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Effects of aqueous fractions of *Uncaria tomentosa* (Willd.) D.C. on macrophage modulatory activities

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ABSTRACT

The D10a fraction obtained from the decoction of *Uncaria tomentosa* was chemically analyzed. An additional aqueous extract (1AERT) from commercial capsules containing powdered cat's claw stem bark was used for comparison purposes. D10a is composed mainly of polyphenols (86%), including the caffeoylquinic derivatives and flavonoid content such as chlorogenic acid, caffeic acid and rutin, quantified by HPLC-PDA.
The polyphenolic composition of the 1AERT fraction was 42%. The arabinogalactan-protein contents of the D10a and 1AERT fractions were 0.3% and 24%, respectively. Both fractions were investigated using macrophage cell culture as an *in vitro* model for immunostimulant screening, which may be a useful approach to evaluate promising candidates for *in vivo* studies. *In vitro* macrophage activation by the D10a and 1AERT fractions was evidenced by the typical cell morphology of activated macrophages and by NO production in the case of 1AERT, although D10a did not affect NO production. Both the D10a and 1AERT fractions increased TNF-α and IL-6 levels, in different proportions, and reduced IL-1β levels. Because of the importance of the polyphenolic contents of aqueous extracts of this species, we suggest that the analysis of the composition of these components could be used as a biomarker for their standardization, as well as, for the design of future *in vivo* studies in the field of pharmacokinetics or nutrikinetic.

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1. Introduction

Uncaria tomentosa (Rubiaceae family), a vine commonly known as the "cat's claw" or "uña de gato," has been widely used in traditional Peruvian medicine to treat several diseases because of its anti-inflammatory and immunostimulatory properties (Sandoval et al., 2000). Decoction is used to obtain its aqueous extract (Keplinger, Laus, Wurm, Dierich, & Teppner, 1999), which is one of the more commonly used forms of *U. tomentosa*, either as an herbal tea or in capsules containing freeze-dried aqueous extract. *U. tomentosa* is also utilized as an alcoholic extract and as a hard gelatin capsule, which is made from the powdered bark of the stem or root of the plant. By focusing on the chemical constituents of *Uncaria*, previous studies have essentially characterized only the plant's oxindole alkaloid components (Laus, 2004). *Uncaria* also contains a series of secondary metabolites, such as polyphenols (flavonoids, proanthocyanidins and tannins), small concentrations of terpenes (quinovic acid, phenylethanoic acid and polyhydroxylated triterpenes) and saponins (Heitzmann, Neto, Winiarz, Vaisberg, & Hammond, 2005; Laus, 2004). However, to the best of our knowledge, no study has analyzed the polysaccharide and/or proteoglycan components of *Uncaria* preparations, including the decoct fractions.

Despite the expected differences in the chemical composition of the different utilized forms of *U. tomentosa*, the alcoholic extracts of *U. tomentosa*, rather than its aqueous extracts, have also been associated with its immunomodulatory activities (Heitzmann et al., 2005; Sheng et al., 2005). Many studies have demonstrated that the overall immunomodulatory activities of extracts obtained from medicinal plant species appear to depend on the combined effects of several constituents, including polysaccharide and/or proteoglycan compounds (Bauer & Wagner, 1991; Paulsen, 2001; Schepetkin & Quinn, 2006). One example of these polysaccharides is arabinogalactans (AGs), complex molecules that exist alone or associated with proteins (arabinogalactan-proteins, AGPs) (Fincher, Stone, & Clarke, 1983; Seifert & Roberts, 2007; Showalter, 2001). AGs and AGPs are significantly water-soluble with a high degree of biocompatibility and are

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used in a number of pharmaceutical and nutraceutical preparations (Alban, Classen, Brunner, & Blaschek, 2002; Fincher et al., 1983; Seifert & Roberts, 2007; Showalter, 2001). In traditional medicine, polysaccharide-containing plant extracts are commonly used for the treatment of skin and mucosal disorders and as wound healing agents (Schepetkin & Quinn, 2006). Furthermore, it is thought that the mechanisms implicated in these effects are due to the modulation of innate immunity, including macrophage function (Schepetkin & Quinn, 2006). Macrophages have been shown to play an essential role in host defenses against microbial agents and neoplasia (Geissmann et al., 2010). Macrophages can be modulated by various compounds, including lipopolysaccharide (LPS), polysaccharides (Schepetkin & Quinn, 2006) and polyphenols (Ho, Hwang, Shen, & Lin, 2007; Kim et al., 2004; Mao et al., 2000; Monobe, Ema, Kato, & Maeda-Yamamoto, 2008). Some polysaccharides have been shown to amplify the cytotoxic activities of macrophages against tumor cells and microorganisms, to activate phagocytic activity, to increase reactive oxygen species (ROS) and nitric oxide (NO) production and to modulate the secretion of cytokines and chemokines, such as tumor necrosis factor (TNF-α), interleukin (IL)-1β, IL-6, IL-10, IFN-α and IFN-β (Schepetkin & Quinn, 2006).

The aim of this work was to analyze the chemical profile of a *U. tomentosa* fraction obtained by decoction (D10a) and to investigate its effects on the modulatory activity of macrophages *in vitro*. An additional aqueous extract from commercial capsules containing powdered cat's claw stem bark was used for comparison purposes. The ability to screen of the macrophage modulatory activity of chemically known plant extracts *in vitro* could be a very useful approach for evaluating potential candidates for other *in vitro* immunostimulant screening tests (anti-complementary activity) and/or *in vivo* studies and could further contribute to the scientific basis for the use of herbal medicine composed of these extracts.

2. Materials and methods

2.1. Chemicals

Amylopectin, amylose, bovine albumin, gallic acid, gum arabic, lipopolysaccharide from *Escherichia coli* (LPS), monosaccharide (Ara, Fuc, Gal, Glc, GlcA, Man, Rha, Xyl) standards, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide), *N*-(1-naphthyl) ethylenediamine dihydrochloride, *p*-aminophenyl β-glucoside, phloroglucinol, rutin, sulfanilamide and trypan blue were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA). Caffeic acid and α-hederin were purchased from Extrasynthese (Genay, RA, France). Chlorogenic acid was obtained from Fluka (Switzerland), KBr from Merck (Darmstadt, HE, Germany) and mitraphylline from ChromaDex (Irvine, CA, USA). Eagle's medium (MEM) was obtained from Cultilab (Campinas, SP, Brazil) and fetal bovine serum (FBS) was obtained from Laborclin (Curitiba, PR, Brazil). Enzyme-linked immunosorbent assay (ELISA Ready-SET-GO!) kits for the quantification of cytokine were obtained from eBioscience (San Diego, CA, USA). All other chemicals or standards were commercial products of the highest available purity.

2.2. Plant material and extraction

Samples of *U. tomentosa* root bark (Sabor da Natureza, Oly & Orty Indústria e Comércio LTDA, Novo Hamburgo, RS, Brazil) were used for the decoction protocol, as shown in Fig. 1A. This protocol followed the Phytotherapeutic Index (EPUB, 2008), which recommends a dose of one tablespoon (10 g) of plant material for each cup of water (200 ml). However, because the plant material was not powdered, the measure of one tablespoon (either by using or not using a balance) can result in either a 5% or a 0.35% (w/v) extraction. Thus, decoction (referred as *D*) was termed "a" for a 5% (w/v) extraction and termed "b" for a 0.35% (w/v) extraction. According to the EPUB (2008), after boiling the water and adding the material, the cooking time may vary from 3 to 20 min. In this work, we chose three distinct times (5, 10 and 15 min), which were all followed by 10 min of rest. After filtration, dark colored aqueous extracts were obtained that had a bitter taste and a pH = 5.2. These aqueous extracts were freeze-dried and six different fractions, termed D5a, D10a, D15a, D5b, D10b and D15b, were obtained (the *number* refers to the length of decoction). In addition, the D5a, D10a and D15a fractions were precipitated using ethanol (3 × vol), which was followed by centrifugation (5000 ×*g*, 20 min, 10 °C) and freeze-drying. This resulted in low (S-D5a, S-D10a, S-D15a) and high (EP-D5a, EP-D10a, EP-D15a) molecular weight fractions.

The stem bark of *U. tomentosa* was donated by the Herbarium Laboratory (Colombo, PR, Brazil). According to the company, this plant material is standardized by its content of oxindole alkaloids (1% as mitraphylline). A 10 g sample of *U. tomentosa* stem bark was added to 200 ml of distilled water (5% w/v extraction), stirred for 4 h at 25 °C (Fig. 1B), and centrifuged at 5000 ×*g* for 20 min at 10 °C, according to methodology described by Rose, Hadfield, Labavitch, and Bennett (1998) with modifications (Fig. 1B). The resulting supernatant was a dark red wine color and its pH = 5.6. After ethanol precipitation (3 × vol), the supernatant was then freeze-dried as usual and labeled as 1AERT (1st aqueous extraction — room temperature). The residue from the first centrifugation step was extracted again using the same conditions described above, except the 2 h stirring procedure was omitted. The resulting solution was termed 2AERT. Finally, the residue from the second centrifugation was extracted with 200 ml of water for 1 h at 60 °C with constant agitation. After centrifugation using the same conditions as described above, the soluble fraction was precipitated using ethanol (3 × vol). The supernatant was then freeze-dried and labeled AEHOT (aqueous extraction — hot).

