days caused significant increases in the neutrophil count in terms of both absolute values (cells/μL) and percentage and promoted recovery from the severe ifosfamide-induced neutropenia. Even animals that received the lower dose, corresponding to 5 mg of *Uncaria tomentosa* dry extract/day, showed a significant increase in neutrophil count compared to controls. The dose tested was equivalent to 1 g/day of dry extract for a 60-kg human. Similar doses have been used for the treatment of rheumatic diseases (Castañeda et al., 1998). In this study (Castañeda et al., 1998), patients took 2 capsules of 150 mg *Uncaria tomentosa* dry extract 3 times daily for 6 months, and their hemogram, creatinine, glucose, transaminase, alkaline phosphatase, and urine were evaluated. Side effects observed include epigastric pain, dizziness, bloating, constipation, diarrhea, and bitterness in the mouth; however, all of these side effects were present only transiently. These data are consistent with those reported by National Center for Complementary and Alternative Medicine (USA) (NCCAM/NIH, 2011a), which has reported few side effects from cat's claw at the recommended dosages. Though rare, side effects may include headaches, dizziness, and vomiting; however, ambiguity exists regarding the values of "recommended dosages". The low toxicity of *Uncaria tomentosa* can be evaluated by the acute median lethal dose (LD50) to mice of an aqueous extract with 3.5% of total POA, which was found to be greater than 16 g/kg bodyweight (Keplinger et al., 1999).

The two treatments (filgrastim and *Uncaria tomentosa*) did not differ in the resulting absolute number of lymphocytes compared to controls. Sheng et al. (2000a), using an aqueous extract of *Uncaria tomentosa* (C-Med100®) in healthy rats, only observed an increase in the total number of leukocytes at doses greater than or equal to 40 mg/kg. In a subsequent study, leukopenia induced by the anticancer agent, doxorubicin, was reversed with subsequent administration of *Uncaria tomentosa*, and significant differences compared to controls at a dose of 80 mg/kg were observed (Sheng et al., 2000b). Using doxorubicin to induce leukopenia, the authors achieved a slight reduction in neutropenia.

As described from the outset, *Uncaria tomentosa* has three properties that are related to cell proliferation, particularly in terms

of WBCs: anti-inflammatory, antioxidant, and CSF-stimulating effects.

4.1. Anti-inflammatory

One of the possible pathways of action of *Uncaria tomentosa* is the prolongation of WBC survival due to the inhibited activation of NF-κB (Sandoval et al., 2000; Fazio et al., 2008), which activates several different signals and can regulate the expression of several pro-inflammatory cytokines, including TNF-α (Groom et al., 2007), interleukin-1 (IL-1), interleukin-2 (IL-2), interleukin-6 (IL-6), and interleukin-8 (IL-8). These effects subsequently decrease oxidative stress (Pilarski et al., 2006) in the cell, which in turn reduces damage to DNA (Mammone et al., 2006). This postulated effect would predict that WBCs survive longer in the circulation due to reduced DNA damage and enhanced DNA repair, thus elevating the number of immune competent cells. In the 1990s, Wurm et al. demonstrated that the POAs from *Uncaria tomentosa* stimulated endothelial cells to release a lymphocyte-proliferation-regulating factor that promoted the proliferation of normal lymphocytes and inhibited the proliferation of lymphoblasts. Akesson et al. (2003) concluded that the increase in lymphocyte counts in animals that received *Uncaria tomentosa* orally for 10 weeks was likely due to increased survival in peripheral lymphoid organs. In our study, there were no changes in the number of lymphocytes, possibly because this study took place during the acute phase.

The inhibition of NF-κB prolongs survival of WBCs; however, it is needed as a mediator for the proliferation of progenitors. Blocking the activation of NF-κB resulted in almost complete suppression of the formation of the CFU-GM, BFU-E and CFU-E progenitors. NF-κB activity is required for normal hematopoiesis and the maintenance of hematopoietic progenitor cell function, and it acts as a second messenger for a number of cytokines known to regulate hematopoiesis, such as IL-3, Epo, IL-6, SCF, and GM-CSF (Bugarski et al., 2006). Similarly, IL-6 aids granulocyte colony maintenance and the viability of mature neutrophils. IL-6 and G-CSF synergistically increase myeloid progenitors (Suzuki et al., 1996) as G-CSF facilitates the expansion of myeloid precursors, likely by stimulating the proliferation of monocytes to secrete IL-6 (Liu et al., 2005). Thus, one possible pathway for stimulating myeloid proliferation by *Uncaria tomentosa* could be the release of IL-1 and IL-6 (Lemaire et al., 1999). In the present study, the animals had a severe reduction in the number of neutrophils and monocytes. A decrease in the number of these cells impairs the production of cytokines (Groom et al., 2007) and other stimulators of the proliferation and maturation of CFU-GM. In the model of the study that we use, it is possible that the stimulation of the proliferation of myeloid tissue occurred through another pathway.

