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Original Article

Immunostimulating activity of *Uncaria tomentosa* in RAW 264.7 macrophages

Atividade imunomoestimulante da *Uncaria tomentosa* em macrófagos RAW 264.7

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Abstract

Uncaria tomentosa is a plant native to the Amazon that has immunomodulatory and antitumor properties due to the alkaloids found in the plant, being able to modify the immune response by potentiating or suspending the action of cytokines secreted by macrophages that induce the immune response, either by the classical route (M1) or through the alternative route (M2). Macrophages activated by M1 convert L-arginine into L-citrulline and nitric oxide (NO), whereas macrophages activated by the M2 pathway use the enzymatic activity of arginase to convert the same substrate into L-ornithine and urea. The aim of this work was to evaluate the immunostimulating activity of the crude hydroalcoholic extract from the bark of the *U. tomentosa* stem in RAW 264.7 macrophages. Concentrations of 0.2, 0.1 and 0.05 mg/mL of *U. tomentosa* extract associated with LPS, INF-γ and IL-4 inducers were tested by determining NO production and arginase enzyme activity. Nitric oxide production was enhanced by the extract when associated with LPS and LPS + INF-γ inducers. In the activity of the arginase enzyme, the extract decreased the stimulation of IL-4 on the enzyme, mainly at 0.2 mg/mL concentration. Therefore, it is concluded that the crude hydroalcoholic extract of the stem bark of *U. tomentosa* in RAW 264.7 cells, at a concentration of 0.2 mg/mL, showed considerable pro-inflammatory activity.

Keywords: arginase, nitric oxide, pro-inflammatory activity.

Resumo

A *Uncaria tomentosa* é uma planta nativa da Amazônia que possui propriedades imunomoduladoras e antitumorais devido aos alcaloides encontrados no vegetal, sendo capaz de modificar a resposta imune ao potencializar ou suspender a ação de citocinas secretadas por macrófagos que induzem a resposta imune, seja pela via clássica (M1) ou pela via alternativa (M2). Macrófagos ativados pela M1 converte L-arginina em L-citrulina e óxido nítrico (NO), já os macrófagos ativados pela via M2 utilizam a atividade enzimática da arginase para converter o mesmo substrato em L-ornitina e ureia. O trabalho teve como objetivo avaliar a atividade imunoestimuladora do extrato hidroalcoólico bruto da casca do caule da *U. tomentosa* em macrófagos RAW 264.7. Foram testadas as concentrações 0.2, 0.1 e 0.05 mg/mL do extrato de *U. tomentosa* associadas aos indutores LPS, INF-γ e IL-4, por meio da determinação da produção de NO e atividade da enzima arginase. A produção de óxido nítrico foi potencializada pelo extrato quando associado aos indutores LPS e LPS + INF-γ. Na atividade da enzima arginase, o extrato diminuiu a estimulação da IL-4 sobre a enzima, principalmente na concentração 0.2 mg/mL. Portanto, conclui-se que o extrato hidroalcoólico bruto da casca do caule de *U. tomentosa* em células RAW 264.7, na concentração 0.2 mg/mL, demostrou atividade pró-inflamatória considerável.

Palavras-chave: arginase, óxido nítrico, atividade pró-inflamatória.

1. Introduction

Uncaria tomentosa (*U. tomentosa*) (Willd. Ex Schult.) DC., is a native Amazonian plant, widely used in folk medicine and the pharmaceutical industry (Honório et al., 2017), due to its medicinal properties. It is locally known as cat's claw and belongs to the Rubiaceae family (Batiha et al., 2020), with geographic distribution in several areas of South and Central America, at altitudes of 800 to 2,500 meters above sea level. It is a climbing shrub, containing adult thorns directed downwards and not twisted, membranous limb of a yellowish-green color and opaque, leaves with seven to 10 veins, sessile flowers and a glabrous corolla (Obregón Vilches, 1997; Pereira and Lopes, 2006).

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There are several medicinal properties attributed to the plant, such as immunomodulation (Domingues et al., 2011), antitumor activity (Santos et al., 2016) and antioxidant action (Miller et al., 2001), with the stem bark and leaves being the most common parts used for the preparation of teas, infusions, geriatric wines, tinctures or alcoholic extracts and capsules (Pereira and Lopes, 2006).