2.3. Isolation and quantification of AGPs from the aqueous U. tomentosa fractions

Dried samples of the 1AERT and 2AERT fractions were resuspended in 500 μl of 1% (w/v) NaCl in 2-ml microcentrifuge tubes. The AGPs were then precipitated using the β-glucosyl Yariv reagent (β-GlcY) according to the method previously described by Gane et al. (1995), which yielded the Yariv precipitation fraction (AERT-YPF). β-GlcY was chemically synthesized as described by Yariv, Rapport, and Graf (1962) using phloroglucinol and a *p*-aminophenyl β-glucoside precursor. The quantification of the arabinogalactan-protein content was determined by radial gel diffusion against β-GlcY, which specifically precipitates AGP (Van-Holst & Clarke, 1985). The calibration curve was prepared using gum arabic as a standard.

2.4. Fourier transform-infrared spectroscopy (FT-IR) analyses

The FT-IR spectra of the samples were obtained using a Bruker Vertex 70 model spectrometer (Bruker BioSpin Corporation, Rheinstetten, BW, Germany) equipped with a KBr beamsplitter and DLaTGS detector. The spectra were recorded from 400 to 4000 cm^{-1}. The samples were analyzed in the form of tablets. To make the tablets, 200 μg of freeze-dried samples was macerated with 250 mg of anhydrous KBr (Lopes, Maurer, Stevan-Hancke, Proença, & Zawadzki-Baggio, 2012).

2.5. ^{13}C Nuclear Magnetic Resonance (^{13}C NMR) spectroscopy analyses

The ^{13}C NMR spectra of the D10a, 1AERT and AEHOT fractions were obtained using a 400 MHz Bruker model DRX Avance spectrometer (Bruker BioSpin Corporation, Rheinstetten, BW, Germany) with a 5-mm inverse probe. ^{13}C NMR (100.6 MHz) analyses were performed at 70 °C in D_2O (99.9 atom%). Chemical shifts were expressed in δ PPM relative to the external standard, acetone (δ 30.2 ppm) (Lopes et al., 2012).

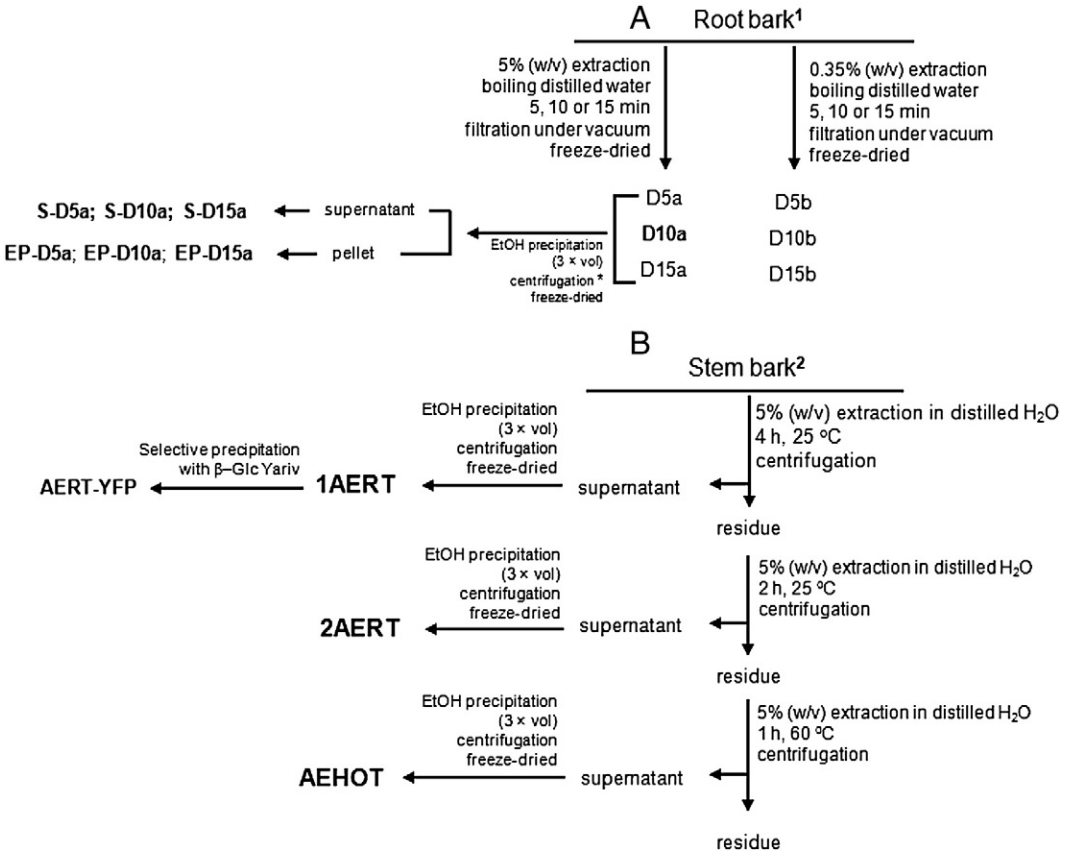

Fig. 1. Schematic of the isolation protocol. (A) Refers to the decoction procedure. (B) Refers to the laboratory sequential extraction. * All the mentioned fractions were centrifuged at 5000 ×*g* for 20 min (10 °C). For more details on the terminology used for the fractions, see Materials and methods section.

2.6. Colorimetric methods

Total carbohydrate levels were determined using a phenol–sulfuric acid microassay (Fox & Robyt, 1991) with Glc as a standard. The dosage of starch was determined using a microassay adapted from Chrastil (1987). The starch percentage was calculated from a calibration curve, which was prepared using standard solutions of amylose with percentages of supplementary amylopectin. The uronic acid content was estimated using the improved *m*-hydroxybiphenyl method (Filizetti-Cozzi & Carpita, 1991) and glucuronic acid (GlcA) as a standard. The protein contents of the samples were determined using the Coomassie Brilliant Blue G-250 method (Bradford, 1976) with bovine albumin as a standard. The total phenol contents were determined using a microassay and the Folin–Ciocalteu reagent, which was adapted from McDonald, Prenzler, Antolovich, and Robards (2001). Calibration curves were prepared using gallic acid and chlorogenic acid as standards.

2.7. Chromatographic methods

The neutral monosaccharide compositions of the samples were determined after total acid hydrolysis using 2 M TFA at 100 °C for 8 h (Albersheim, 1967). The resulting digest was evaporated to dryness, and the sugars were then reduced with $NaBH_4$ (10 mg, 2.5 h, at 25 °C) and acetylated with acetic anhydride:pyridine (1:1, v/v) for 16 h (25 °C). The alditol acetates were analyzed using a Varian model 3300 gas chromatograph (GC, Varian, Walnut Creek, CA, USA) coupled to a Finnigan ion-trap mass spectrometer (MS; model 810 R-12, Finnigan Mat, San Jose, CA, USA) equipped with a DB-225 capillary column (30 m × 0.25 mm i.d., Agilent Technologies, Palo Alto, CA, USA). The temperature of the column was maintained at 50 °C during injection and then raised by 40 °C/min to 220 °C (constant). The carrier gas was He with a flow rate of 2 ml/min. The alditol acetates were identified based on their typical retention times and electron impact profiles (Jansson, Kenne, Liedgren, Lindberg, & Lonngren, 1976).

The oxindole alkaloid contents of the D10a, 1AERT and AEHOT fractions were determined using high performance liquid chromatography (HPLC-PDA; Shimadzu's Prominence, Tokyo, WK, Japan) and mitraphylline as an external standard, as previously described by Bertol, Franco, and Oliveira (2012). Quinovic acid glycoside content was analyzed using HPLC-PDA and α-hederin as an external standard according to the method described by Pavei, Kaiser, Verza, Borré, and Ortega (2012). The specific polyphenol content (caffeoylquinic derivatives and flavonoids) was analyzed by HPLC-PDA using chlorogenic acid, caffeic acid and rutin as external standards according to the method described by Pavei, Kaiser, Borré, and Ortega (2010). The aqueous extract of the *U. tomentosa* stem bark (control 1) and the aqueous extract of the *U. tomentosa* root bark (control 2) were obtained as described by Pavei et al. (2010) and were used for comparison purposes.

2.8. Animals

Female Swiss mice (two months old, 50 ± 5 g) were maintained at 21–23 °C under a 12 h/12 h light-dark cycle and received a standard laboratory diet (Purine®) and water *ad libitum*. Experimental procedures involving animals and for obtaining peritoneal macrophages were approved by the Committee on Animal Experimentation and Ethics of Life Sciences of Federal University of Paraná (UFPR; Ethics Committee Number 452/2010).