4.2. Antioxidant properties

ROS interfere with erythropoiesis because levels of reduced glutathione are directly related to mitosis. The ifosfamide used in *in vivo* tests is a DNA-alkylating agent frequently used in chemotherapy against human malignancies. Ifosfamide and its major decomposition products deplete intracellular glutathione (GSH). GSH is the major intracellular thiol reductant, protecting cells against oxidative injury. Ifosfamide depletion of intracellular GSH in human dendritic cells (DC), T cells and natural killer (NK) cells impairs their functional activity, which can be restored by reconstituting GSH (Kuppner et al., 2008). The assay of non-protein thiols was not different in animals treated with *Uncaria tomentosa*, but all groups had lower values (0.73 mmol/mL blood) than those found in the control rats of other studies (1.20 ± 0.10 mmol/mL of plasma; Spanevello et al., 2009). Also ascorbic acid, drug known to have antioxidant proprieties, did not restored non-protein thiols levels.

The use of antioxidants during cancer treatment is still controversial. This duality is true because if some antioxidants scavenge the ROS integral to the activity of certain chemotherapy drugs, which would diminish treatment efficacy, another antioxidant might mitigate toxicity and thus allow for uninterrupted treatment schedules and a reduced need for lowering chemotherapy doses (NCCAM/NIH, 2011b). Moreover, the various ROS can exert different effects according to their nature and to their intracellular level. The same phenomena occur for antioxidants, e.g., N-acetylcysteine, which decreases H_2O_2 levels, inhibits normal cell (fibroblast) proliferation but increases tumor cell proliferation (CT26 and Hepa 1–6 cells). In contrast, antioxidant molecules that mimic SOD (CuDIPS or MnTBAP) increase *in vitro* proliferation of normal cells but kill tumor cells (Laurent et al., 2005).

Even in relation to oxidative stress, levels of H_2O_2 interfere with the proliferation of myeloid precursors. Neutralization of H_2O_2 by catalase inhibits the proliferation of immature myeloid cells and also stimulates their differentiation into mature myeloid cells. SOD slightly decreases proliferation (Kusmartsev and Gabrilovich, 2003). Other studies have shown that *Uncaria tomentosa* has antioxidant activity, which is related to the reduction of tumor growth (Dreifuss et al., 2010); however, contradictory results in which a mild reduction of H_2O_2 (Gonçalves et al., 2005) and an increased activity of SOD have been observed (Pilarski et al., 2006). However, in our study, no significant differences in catalase or SOD activities were registered between treatments.

4.3. Effect on myeloid progenitors

In the case of neutropenia due to chemotherapy, when the number of WBCs is severely diminished and the scavenging activity of GSH is decreased, it is necessary to understand the route that would preferentially activate recovery from neutropenia. Therefore, to assess whether *Uncaria tomentosa* has a stimulating effect on myeloid progenitors, CFC assays were conducted using hHSPCs obtained from UCB. The addition of dry *Uncaria tomentosa* extract resulted in an increase in CFU-GM size and CFU-GEMM size at the final concentrations of 100 and 200 μg extract/mL.

It is suggested that *Uncaria tomentosa* extract may have positive effects on myeloid progenitor proliferation. These data are consistent with results obtained by Eberlin et al. (2005), who documented an increase in marrow CFU-GM and an increase in serum colony-stimulating factor CSFs in mice treated with *Uncaria tomentosa* extract (50 and 100 mg/kg).

To compare the effect on myeloid progenitors with the antiproliferative effect of neoplastic cells described above, the concentration of mitraphylline was transformed from μmol to μg/mL. Mitraphylline ($C_{21}H_{24}N_2O_4$, Molar mass 368.1736 g/mol) was used at 5–40 μmol (Garcia Prado et al., 2007; García Gimenez et al., 2010), which corresponds to 1.8–14.7 μg/mL. In the present study, 100–200 μg/mL of dry hydro-alcoholic extract containing POA 2.6–5.1 μg/mL was used, of which 0.8–1.6 μg/mL was mitraphylline. Bacher et al. (2005) used larger concentrations of four POAs (50–200 μmol); therefore, the concentration needed to stimulate the proliferation of myeloid precursors may be less than the concentration needed to induce apoptosis of neoplastic cells.A qualitative reduction in hemoglobin content was observed macroscopically and microscopically. Pilarski et al. (2009), using a chicken embryo model injected with *Uncaria tomentosa* extracts, detected unfavorable changes in Mean Corpuscular Volume (MCV), Mean Cellular Hemoglobin (MCH) and Mean Cellular Hemoglobin Concentration (MCHC). Further studies are necessary to evaluate if a toxic effect occurred and to identify the possible route of action. The leading possibility is inhibition of enzymes that participate in

the formation of heme and hemoglobin or metabolic deficiencies of Vitamin B12 and folic acid, as proposed by Pilarski et al. (2009).

5. Conclusion

At the doses tested, *Uncaria tomentosa* is promising for use in conjunction with chemotherapy because it minimizes the adverse effects of this treatment and attenuates neutropenia. More studies are needed to evaluate the interaction of *Uncaria tomentosa* with various chemotherapeutic agents and cell types.

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