The pentacyclic oxindolic alkaloid metabolites contained in *U. tomentosa* stem bark extracts already described in the literature are specophylline, mitraphylline, uncarin F, pteropodin, isomitraphyline and isopteropodin (uncarin E) (Sandoval et al., 2002; Pereira et al., 2008; Zeferino et al., 2021), being the biological activities of the plant related to the immune system attributed to these compounds.

There are several ways to evaluate the immunomodulatory effect of plant extracts on cells linked to the immune system such as immortalized cell lines of macrophages, since the RAW 264.7 cell is widely used (Lemaire et al., 1999). Macrophages have strong plasticity, heterogeneity, pluripotency and in different phases of the inflammatory response cytokines secreted by macrophages are important, as they induce and mediate inflammatory responses (Stuehr and Marletta, 1987). Once being activated, macrophages can differentiate into M1 or M2 phenotype and perform specialized functions in different microenvironments (Li et al., 2021). Macrophages can be polarized into classically activated macrophages (M1) and alternatively activated macrophages (M2), performing pro-inflammatory or anti-inflammatory functions, respectively (Li et al., 2021; Lv et al., 2017).

Upon activation via the M1 pathway, large amounts of the enzyme nitric oxide synthase (NOS) are produced, which uses the substrate L-arginine to produce nitric oxide (NO) and the activation is mainly induced by lipopolysaccharide (LPS), interferon-gamma (IFN-γ) and tumor necrosis factor (TNF). M1 macrophages participate in immune responses, secreting pro-inflammatory cytokines and chemokines and play a role in immune surveillance (Li et al., 2021). Stimulation of this pathway kills intracellular microorganisms, T helper type 1 (Th1) response, and potent effector macrophages that eliminate microorganisms and tumor cells. It may also induce the increase and secretion of pro-inflammatory mediators and cytokines such as tumor necrosis factor alpha (TNF-α), interleukin 6 (IL-6), interleukin 12 (IL-12) and interleukin 23 (IL-23) (Bogdan et al., 2000; Huppes, 2013; MacMicking, 2009).

Activation of the M2 pathway is induced by interleukin 4 (IL-4) and interleukin 13 (IL-13), which promotes T helper type 2 (Th2) response and type II inflammation, helminth death, tumor development, in addition to promoting immunoregulation and repair of tissue damage. This macrophage metabolizes L-arginine by the enzyme arginase, producing L-ornithine and urea as a product. M2 macrophages reduce the expression of inflammatory cytokines, secrete large amounts of anti-inflammatory cytokines such as IL-10 and TGF-β, and downregulate immune responses that promote tissue remodeling (Huppes, 2013; Li et al., 2021).

Due to the immunological potential of *U. tomentosa*, this study aimed to evaluate the immunostimulant activity of the crude hydroalcoholic extract of the stem bark of *U. tomentosa* in murine macrophages of the lineage RAW 264.7, by evaluating NO production and activity of the arginase enzyme.

2. Material and Methods

2.1. Characterization of vegetable raw material

For the characterization of the vegetable raw material, the foreign matter determination tests were carried out, as well as the determination of desiccation loss, determination of total ash and acid-insoluble ash, determination of the swelling index and determination of the particle size of the powders, according to the methodology described in Brasil (2010).

2.2.1. Determination of foreign materials

A 50g sample of plant material was spread in a thin layer on a flat surface and analyzed with the naked eye. Foreign materials were separated from the drug, weighed and their percentages determined based on the weight of the sample submitted to the test.

2.2.2. Determination of loss on desiccation

The determination of the amount of volatile substance present in the plant bark powder was carried out according to a procedure that uses an infrared scale, an alternative method to the gravimetric method. For this, about 1 g of sample was placed on an Ohaus® model MB35 balance and subjected to high temperature (105ºC). Finally, assays were performed in triplicate and the result was recorded as a percentage on the scale display.