2.8.1. Preparation of peritoneal macrophages and decoction samples for tissue culture treatment

Mouse peritoneal macrophages were collected by peritoneal cavity infusion with 10 ml of ice-cold, sterile phosphate-buffered saline (PBS)

solution, pH 7.4. After centrifugation, the cell pellet was washed twice with cold, sterile PBS and then resuspended in the same solution. Determinations of cell number and cell viability were performed using a Neubauer hemocytometer and the trypan blue assay (Phillips, 1973) with an inverted microscope (Bel Engineering, model INV 100, Monza, LO, Italy). All experiments were performed using cell preparations with at least 95% cell viability.

The D10A and 1AERT fractions were previously prepared in PBS under overnight agitation at a concentration of 10 mg/ml (w/v). These solutions were filter-sterilized using a sterile 0.22-μm membrane and serial dilutions were performed in sterile MEM in 10% (v/v) FBS, yielding the sample solutions used to treat macrophage cultures.

2.8.2. Cell viability

Macrophages were plated at a density of 5×10^5 cells/ml in 96-well tissue culture plates and incubated in MEM supplemented with 5% (v/v) FBS in the absence (negative control) or presence of the D10a and 1AERT fractions at different concentrations (0.32, 1.25, 5, 20, 80, 160 and 320 μg/ml). The cells were incubated at 37°C in a 5% CO_2 incubator (Thermo Scientific, San Jose, CA, USA) for 24 or 48 h. After this period, cell viability was evaluated using the MTT method (Reilley, Bellevue, Woster, & Svesson, 1998).

2.8.3. Nitric oxide (NO) production assay

Macrophages were plated at a density of 5×10^5 cells/ml in 96-well tissue culture plates and incubated in MEM supplemented with 5% (v/v) FBS in the absence (negative control) or presence of the D10a and 1AERT fractions (0.32, 1.25, 5, 20, 80, 160 and 320 μg/ml). LPS (50 ng/ml) was used as a positive control. Cells were incubated at 37 °C in a 5% CO_2 incubator (Thermo Scientific, San Jose, CA, USA) for 48 h. The supernatants were then removed, transferred to 96-well plates and assayed for NO. Nitrite ion (NO_2^-) concentration was used as an indication of NO production, and the amount of NO_2^- in the culture medium was determined according to the colorimetric method using the Griess reagent [0.1% (w/v) *N*-(1-naphthyl) ethylenediamine dihydrochloride and 1% (w/v) sulfanilamide in 5% (v/v) phosphoric acid] and $NaNO_2$ as a standard (Grenn et al., 1982). The absorbance was measured at 550 nm using a microplate reader spectrophotometer (Bio Tek, model EPOCH, Winooski, VT, USA).

2.8.4. IL-1β, IL-6, IL-10 and TNF-α quantification

The adherent macrophages (1×10^6 cells/well) were incubated in 24-well tissue culture plates with MEM enriched with 5% (v/v) FBS in the absence (negative control) or presence of varying concentrations of the D10a and 1AERT fractions (5, 20, 80 and 160 μg/ml). LPS (50 ng/ml) was used as a positive control. After 24 h, the culture supernatant was collected and stored at −80 °C until utilization. The IL-1β, IL-6, IL-10 and TNF-α concentrations in the supernatant were measured using enzyme-linked immunosorbent assay kits (ELISA Ready-SET-GO!) following the manufacturer's instructions.

2.8.5. Statistical analyses

All experiments were performed at least in triplicate and were performed independently at least twice. The results are expressed as the means ± S.D. Tukey's test was used to determine the statistical significance ($^*P < 0.05$) using R software.

3. Results and discussion

3.1. The carbohydrate, protein and AGP contents of the aqueous U. tomentosa fractions

The D10a fraction is the extract of *U. tomentosa* obtained by decoction, which is one of the most popular forms of utilization of this plant. The EP-D10a and S-D10a fractions were obtained after an ethanol precipitation of D10a. Thus, the EP-D10a fraction is rich in high molecular weight compounds, whereas the S-D10a fraction is rich in low molecular weight compounds. The 1AERT, 2AERT and AEHOT fractions were obtained using a laboratory protocol (Fig. 1B) that isolates high molecular weight and polar compounds by sequential aqueous extractions at 25 °C and 60 °C.

The D10a, EP-D10a S-D10a, 1AERT, 2AERT and AEHOT fractions were analyzed for their total contents of carbohydrates, starch, protein and AGPs (Table 1). The AEHOT fraction contained the highest levels of total carbohydrate content in relation to the other fractions. This result was expected because of the extraction conditions for this fraction, which included a longer extraction time and a greater increase in temperature. As has been previously demonstrated (Wu, Cui, Tang, & Gu, 2007), these conditions increase the extraction potential for carbohydrates. The amount of starch was greater in the samples extracted with hot water (the decoctions and the AEHOT fraction) and even greater when the samples were submitted to polymer ethanol precipitations (the EP-D10a and AEHOT fractions). These results correlate well with the properties of starch, which possesses a higher solubility in higher temperature water (Tan, Li, & Tan, 2009). The amount of protein contained in the analyzed fractions was lower than 11%.

The monosaccharide composition of the analyzed fractions indicated that Glc was the major component of all the studied fractions (D10a, EP-D10a, S-D10a, 1AERT, 2AERT and AEHOT; Table 1). In addition to Glc, the D10a, EP-D10a and S-D10a fractions contained significant amounts of Rha, and the 1AERT fraction contained significant amounts of Ara and Gal. The presence of Glc as a major component of the AEHOT fraction is in agreement with the results showing the amount of starch contained in this fraction (Table 1). Comparative analyses of the D10a, S-10a and EP-D10a fractions relative to the other fractions (D5a, S-D5a, EP-D5a, D15a, S-D15a, EP-D15a, D5b, D10b, D15b), which were obtained after different decoction times (5, 10 and 15 min) and from different concentrations of starting material [5% or 0.35% (w/v)], indicated that these variations did not interfere with the neutral monosaccharide composition of the fractions (Table S1, Supplementary data section). The percentage of acid monosaccharide in the studied fractions was determined by colorimetric dosage (Filizetti-Cozzi & Carpita, 1991). Whereas the 1AERT and 2AERT samples contained relatively high amounts of uronic acid, an average of 25% relative to the total carbohydrate content, the other fractions did not contain more than 10% of this particular monosaccharide. In addition, corroborating the results from the neutral monosaccharide compositions, the 1AERT and 2AERT fractions contained higher percentages of AGP, with 24% and 19%, respectively, whereas the AEHOT, D10a and EP-D10a samples contained less than 1% AGP.

Because of the high percentage of AGPs found in the 1AERT fraction, the β-GlcY reagent was used to selectively precipitate the AGPs (Gane et al., 1995) that originated from the AERT-YPF fraction, which produced a yield of 2.5%. This low yield may have been due to the presence of a large amount of phenolic compounds in the 1AERT sample (42%; Table 2). These compounds are structurally similar to the Yariv reagent (Nothnagel, 1997) and may inhibit the interaction between β-GlcY and the AGPs (Jermyn, 1978). Another possibility that may explain the phenolic content in the 1AERT fraction is the presence of ferulic acid, which is the most common covalently linked insoluble phenolic compound and may be esterified to the Ara or Gal present in arabinogalactans of plant cell walls (Carpita & Gibeaut, 1993). Analysis of the monosaccharide composition of the AERT-YPF fraction (Table 1) showed the presence of four monosaccharides, Rha (7%), Ara (39%), Gal (24%) and Glc (28%). Compared with the 1AERT fraction from which it originated, the AERT-YPF fraction contained a higher percentage of Ara and a lower percentage of Gal. However, both monosaccharides were still present in large amounts, which is typical of AGPs (Fincher et al., 1983; Showalter, 2001). The presence of Rha, which can be located at the end of side chains, is also common in AGPs (Nothnagel, 1997). Thude, Classen, Blaschek, Barz, and Thude (2006)

Table 1
Analysis of the carbohydrate and protein contents of the fractions obtained from the aqueous *U. tomentosa* extracts by decoction and sequential laboratory extractions.