2.2.3. Determination of total ash

For the determination of total ash, 3 g of pulverized sample was transferred to a crucible previously calcined in a muffle (EDG Equipment's® - Edgcon1P) at 450°C, cooled, weighed, and tared. The sample was uniformly distributed in the crucible and incinerated in a muffle, gradually increasing the temperature to a maximum of 600±25°C until all the charcoal was eliminated. Subsequently, the sample was cooled in a desiccator and weighed again. The total ash content was determined in percentage by the average of three determinations in relation to the air-dried drug.

2.2.4. Determination of acid-insoluble ash

To determine the acid-insoluble ash content, the residue obtained in the test of item 2.2.3 was boiled for 5 minutes with 25 mL of 7% hydrochloric acid (w/v), in a crucible covered with a watch glass. The watch glass was washed with 5mL of hot water and the samples (residue + watch glass wash) were pooled and filtered through ash-free filter paper. Then, the filter paper was washed with hot water until neutral pH and transferred to the original crucible, whose contents were dried under a hot plate and incinerated in a muffle at 500°C until constant weight. The acid-insoluble ash content was determined as a percentage by the average of three determinations related to the air-dried drug.

2.2.5. Determination of the swelling index

To determine the swelling index, 1 g of the powder was weighed and placed in a 25 mL test tube with a ground-top lid and then 25 mL of distilled water was added, shaking the test tube every 10 minutes for 1 hour. After resting for three hours at room temperature, the final volume occupied by the plant material was verified and subtracted from the initial volume of the drug. The swelling index was obtained from individual determinations, and the experiment was carried out in triplicate.

2.2.6. Determination of powder granulometry

For granulometric determination, a set of stainless-steel sieves with 710, 355, 300, 250, 180 and 125 µm mesh, provided with a lid and container for collecting dust, were previously weighed and assembled. About 25 g of Uncaria tomentosa stem bark powder was weighed in a semi-analytical balance and then uniformly distributed in the upper sieve. The set of sieves was subjected to vibrations at a standardized speed for approximately 15 minutes in a vibrating sieve (Bertel). After the procedure, the portions of powder retained in each sieve and also in the collection flask were weighed separately. The analysis was performed in triplicate and the percentage of powders related to each sieve was calculated to classify the powders according to the parameters described in the aforementioned literature.

2.2. Obtaining the crude hydroalcoholic extract of Uncaria tomentosa

The powder obtained was subjected to dynamic maceration with a 40% (v/v) hydroalcoholic solution, for 12 hours, and transferred to a percolator, whose volume was completed with enough alcohol to reach a ratio of 1:10 (m/v). The extract was then repercolated 10 times and finally concentrated in a rotoevaporator (Buchi® – model R-220 SE), under controlled conditions of pressure (100 mbar), temperature (40°C) and rotations per minute (70rpm). This step is essential for the evaporation of all solvent contained in the sample. After obtaining the crude hydroalcoholic extract powder, it was diluted to obtain the test concentrations.

All tests for the characterization of the vegetable raw material, such as obtaining the crude extract of the bark of the *U. tomentosa* stem, were carried out at the Laboratory of Research, Development and Innovation of Bioproducts / Natural Products Research Laboratory (PD&I of Bioproducts / LPPN) of the Faculty of Pharmacy at UFG.

2.3. Cytotoxicity test

Macrophages from RAW 264.7 mice were cultured in a flat-bottomed culture dish ((Corning-Costar - Kennebunk, ME, USA) in RPMI 1640 medium (Sigma-Aldrich) supplemented with 50 µM 2-mercaptoethanol (Sigma-Aldrich), 1 M HEPES, 100 U/mL penicillin (Sigma-Aldrich) and 100 µg/mL streptomycin (Sigma-Aldrich) at 37ºC, in a 5% CO2 oven.

For the test, 15 concentrations (12.8; 6.4; 3.2; 1.6; 0.8; 0.66; 0.44; 0.3; 0.2; 0.15; 0.1; 0.07; 0.05; 0.03 and 0.02 mg/mL) were prepared, the powder being weighed and then dissolved in RPMI medium for better solubilization. Afterwards, the concentrations were filtered with a 0.22µm filter and stored in a polystyrene tube at 4ºC. A cell suspension at a concentration of $5x10^5$ cells was prepared and added to a 24-well polystyrene plate, along with each concentration of extract. In one well, only cell and RPMI medium were added, being considered the test control. Again, the plates were incubated at 37°C, in a 5% CO2 oven, for 48 hours.