	D10a	EP-D10a	S-D10a	1AERT	2AERT	AEHOT	AERT-YPF
Yield (%)[a]	4.3	26	66	0.5	0.6	0.1	0.25
Total carbohydrate (%)[b]	45 ± 2.70	47 ± 1.97	43 ± 1.73	28 ± 1.48	11 ± 0.40	82 ± 1.32	ND
Starch (%)[c]	13	36	ND	3	ND	30	ND
Protein (%)[d]	7 ± 1.82	11 ± 0.44	7 ± 1.11	5 ± 1.01	2 ± 0.97	4 ± 0.05	ND
AGP (%)[e]	0.3 ± 0.01	0.2 ± 0.01	ND	24 ± 0.20	19 ± 0.20	1 ± 0.01	ND
Monosaccharide composition (mol%)[f]							
Rha	28	16	38	8	3	tr	7
Fuc	tr	0	tr	tr	tr	tr	tr
Ara	4	3	6	11	7	2	39
Xyl	2	2	1	3	5	tr	tr
Man	3	4	6	2	2	tr	tr
Gal	3	5	2	23	9	3	25
Glc	54	65	47	27	49	85	29
Uronic acid[g]	6 ± 1.60	5 ± 1.70	0	26 ± 2.46	25 ± 3.41	10 ± 1.49	ND

Tr, traces (<0.5 mol%). ND, not determined. For more details on the terminology used for the fractions, see Materials and methods section.
[a] Average of duplicate determinations calculated as dried weight in relation to the original material.
[b] Determined using the phenol–sulfuric acid microassay (Fox & Robyt, 1991).
[c] Average of duplicate determinations by using the microassay adapted from Chrastil (1987), % in relation to the total carbohydrate content.
[d] Determined according to Bradford (1976).
[e] Determined by radial gel diffusion against the β-glucosyl Yariv reagent (β-GlcY) (Van-Holst & Clarke, 1985), % in relation to the total carbohydrate content.
[f] Average of duplicate determinations by GC–MS of acetate alditol derivatives (Albersheim, 1967).
[g] Determined by using colorimetric assay (Filizetti-Cozzi & Carpita, 1991), % in relation to the total carbohydrate content.

isolated an AGP from *Echinacea purpurea* that also contained neutral carbohydrates such as Ara, Gal, Glc and Rha. However, the amount of Glc (6.9%) isolated from that AGP was lower than the amount isolated from the AGPs in this work. The large amount of Glc in the AERT-YPF fraction might be from the β-GlcY reagent, which was not completely extracted from the sample after the precipitation of the AGPs. However, this possibility is unlikely because a large amount of Glc was already present in the 1AERT fraction before the reagent was added. Thus, it is likely that this high level of Glc was directly linked to the AGPs. In addition, it is known that AGPs possess the capacity to specifically link to certain types of β-linked glucans, which are present in plant cell walls (Carpita & Gibeaut, 1993). However, AGPs can interact with glycosylated phenols after they present certain types of phenols similar to β-GlcY as natural ligands (Nothnagel, 1997). Thus, Glc may have been derived from these molecules. Currently, no data in the literature have been found that report the quantification and chemical analyses of the AGPs present in *U. tomentosa* extracts.

3.2. The oxindole alkaloid, quinovic acid glycoside and polyphenol contents of the aqueous U. tomentosa fractions

Using mitraphylline as an external standard, the alkaloid content of the D10a, 1AERT and AEHOT fractions was analyzed by HPLC-PDA and was determined to be lower than 0.01% (Table 2). However, these results were expected because alkaloids are typically better extracted in alcohol solutions (Aniszewski, 2007) than in aqueous solutions. As previously mentioned, Sheng et al. (2005) obtained aqueous *U. tomentosa* extracts (C-Med 100®) with significant biological activities. The C-Med 100® did not contain significant amounts of alkaloids (<0.05%), however, it contain quinovic acid glycosides. The amount of quinovic acid derivatives in the D10a fraction was lower than 0.01%, and these derivatives were not detected in the 1AERT fraction (Table 2).

In contrast to their oxindole alkaloid and quinovic acid glycoside contents, the percentage of total phenol was higher in the D10a, EP-D10a and S-D10a fractions, varying from 80 to 89% (Table 2). The higher percentage of extracted total phenols at high temperatures (90–100 °C) compared with lower temperatures (20–40 °C) has previously been reported during the extractions of *Phyllanthus amarus* (Lim & Murtijaya, 2007) and in the decoctions of *Aspalathus linearis* (Joubert, 1990). The analysis profiles of the polyphenols of the D10a and AERT fractions by HPLC-PDA are shown in Fig. 2. For the 1AERT fraction, the characteristic peaks indicating the presence of specific polyphenols (such as caffeoylquinic derivatives and flavonoids) were not detected under the conditions tested. However, for the D10a fraction, it was possible to verify the presence of chlorogenic acid, caffeic acid and rutin through comparisons with the retention times of standard compounds (Fig. 2A). To get a clearer picture of the polyphenol composition of the D10a fraction, HPLC-PDA profiles obtained from aqueous extracts from both root and bark were compared (Fig. 2B). Four additional compounds, one flavone, one flavonol and two derivatives of quercetin or isorhamnetin, were verified, which corroborates the results obtained by Pavei et al. (2010). Due to their intensities, the unknown peaks with absorption maxima at 271–273 and 339–346 nm that were observed in only the D10a fraction (Fig. 2C) are likely two polyphenol derivatives belonging the flavonoid class (Rijke et al., 2006). The concentration of polyphenols by HPLC-PDA in the D10a fraction was calculated relative to the standards' calibration

Table 2
Analysis of the oxindole alkaloid, quinovic acid glycoside and polyphenol contents of the fractions obtained from the aqueous *U. tomentosa* extracts by decoction and sequential laboratory extractions.

	D10a	EP-D10a	S-D10a	1AERT	2AERT	AEHOT
Oxy-indolic alkaloids (%, w/w)[a]	<0.01	ND	ND	<0.01	ND	<0.01
Quinovic acid glycosides (%, w/w)[b]	<0.01	ND	ND	0	ND	ND
Total phenol (%, w/w)[c]	86 ± 3.55	80 ± 1.96	89 ± 2.41	42 ± 2.14	16 ± 0.27	17 ± 0.43

The total phenol content (%, w/w) of D10a and 1AERT was 84 ± 5.19 and 40 ± 0.48, respectively, with chlorogenic acid as a standard. ND, not determined. For more details on the terminology used for the fractions, see Materials and methods section.
[a] Determined by HPLC-PDA (Bertol et al., 2012).
[b] Determined by HPLC-PDA (Pavei et al., 2012).
[c] Determined using a microassay and the Folin–Ciocalteu reagent, adapted from McDonald et al. (2001), with gallic acid as a standard.