After the incubation period, a 10 µL aliquot of 0.1% trypan blue was added to each well in order to assess cytotoxicity by counting viable and non-viable cells. This evaluation was performed by placing the plate under an inverted optical microscope. The test was performed in triplicate and the average was used to determine the cell viability curve of the *U. tomentosa* extract. Among the concentrations evaluated, three concentrations that had cytotoxicity less than 25% were selected for evaluation of immunostimulating activity.

To determine the cell viability curve, a relationship with the control was established, being calculated as shown in Equation 1:

$$Cell\ viability(\%) = \frac{\text{Number of live cells at each concentration}}{\text{Number of live cells in control}} \times 100 \quad (1)$$

2.4. Assessment of immunostimulant activity

To evaluate the immunostimulating activity, $5x10^5$ RAW 264.7 cells were added in a 24-well plate, as well as the stimuli LPS, INF-γ, IL-4; and the crude extract of the stem bark of *U. tomentosa* at concentrations of 0.05; 0.1 and 0.2 mg/mL, with previous addition of polymyxin B (50 µg / mL) to inactivation of endotoxin contaminants. A well containing only cells in RPI medium was used as a control (Figure 1).

After distribution, the plates were incubated for 48 hours at 37°C in an oven with 5%. CO2. At the end of this period, the supernatant and adhered cells from each well were collected separately in a polystyrene tube, for the measurement of nitric oxide (NO) and evaluation of the arginase enzyme activity, respectively.

2.5. Nitric oxide dosage

The measurement of NO was performed by detecting nitrite in the cell culture supernatant, using the Griess method. For this purpose, 50µL of the sample supernatant was distributed in 96-well plates, in duplicate.

To determine the nitrite concentration, a standard curve of nitrite in complete RPMI from 100µM was built, distributed on the plate in duplicate along with the samples.

Once the sample and curve were distributed, 50µL of Griess reagent (0.2% Naphthyl ethylenediamine dihydrochoride-NEED, 2.0% sulfanilamide in 2.5% phosphoric acid) was added to each well.

After 10 minutes, the plate was read in a plate reader (MULTISKAN), with a 550nm filter. The determination of nitrite concentration was calculated by the equation of the straight line (y= ax + b) obtained by the absorbance of the standard curve.

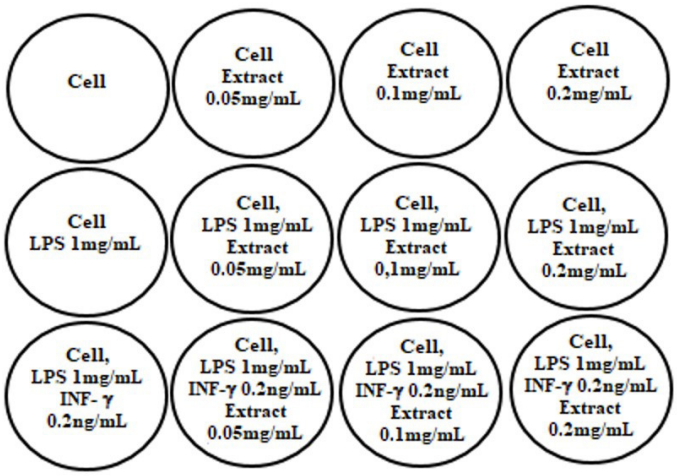

Figure 1. Methodology used to evaluate the immunostimulant activity of the crude hydroalcoholic extract of *Uncaria tomentosa* in RAW 264.7 cells, together with the stimuli lipopolysaccharide (LPS), interferon-gamma (INF-γ), interleukin-4 (IL-4).