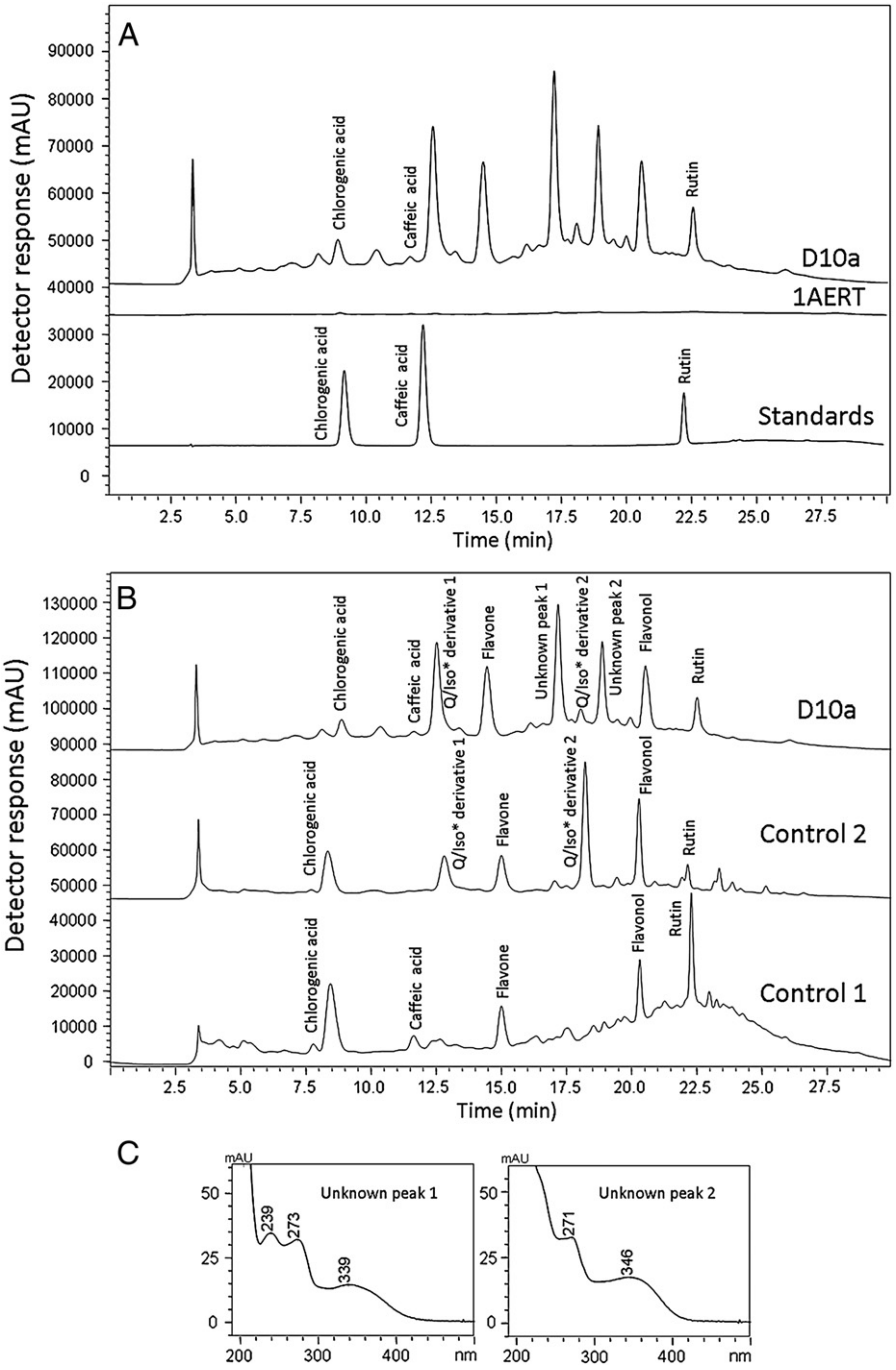

Fig. 2. HPLC-PDA polyphenol profiles for the D10a and 1AERT fractions at 325 nm. (A) Comparison among the D10a and 1AERT fractions, and standard solution composed of chlorogenic acid, caffeic acid and rutin. (B) Comparison among the D10a, control 1, aqueous extract of *U. tomentosa* stem bark, and control 2, aqueous extract of *U. tomentosa* root bark. (C) UV spectra (190–500 nm) of unknown peaks 1 and 2 of D10a fraction; * Q/Iso: quercetin/isorhamnetin.

curve (chlorogenic acid, caffeic acid and rutin) and is shown in Table 3. Additionally, the flavone, flavonol and quercetin/isorhamnetin derivatives 1 and 2 were quantifying thoroughly the chlorogenic acid calibration curve. Therefore, it can be concluded that the D10a fraction contained moderate concentrations of these specific polyphenols (caffeoylquinic derivatives and flavonoids). It should be noted that the HPLC-PDA method used was intended to quantify the caffeoylquinic derivatives and flavonoids present in *U. tomentosa*, which represent a minority fraction compared to the polymeric polyphenols, such as proanthocyanidins (Gonçalves, Dinis, & Batista, 2005; Heitzmann et al., 2005).

3.3. Fourier transform-infrared spectroscopy (FT-IR) analyses of the aqueous U. tomentosa fractions

During the analysis of the FT-IR spectra of the *U. tomentosa* fractions (D10a, S-D10a, EP-D10a, 1AERT and AERT-YPF; Fig. 3), emphasis was placed on the bands representing the characteristic functional groups of carbohydrates, proteins and phenols. The polysaccharide OH group in the 3400 cm^{-1} region, CH_3 or CH_2 groups at approximately 2934 to 2850 cm^{-1}, and carbohydrates (C–H and C–O–C) (Zhou, Sun, Bucheli, Huang, & Wang, 2009) in the 1200–900 cm^{-1} region are *fingerprints* where the position and intensity of the bands are specific

Table 3
HPLC-PDA analysis of caffeoylquinic derivatives and flavonoids in the D10a fraction.

Compound	Concentration (%, w/w); mean ± S.D.[a]
Chlorogenic acid	0.21 ± 0.01
Caffeic acid	0.01 ± 0.01
Rutin	0.33 ± 0.01
Flavone[b]	0.40 ± 0.01
Flavonol[b]	0.48 ± 0.01
Quercetin/isorhamnetin derivative 1[b]	0.57 ± 0.01
Quercetin/isorhamnetin derivative 2[b]	0.17 ± 0.01
Unknown peak 1[b]	0.56 ± 0.01
Unknown peak 2[b]	0.40 ± 0.01
Total	3.12 ± 0.01

[a] Mean of three independent determinations.
[b] Quantified through chlorogenic acid standard curve.

to each polysaccharide, thus allowing for their identification (Filippov, 1992). The spectra of the D10a, S-D10a, EP-D10a, 1AERT and AERT-YPF fractions (Fig. 3) all contained bands in these regions. Specific bands are used as the protein characteristics of primary amides, between 1720 and 1600 cm^{-1}; secondary amides, between 1500 and 1600 cm^{-1}; and tertiary amides, between 1200 and 1450 cm^{-1} (Zhou et al., 2009). The D10a, S-D10a and EP-D10a fractions presented characteristic bands in the regions of primary, secondary and tertiary amides. The AERT-YPF fraction only contained primary amide bands, while 1AERT contained bands representing both primary and tertiary amides. A similar result was obtained by Zhou et al. (2009), who analyzed the AGPs extracted from *Camellia sinensis* by FT-IR. Uronic acids are characterized by carboxylic groups, which can generate three different absorbance peaks. The band at approximately 1750 cm^{-1} is assigned to the vibration of the C═O stretch in methyl-esterified carboxyl groups or to protonated carboxyl (–COOH) groups (Manrique & Lajolo, 2002). Two peaks at approximately 1630 and 1425 cm^{-1} have been assigned to the absorbance of the non-protonated carboxylic group (COO^-) (Manrique & Lajolo, 2002; Marry et al., 2000; Monsoor, Kalapathy, & Proctor, 2001). The D10a, S-D10a and EP-D10a fractions contained the characteristic band (at 1630 cm^{-1}) corresponding to the non-protonated carboxylic group. The 1AERT fraction contained a larger percentage of uronic acid, according to our results for uronic acid determination (Table 1), and presented two peaks (1630 and 1425 cm^{-1}) corresponding to the absorbance of the non-protonated

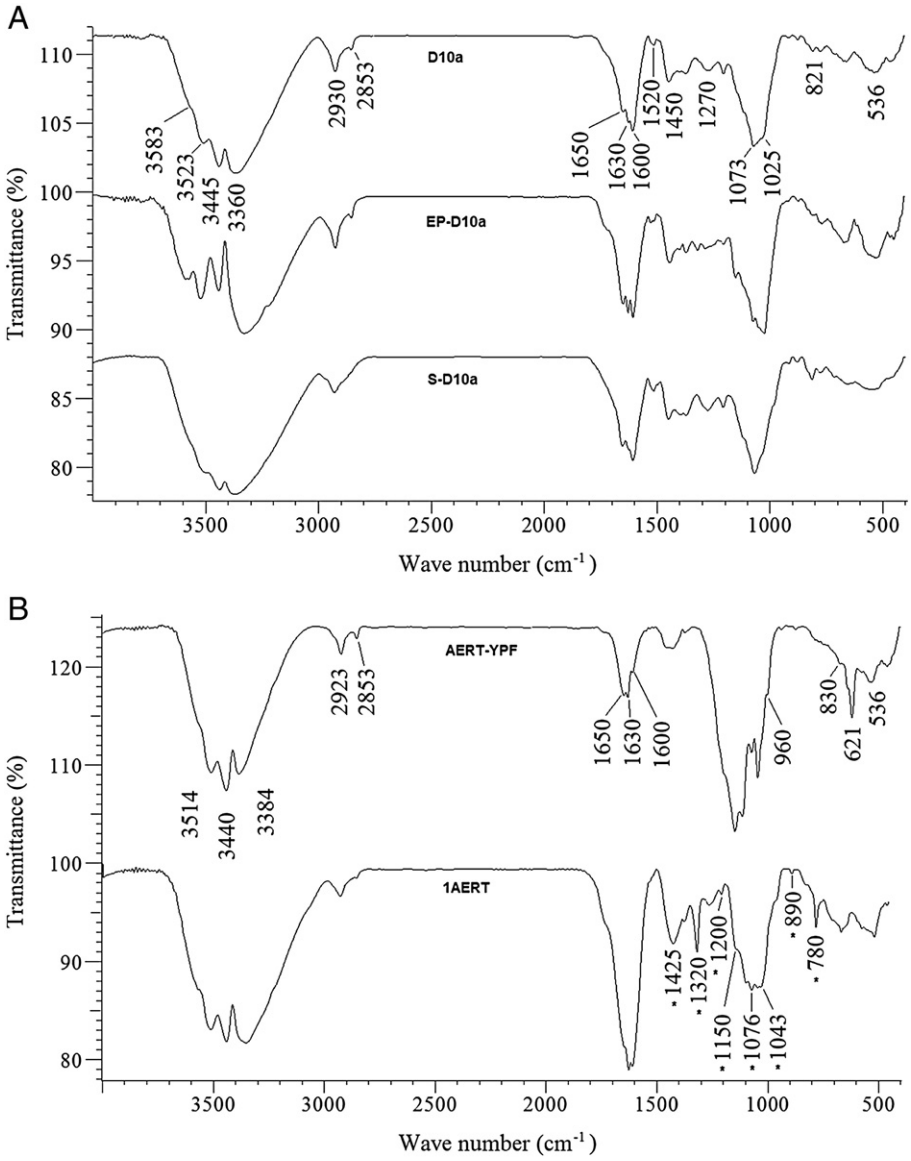

Fig. 3. FT-IR spectra of D10a, EP-D10a and S-D10a (A) and 1AERT and AERT-YPF (B). The freeze-dried samples were analyzed in the form of tablets (200 μg in 250 mg of anhydrous KBr). The peaks indicated in the D10a spectrum are also presented in the EP-D10a and S-D10a spectra. The peaks indicated in the AERT-YPF spectrum are also presented in the 1AERT spectrum. The peaks indicated with (*) in the 1AERT spectrum are presented only in this fraction.

carboxylic group (COO^-) (Fig. 3). As expected, the band features of phenolic compounds were also identified in the analyzed fraction. These compounds exhibit noticeable bands at 800 and 920 cm^{-1} corresponding to "wag" C–H vibrations as well as 1270–1180 cm^{-1} bands originating from C–C–O groups (Schulz & Baranska, 2007). In the spectra obtained during this study, peaks for the phenolic compounds were observed in the 800–920 cm^{-1} and 1270–1180 cm^{-1} regions for all the samples. The AEHOT spectrum (data not shown) presented a typical starch (amylose) spectrum, with characteristic absorption peaks at 1155, 1108, 1080, 1026 (the largest), 933 and 858 cm^{-1} (Kacuráková, Smith, Gidley, & Wilson, 2002).

3.4. ^{13}C Nuclear Magnetic Resonance (^{13}C NMR) spectroscopy analyses of the aqueous U. tomentosa fractions

The D10a and 1AERT fractions were analyzed by ^{13}C NMR. In the ^{13}C NMR spectrum for the D10a fraction (Fig. 4A), the C-1 regions presented signals at δ 100.6 > 101.1 and at 100.0, which were marked for α-Rha and α-Glc, respectively. In the higher field region, the signals at δ 60.9 and at 16.5 > 16.9 were marked for the C-6 of Glc and C-6 of Rha, respectively. In addition, polyphenol signals (tannins) were observed at δ 143.9, 131.3, 40.6 and 37.7 (Souza, Cipriani, Iacomini, Gorin, & Sassaki, 2008). When analyzing the ^{13}C NMR spectrum of the 1AERT fraction (Fig. 4B), the signal at δ 178.6 in the lower field region was marked for the C-6 of uronic acid (–COOH) (Menestrina, Iacomini, Jones, & Gorin, 1998). This result, together with the marking at δ 99.9 for the C-1 of α-Gal*Ap*, agrees with the percentage of acidic sugars found in this fraction (26%; Table 1). In addition, other signals were also obtained in the C-1 region: δ 109.3 > 110.4, marked for α-Ara*f*; δ 103.7 > 104.6, marked for β-Gal*p*; and δ 98.5 > 99.9, marked for α-Glc*p* (Gorin & Mazurek, 1975; Menestrina et al., 1998). The signals at δ 103.7 > 104.6 can also be marked for the C-1 of β-GlcA*p* (Menestrina et al., 1998). Due to the complexity of the spectrum obtained for the 1AERT fraction, with possible signal overlapping (the anomeric carbons of β-glucuronic acid with β-Gal*p* and α-Gal*Ap* with α-Glc*p*) and the shortage of experimental data on the carboxy reduction of this fraction, the types of uronic acid present in the 1AERT fraction remain to be elucidated. In the C-2 to C-5 region, the obtained signal at δ 81.6 is characteristic of the C-3 of β-linked Gal*p* units (Menestrina et al., 1998), which are characteristic of arabinogalactan type II [galactan β-(1 → 3) replaced in *O*-6 for Gal*p* and Ara] and constitute the carbohydrate portion of the AGPs (Fincher et al., 1983). This signal, together with the quantification data for the AGPs that were obtained through radial diffusion (Table 1), confirms the presence of AGPs in the 1AERT sample. Finally, in the higher field region, two signals were marked δ 61.0 and δ 16.8, corresponding to the C-6 of free Glc*p* or Gal*p* and the C-6 of Rha*p*, respectively. The ^{13}C NMR spectrum obtained for the AEHOT fraction (spectrum not shown) is characteristic of a linear α-(1 → 4) linked glucan (Choi, Kim, & Park, 1999), confirming the quantification analyses of the starch and monosaccharide compositions (Table 1) and the FT-IR analysis. The ^{13}C NMR spectrum of the EP-D10a fraction was identical to the AEHOT spectrum (data not shown).

3.5. Biological activity of the D10a and 1AERT fractions in a Swiss mouse peritoneal macrophage culture model

Peritoneal macrophage cultures were grown in MEM in the absence (the control) or presence of the D10a and 1AERT fractions. The parameters evaluated were the production of NO and cytokines (IL-1β, IL-6, IL-10 and TNF-α). The *in vitro* test model for screening immunostimulants was the protocol chosen for the present work. Thus, the macrophage cell cultures were not previously stimulated by LPS (or any other activator compound) (Wagner, 1990). A cell viability assay using MTT and morphological analyses of the macrophage cultures were also evaluated. An analysis of the data obtained

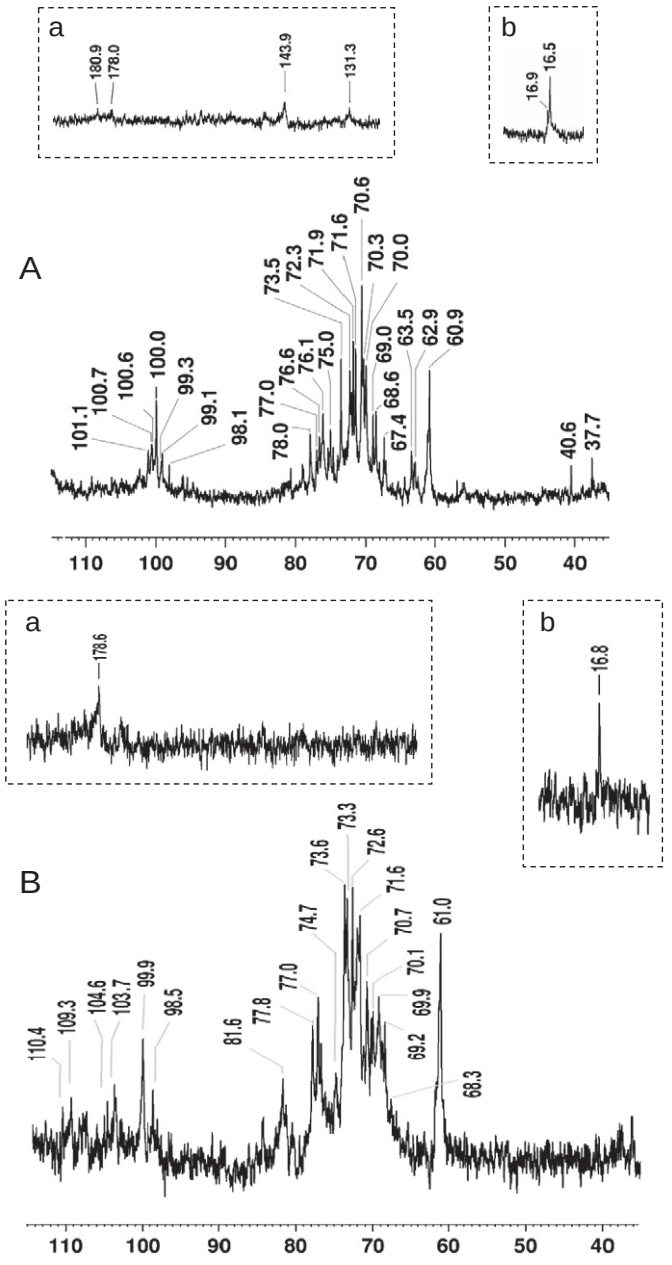

Fig. 4. ^{13}C NMR spectra (115 to 35 ppm) of D10a (A) and 1AERT (B). Solvent D_2O at 70 °C, with numerical values expressed in δ (PPM) relative to the external standard, acetone (δ 30.2 ppm). Insert: a) δ 180 to 125 ppm region and b) CH_3 region.

from the MTT assay indicated that neither of the fractions (D10a or 1AERT) produced cytotoxic effects at the tested concentrations during 24 h or 48 h of incubation (data not shown). The morphological analysis (Fig. S1, Supplementary data section) demonstrated that both fractions (D10a and 1AERT) were able to activate the cells grown in their presence, producing the typical cell morphology of an activated macrophage with increases in membrane projections and number of vacuoles. The macrophages grown in the presence of LPS (the positive control) showed the most evident and typical morphological characteristics of activated macrophages in comparison with the cells grown in the medium only (the negative control).