2.6. Assessment of arginase enzyme

Adhered cells were removed from the plate using lysis buffer (Tris-HCl 500 mM pH 7.5 containing 1% Triton). This cell lysate was transferred to a polystyrene tube, followed by the addition of 50 µL of $MnCl_2$ (10 mM). The tube was then incubated for 10 min at 56°C for enzyme activation. Then, 100µL of L-arginine (5M pH 9.7) was added, and a new incubation for 60 min at 37°C.

The reaction was stopped by adding 200 µL of sulfuric acid/phosphoric acid/water solution (1:3:7), followed by the addition of 25 µL of α-isonitropropiophenone solution in absolute ethanol, with another incubation at 100°C for 45 min to produce pink color, which indicates the presence of urea. The urea concentration was compared with a standard curve starting at 800mg/dL.

The 120µL volume of each sample and curve solution was then transferred (in duplicate) to a "u" bottom plate and analyzed in a plate reader (MULTISKAN) with a 550nm filter. The determination of the urea concentration was calculated by the equation of the straight line (y= ax + b) obtained by the standard curve absorbance.

2.7. Statistical analysis

The significance of the results was compared using the one-way ANOVA test of the Graph-Pad Prism Software 5.0 program (Inc. San Diego, CA, USA), followed by Tukey, whose results were presented in graphs and expressed as mean and standard deviation. Values of $p < 0.05$ were considered significant.

3. Results

In the determination of foreign matter from the scratched bark of the *U. tomentosa* stem, no impurity was observed with the naked eye. The desiccation loss in plant material was 9.8 ± 0.08%. The total ash content found for the analyzed samples was 3.74 ± 0.014%, while for acid-insoluble ash it was nil. The tumescence index, in turn, was 4.26 ± 0.094 mL.

In determining the particle size distribution, 86.32% of the powder was retained in the 710 µm mesh size (Figure 2), being classified as coarse.

In the cytotoxicity test, the extract was shown to be 100% cytotoxic at concentrations from 12.8 to 1.6 mg/mL. At concentrations 0.8 to 0.3 mg/mL, cell viability was observed, although cytotoxicity was above 49%. Below the concentration of 0.2 mg/mL, cell cytotoxicity was low, with viability greater than 90% ($p<0.05$, ANOVA), as shown in Figure 3.

Based on the results of cytotoxicity presented, the dosage of nitric oxide production and evaluation of the activity of the arginase enzyme were carried out with 0.2, 0.1 concentrations and 0.05 mg/mL of the crude hydroalcoholic extract of *U. tomentosa*, as they demonstrate cell viability above 85%.

The association of LPS (0.1 µg/mL) + INF-γ (0.2 ng/mL) was able to induce the significant production of NO by macrophages RAW 264.7, unlike IL-4, (Figure 4).

When extract concentrations were added, an increase in NO production was observed in cells stimulated with 0.2mg/mL of extract with LPS or LPS+IFN-γ. However, only in the last case the increase was significant (Figure 5). NO production was not altered when the extract was added to IL-4 stimulated or unstimulated (CT) cells.

As for the arginase enzyme activity test, no amount of LPS tested alone or in association with INF-γ was able to significantly increase arginase activity, although with the addition of 1 µg/mL of LPS it induced a detectable arginase activity in some assay. On the other hand, the cytokine IL-4 at 5 ng/mL induced significant arginase activity (Figure 6).

As arginase activity significantly increased after stimulation of RAW 264.7 cells with 5 ng/mL of IL-4, the capacity of the crude hydroalcoholic extract of *U. tomentosa* to alter the enzyme activity was evaluated.

Arginase activity was not detected when *U. tomentosa* extract was added directly to RAW 264.7 cells at different concentrations. However, arginase activity was significantly inhibited in the presence of IL-4, according to the extract concentrations, being more accentuated in the 0.2 mg/mL extract concentration (Figure 7).

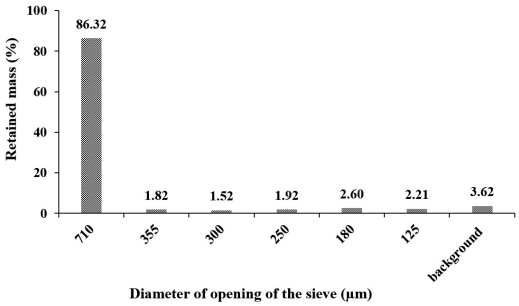

Figure 2. Result of the granulometric analysis test of the powder of the stem bark of *Uncaria tomentosa*. Retained percentage of powder according to the diameter (µm) of opening of each sieve.