NO is the main molecular mediator of macrophages, and nitric oxide synthase (iNOS) is the enzyme responsible for the synthesis of NO (Kiechele & Malinski, 1993). iNOS expression is mediated by LPS and cytokines (Kiechele & Malinski, 1993). The cells grown in

the presence of the 1AERT fraction (80 and 160 μg/ml) increased their production of NO by 45% and 50%, respectively, relative to the negative control. The D10a fraction did not alter the production of NO, whereas the cells grown in the presence of LPS (the positive control) increased their production of NO by 105% relative to the negative control (Fig. 5). It is important to emphasize that in the current work, the mouse peritoneal macrophages were treated only with the aqueous *U. tomentosa* extracts D10a and 1AERT without the addition of or previous stimulation by LPS. Thus, the obtained results represent NO production effects that were exclusively caused by the aqueous extracts and not due to LPS interference. The direct action of *U. tomentosa* extracts on NO production (without previous LPS stimulation) has also been reported by Sandoval-Chacón et al. (1998). These authors did not observe any alteration in NO production relative to their negative control. Despite the similar results obtained for the D10a fraction and the extracts used by Sandoval-Chacón et al. (1998), there are significant differences in the chemical compositions of these fractions. The extracts tested by Sandoval-Chacón et al. (1998) contained oxindole alkaloids, which was not the case for the D10a fraction (Table 2). However, this difference does not appear to have had a significant effect on the results. In a subsequent work, Sandoval and co-workers demonstrated that the anti-inflammatory and anti-oxidative activities of the *U. tomentosa* extracts are independent of their alkaloid contents (Sandoval et al., 2002).

The increase in NO production induced by the 1AERT fraction could be due to higher amounts of AGP and uronic acids and other differential characteristics compared with the D10a fraction. Among other reasons, AGPs are thought to induce NO production because of their immunostimulant activity. Classen, Thude, Blaschek, Wack, and Bodinet (2006) used AGPs extracted from *Baptisia tinctoria* and *Echinacea pallida*, and Schepetkin, Faulkner, Nelson-Overton, Wiley, and Quinn (2005) used *Juniperus scopulorum* extracts containing type II AGs. Those AGPs were all proven to be capable of stimulating NO production in mice macrophages *in vitro*. However, the mechanism of how AGPs modulate NO production remains to be elucidated (Schepetkin & Quinn, 2006).

Although the classic stimulus for cellular production of cytokines is LPS, many pathogen components are also able to activate toll-like receptors (TLRs) on the surface (Medzhitov, Preston-Hurlburt, & Janeway, 1997; Takeuchi et al., 1999). TLRs trigger a cascade of intracellular signals that activate NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells), resulting in the transcription of cytokines such as IL-1, IL-6 and TNF-α. IL-1 and TNF-α, which are predominantly produced by monocytes and macrophages, are important mediators and play key roles during the inflammatory and immune responses in humans (Baugh & Bucala, 2001). IL-1 and TNF-α are both capable of activating pathways similar to those activated by LPS (May & Ghosh, 1998; Shalaby, Waage, Aarden, & Espevik, 1989). IL-6 is a multifunctional cytokine produced by both lymphoid and non-lymphoid cells, which include macrophages, fibroblasts and endothelial cells. This interleukin is involved in antigen-specific immune responses and inflammatory reactions (Matsuda et al., 1989). IL-10 inhibits cytokine synthesis and macrophage activity (Bogdan, Vodovotz, & Nathan, 1991).

The results for the release of the cytokines IL-6, TNF-α, IL-1β and IL-10 by the macrophages after treatment for 24 h with the D10a and 1AERT fractions are presented in Fig. 6. Both tested fractions promoted a significant increase in IL-6 production at 80 and 160 μg/ml (Fig. 6A), and the D10a fraction increased IL-6 production by 13-fold compared to the negative control (medium alone) at a concentration of 160 μg/ml. The greatest increase in IL-6 production (725%) was induced by the 1AERT fraction at a concentration of 80 μg/ml. Both the D10a and 1AERT fractions promoted dose-dependent increases in the production of TNF-α (Fig. 6B). The 1AERT fraction (160 μg/ml) increased TNF-α by 230% relative to the negative control, whereas the increase induced by the D10a fraction was only 136%. However, in both cases, the increases were lower than the positive control, which increased TNF-α production by 381%. Both fractions decreased the production of IL-1β (Fig. 6C), whereas the positive control caused an increase of approximately 180% relative to the negative control. The D10a fraction inhibited cytokine production by 100% at 5 and 20 μg/ml and by approximately 85% at 80 and 160 μg/ml. On average, the 1AERT fraction inhibited IL-1β production by 60%. The effects caused by the D10a and 1AERT fractions on IL-10 production are shown in Fig. 6D. On average, the D10a fraction inhibited the production of IL-10 at 5 and 20 μg/ml by 30% relative to the negative control, and at higher concentrations, the production was similar to the negative control. The 1AERT fraction did not significantly alter IL-10 production at any of the tested concentrations.

Other studies of *U. tomentosa* extracts and the production of interleukins by immune system cells have previously been performed. Lemaire, Assinewe, Cano, Awang, and Arnason (1999) demonstrated that aqueous extracts (at concentrations from 25 to 100 μg/ml)

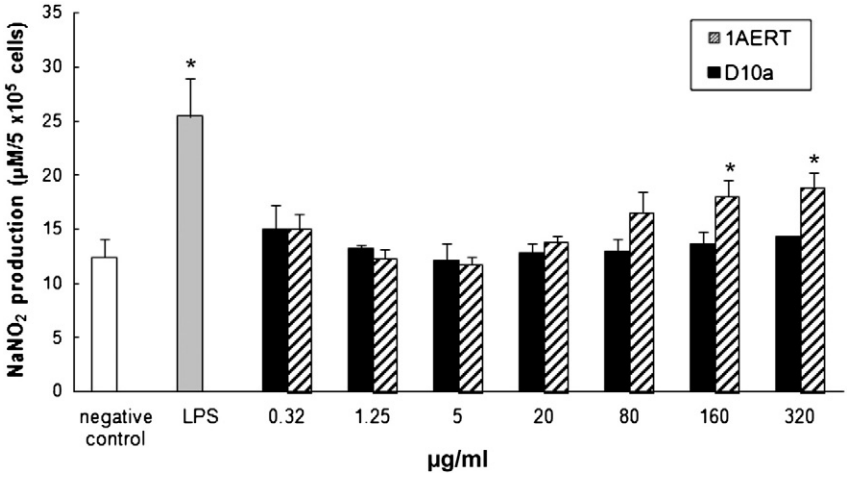

Fig. 5. Effects of the D10a and 1AERT fractions on nitric oxide (NO) production in macrophages. Adherent macrophages were incubated for 48 h under 5% CO_2 (37 °C) with the indicated concentrations of D10a and 1AERT or LPS (50 ng/ml) (positive control). Subsequently, 100 μl of culture supernatant was removed, transferred to 96-well plates and assayed for NO. Nitrite ion (NO_2^-) concentration was used as an indication of NO production, and this content was determined according to the method of Grenn et al. (1982) using $NaNO_2$ as a standard. Values are the means ± S.D. of triplicate determinations from two independent experiments. (*) Significant differences were determined using Tukey's test (*$P < 0.05$) and were compared to the negative control (cells incubated with MEM only; white bar).