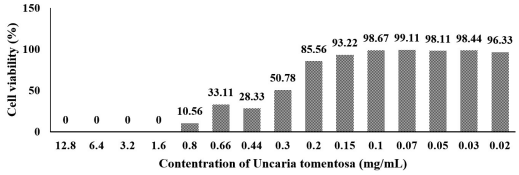

Figure 3. Viability of RAW 264.7 cells after 48 hours of exposure to different concentrations of *Uncaria tomentosa* extract by the trypan blue exclusion method, represented by the mean and standard deviation of the triplicate.

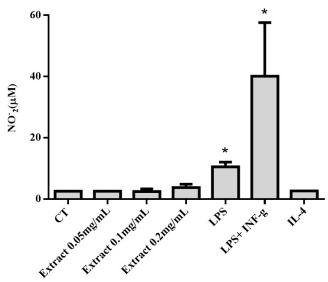

Figure 4. Production of nitric oxide by RAW 264.7 macrophage lineage. RAW 264.7 cells (5 x 10^5 cells) were stimulated with LPS (1 µg/mL), LPS (0.1 µg/mL) + INF-γ (0.2 ng/mL), IL-4 (5 ng/mL) or not stimulated (CT - Control) for 48h. After this time, the cell supernatant was collected and the amount of nitrite detected by the Griess method. The bars represent the mean ± standard deviation of nitrite production from 5 independent experiments. *Indicates significant difference by one way ANOVA test followed by Tukey (p<0.05).

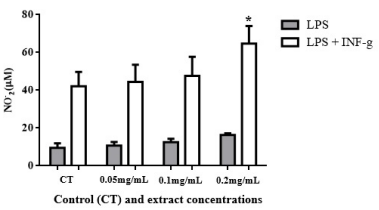

Figure 5. Production of nitric oxide by RAW 264.7 macrophage strain treated with Uncaria tomentosa extract. RAW 264.7 cells (5 x 10^5 cells) were stimulated with LPS (1 µg/mL) or LPS (0.1 µg/mL) + INF-γ (0.2 ng/mL) in the presence of different concentrations of crude hydroalcoholic extract of Uncaria tomentosa per 48h. Afterwards, the cell supernatant was collected and the amount of nitrite detected by the Griess method. The bars represent the mean ± standard deviation of nitrite production from 3 independent experiments. *Indicates significant difference by the one way ANOVA test followed by Tukey between cells without stimulus (CT - Control) or with addition of extract (p<0.05).

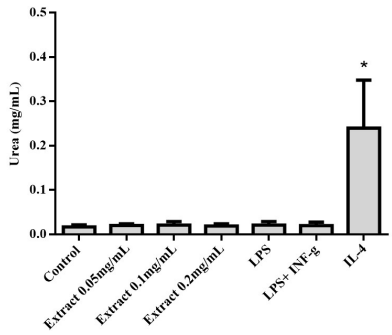

Figure 6. Arginase activity by RAW 264.7 macrophage lineage. RAW 264.7 cells (5 x 10^5 cells) were stimulated with LPS (1 µg/mL), LPS (0.1 µg/mL) + INF-γ (0.2 ng/mL) or IL-4 (5 ng/mL) or not stimulated (Control) for 48h. Afterwards, the culture supernatant was discarded and the cells remaining on the plate were used to assess arginase activity. The bars represent the mean ± standard deviation of urea production in 5 independent experiments. *Indicates significant difference by one way ANOVA test followed by Tukey (p<0.05).

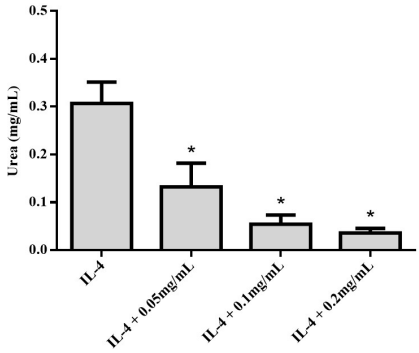

Figure 7. Arginase activity by RAW 264.7 macrophage strain treated with Uncaria tomentosa extract. RAW 264.7 cells (5 x 10^5 cells) were stimulated with IL-4 (5 ng/mL) in the absence (Control) or presence of different amounts of Uncaria tomentosa crude hydroalcoholic extract for 48h. Afterwards, the cell supernatant was discarded and the cells remaining on the plate were used to assess arginase activity. The bars represent the mean ± standard deviation of urea production from 8 independent experiments. *Indicates significant difference by one way ANOVA test followed by Tukey when p<0.05.

4. Discussion

The results of the tests used for the characterization of the vegetable raw material were within the recommended by the literature, which indicates that the samples were not adulterated. As a result of the particle size analysis, the size of the powder of the *U. tomentosa* bark was close to that recommended for plant drugs, which is moderately coarse, and the result obtained was coarse (Brasil, 2010; USP, 2015).

The granulometric evaluation of the plant drug is an important parameter to be established, as the degree of division of the raw material has a direct influence on the extractive process. When a plant extract is produced from a whole or divided plant drug, there is poor and slow penetration of the solvent into plant tissue. On the other hand, excessive division can cause problems, such as compaction of the powder, which makes it difficult for the solvent to pass through the percolation or the passage of finer particles to the extract, giving a cloudy appearance in the maceration.

Thus, the use of moderately coarse powder is recommended for the vast majority of plant drugs (Novais, 2017). In the present study, the hydroalcoholic extraction was satisfactory, after percolation for 10 times.

Cytotoxicity testing in cell culture is performed to check whether a test substance is capable of interfering with cell attachment, morphology, growth rate or even causing cell death (Horváth, 1980). The test is especially valid in screening the biological activity of plant extracts and active compounds isolated from plants. Basal cytotoxic mechanisms are studied using undifferentiated finite or continuous cell lines (Barile, 2013), such as RAW 264.7 cells, and cell viability above 90% is recommended for testing (Fuentes et al., 2014). This feasibility, in the present study, was achieved at concentrations below 0.2 mg/mL of the crude hydroalcoholic extract of *U. tomentosa*. However, the 0.2mg/mL concentration was also used, as it presented viability above 85%, which is acceptable.

According to the literature, the high cytotoxicity of the ethanol extract of *U. tomentosa* can be explained by the high amount of polyphenols found in the plant, and that the ethanol extract of the bark is capable of inducing apoptosis in human mononuclear blood cells (Bors et al., 2012). In the present study, this effect was also observed in RAW 264.7 cells, mainly at concentrations from 1.6 to 12.8 mg/mL, with 100% cell mortality.

In immunological tests, the significant induction of NO production by RAW 264.7 macrophages after stimulation with LPS and INF-γ was already expected, as well as the non-induction by IL4. This is because the activation of macrophages takes place in two ways, the classic (M1) and the alternative (M2). Activation of the M1 pathway occurs after macrophage stimulation by INF-γ and LPS, with NO production, which is important for killing intracellular pathogens. Activation via M2 occurs when macrophages are stimulated by IL-4, being characterized by the production of arginase. They are highly effective in clearing helminth infections and also important in tissue repair (Fairweather and Cihakova, 2009).

Although LPS and INF-γ separately induced NO production by RAW 264.7 macrophages, NO production was significantly higher when LPS and INF-γ were administered together (Figure 4), indicating a more powerful pro-inflammatory effect. This effect was even greater when the crude hydroalcoholic extract of the stem bark of *U. tomentosa* was added at a concentration of 0.2 mg/mL, as seen in Figure 5, with this increase being significant.

In a similar study, but with two aqueous extracts of the stem bark of *U. tomentosa*, obtained from different regions of Peru, in alveolar macrophages of rats, at concentrations of 0.025 – 0.5 mg/mL, the authors observed a significant increase in IL production -1 (10 x) and IL-6 (7.5 x) by macrophages when cells were stimulated only with the extracts, with the best results obtained at concentrations from 0.05 to 0.1mg/mL. When LPS was used as a stimulus, the addition of extract concentrations potentiated the LPS response, with IL-1 stimulation being 5.2x greater than LPS control and IL-6 was >2x greater than LPS control (Lemaire et al., 1999). In the present study, however, there was no quantification of the interleukins studied by Lemaire et al. (1999) but NO production.

The macrophage phenotypes are defined by expression of markers. M1 markers include inducible NO synthase (iNOS or iNOS2), while M2 markers include arginase-1 (Arg-1), found in inflammatory zone 1 (Fizz1), and chitinase-like molecule (Ym1) (Brito et al., 2020). The detection of these markers allows defining the type of profile to be developed by the macrophage, and its detections is important.

IL-1 is an inflammatory cytokine, considered to be a key regulator of inflammation, as it controls a variety of innate immune processes. Classified as IL-1α and IL-1β, they are expressed by various tissues and cells, especially macrophages (Kaneko et al., 2019). IL-6 is often associated with pro-inflammatory processes, as it is found in abundance in inflammatory environments. It has functions similar to those of tumor necrosis factor and IL-1 β (Mauer et al., 2015), being one of the pro-inflammatory cytokines produced by macrophages.

As for NO, it is considered a signaling molecule that plays a fundamental role in the pathogenesis of inflammation. M1 macrophages express the enzyme nitric oxide synthase, which metabolizes arginine to NO and citrulline. NO can be metabolized to other reactive nitrogen species, while citrulline can be reused for efficient NO synthesis through the citrulline-NO cycle (Rath et al., 2014).

The pro-inflammatory effects of NO include vasodilation, edema, cytotoxicity and the mediation of cytokine-dependent processes that can lead to tissue destruction (Abramson et al., 2001), thus acting as a pro-inflammatory mediator that induces inflammation by being produced in large quantities in abnormal situations (Sharma et al., 2007).

Another study carried out with the roots of *U. tomentosa* showed a significant increase in phagocytosis. The authors isolated and identified four pentacyclic alkaloids (isopteropodin, pteropodin, mitraphylline, isomitraphylline) and two tetracyclic alkaloids (rhincophyllin and isorchophyllin) by the material tested. After the isolation of these components, the authors found that the mixture of alkaloids and the ethanol extract showed an increase in phagocytosis between 20 and 30%. When the test was performed with the isolates, isopteropodin showed the greatest efficacy. A similar result was obtained when the alkaloids pteropidine, isomitraphylline and isorchophyllin were tested separately. In contrast, mitraphylline and rhincophyllin showed no effect. The authors concluded that the type of alkaloid and the presence of accompanying substances are fundamental to the effectiveness of the extract (Wagner et al., 1985).

In the present study, the pro-inflammatory effect was detected with the hydroalcoholic extract of the stem bark, which reaffirms that *U. tomentosa* has an immunostimulant effect, depending on the type of extract and concentration.

As for arginase activity, its increase occurred after the activation of RAW 264.7 cells with IL-4, which results in the generation of the M2 profile of macrophages, being oriented to tissue remodeling and repair, resistance to parasites, regulation of immunity and tumor promotion (Mantovani and Locati, 2009). IL-4 alters macrophage arginine metabolism by inducing arginase I expression and competing for the substrate L-arginine also used for NO production (Gray et al., 2005).

In the present study, arginase activity was significantly inhibited (Figure 7) when the RAW 264.7 cell was stimulated with IL-4 in the presence of the three tested concentrations of the hydroalcoholic extract of *U. tomentosa*, with emphasis on the concentration of 0.2mg/ mL.

Considering the results obtained in the IL-4 test with the extract concentrations, it can be observed that there was inhibition of arginase production, which is also a pro-inflammatory effect, and the inhibition of arginase results in greater availability of arginine for the synthesis of nitric oxide (Tommasi et al., 2018), which reinforces the pro-inflammatory effect of the hydroalcoholic extract of *U. tomentosa* in this study.

The results obtained in the present study, including the data found in the literature, indicate a stimulating action of the hydroalcoholic extract of *U. tomentosa*, being important more detailed studies on which active principles, single or together, are able to promote the effect found.

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