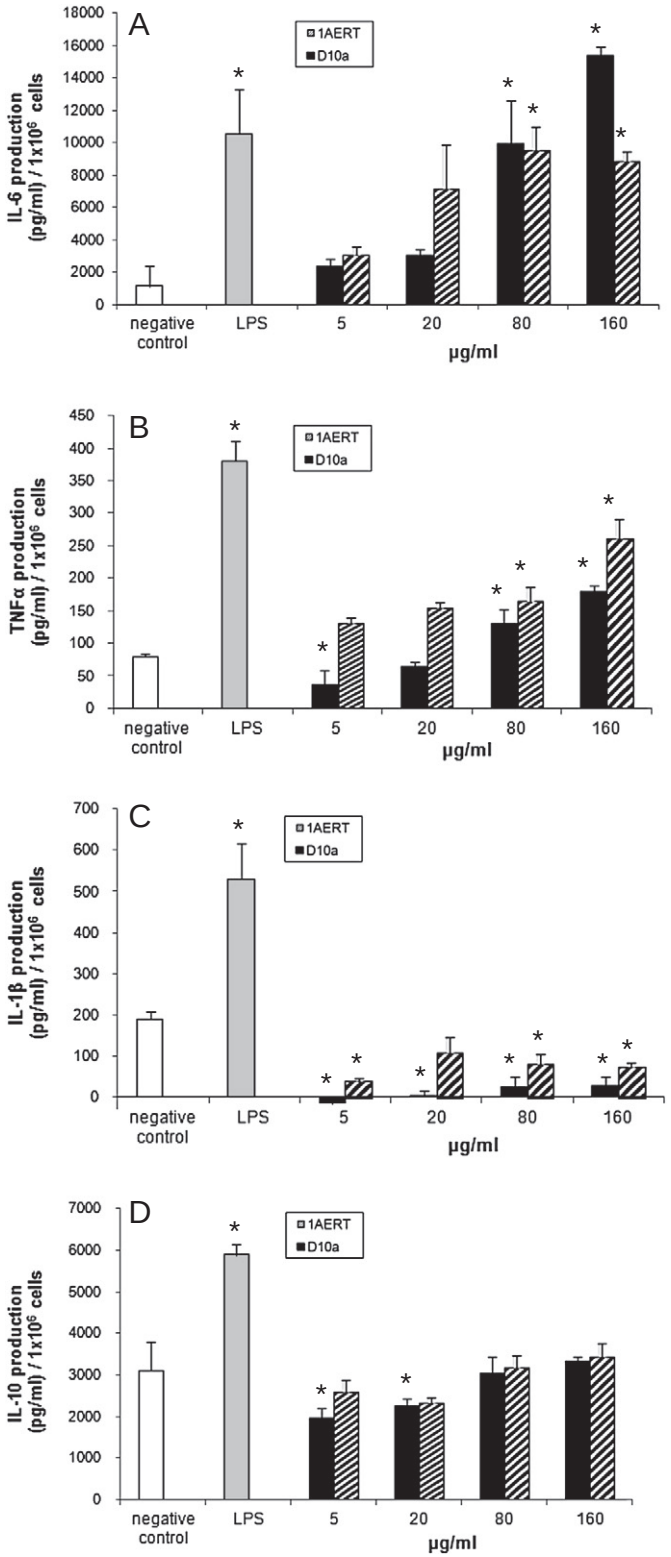

Fig. 6. Effects of the D10a and 1AERT fractions on cytokine production in macrophages. Adherent macrophages were incubated for 24 h under 5% CO_2 (37 °C) with the indicated concentrations of D10a and 1AERT or LPS (50 ng/ml) (positive control). The concentrations of cytokines [IL-6 (A); TNF-α (B); IL-1β (C); IL10 (D)] in the cell-free supernatants were determined by using mouse ELISA Ready-SET-GO! Kits (eBioscience) according to the manufacturer's instructions. Values are the means ± S.D. of quadruplicate determinations from two independent experiments. (*) Significant differences were determined using Tukey's test (*$P < 0.05$) and compared to a negative control (cells incubated with MEM only; white bar).

containing 6 mg g^{-1} of oxindole alkaloids were able to increase the production of IL-1 and IL-6 by rat alveolar macrophages by approximately 10-fold *in vitro*. In addition, these same extracts increased the effect of LPS on the production of IL-1 and IL-6 by approximately 5- and 2-fold, respectively, in macrophages previously treated with LPS.

In another study, Sandoval et al. (2002) demonstrated that TNF-α production, which is dependent on LPS, is inhibited by aqueous *U. tomentosa* extracts. Other authors (Allen-Hall et al., 2007) have shown that alcoholic extracts containing *U. tomentosa* alkaloids promote the increased production of IL-1β by THP1 cells (human acute monocytic leukemia cell line); however, these extracts did not alter TNF-α production. When added to THP1 cells along with LPS, the alcoholic extracts were able to potentiate the effect of LPS on the production of IL-1β by 2.4-fold and to inhibit TNF-α production by 5.5-fold.

Classen et al. (2006) evaluated the effect of three different AGPs on IL-6 production levels using murine alveolar macrophages. Only the AGPs extracted from the roots of *B. tinctoria* and *E. pallida* effectively stimulated IL-6 production, whereas the AGPs from *E. purpurea* cell cultures had minimal effects on IL-6 stimulation. Another study involving type II AGs was performed by Schepetkin et al. (2005). Five fractions were extracted from the seeds and leaves of *J. scopulorum* and were used to evaluate the cytokine production of murine peritoneal macrophages. In this study, the fractions with larger molecular masses ($>2 \times 10^5$ g/mol), tested only at a concentration of 200 µg/ml, stimulated the production of IL-6, TNF-α and IL-12 as well as the anti-inflammatory cytokine IL-10. However, the mechanisms underlying the modulation of cytokine production by AGPs remain to be elucidated (Schepetkin & Quinn, 2006).

The elicited immunomodulatory responses of the D10a and 1AERT fractions were of different intensities, which may be attributed to differences in the polyphenol content and the carbohydrate polymer components that were present in distinct proportions of both fractions. It has been demonstrated that the biological activities of herbal tea extracts correspond to the sum of the independent activities of each of the compounds present or even to the synergism between the compounds (Schepetkin & Quinn, 2006).

Table 4 shows a general comparison of the different extracts obtained from *U. tomentosa* to evaluate the relationship between chemical composition and the biological activities observed. The main types of aqueous extract cited in the literature were: (a) extract obtained by decoction protocol (2 or 5 g% w/v, 5 to 30 min in boiling water); (b) extract obtained by decoction prepared according to traditional Peruvian medicine (0.5 g% w/v, 40 min in boiling water) and (c) the C-Med 100®, which is a hot water-soluble extract that is ultra-filtered to remove high molecular weight conjugates (>10 kDa) such as tannins and polysaccharides. Many studies also used alcoholic extracts obtained with 80% or 95% aqueous ethanol. It is difficult to evaluate the components directly involved in biological activities, partly because of the lack of a comprehensive chemical analysis of extracts and also because all of the components present in the *U. tomentosa* extracts are potentially biologically active molecules.

Despite the *in vitro* immunomodulatory activities observed for the D10a and 1AERT fractions, it is not possible to conclude that these activities will occur *in vivo*. First, considering our technical experience, the concentrations used in the *in vitro* studies with macrophage cultures are usually on the order of µg/ml. In general, a concentration greater than 500 µg/ml for this type of experimental model could cause cytotoxicity and therefore may not be used in evaluating the functional parameters of these cells. In contrast, for *in vivo* experimental models using oral treatment, the concentration can vary from 1 to 100 mg/kg; however, the concentrations should unquestionably be standardized according to the particular characteristics of each test sample.

Table 4
Overall chemical composition and biological activities of different extracts obtained from *U. tomentosa*.

	D10a[a]	1AERT[b]	Ext 1[c]	Ext 2[d]	Ext 3[e]	Ext 4[f]	Ext 5[g]
Chemical compounds							
Oxy-indolic alkaloids	−	−	+	+	ND	−	+
Quinovic acid glycosides	−	−	ND	ND	ND	+	ND
Phenolic acids, flavonoids and/or flavones	+	+	ND	ND	+	ND	ND
Flavan-3-ol related compounds (proanthocyanidins and catechins)	ND	ND	+	ND	+	ND	ND
Tannins	*sg*	ND	ND	ND	*sg*	ND	ND
Polysaccharides and/or glycoconjugates	+	+	ND	ND	ND	ND	ND
Biological activities							
Anti-tumoral	ND	ND	ND	ND	ND	+	+
Immunomodulatory	+	+	ND	+	ND	+	+
Anti-inflammatory	ND	ND	+	ND	ND	ND	+
Anti-oxidant	ND	ND	+	ND	+	ND	ND
References	Present work	Present work	Sandoval-Chacón et al. (1998), Sandoval et al. (2000, 2002)	Lemaire et al. (1999)	Gonçalves et al. (2005)	Sheng et al. (2005)	Aguilar et al. (2002), Heitzmann et al. (2005), Allen-Hall et al. (2007), Allen-Hall, Arnason, Cano, and Lafrenie (2010)

The information regarding Ext1 to Ext5 was added for comparison purposes. (+), chemical composition or biological analyses were performed with positive results; (−), chemical composition or biological analyses were performed and the results were negative or not significant; (sg), the results obtained suggested the presence of the chemical compound but without quantification; (ND), not determined.

[a] Aqueous extract obtained by decoction according to the Phytotherapeutic Index (EPUB, 2008) (5 g% w/v, 10 min in boiling water).
[b] Aqueous extract produced using the laboratory protocol for high molecular weight and polar compounds (Rose et al., 1998) (5 g% w/v, 4 h, 25 °C, followed by ethanol precipitation).
[c] Aqueous extract obtained by decoction protocol (2 or 5 g% w/v, 30 min in boiling water).
[d] Aqueous extraction obtained by water extraction, followed by freeze-drying or atomization procedure.
[e] Aqueous extract obtained by decoction prepared according to traditional Peruvian medicine (0.5 g% w/v, 40 min in boiling water).
[f] The C-Med 100®.
[g] Alcoholic extract obtained with 80% or 95% aqueous ethanol.

Moreover, to evaluate the *in vivo* potential therapeutic value of these fractions, factors other than concentration, such as bioavailability, metabolism and distribution, should be taken into account. Very few reports of these characteristics of *U. tomentosa* have been found in the literature; however, data on the bioavailability of polyphenols are available (Crozier, Rio, & Clifford, 2010). These data will contribute to the design of future experiments in the field of herbal tea extracts of this medicinal species.

4. Conclusion

In the present work, a chemical analysis of D10a, a decoction fraction of *U. tomentosa*, was performed. Decoction is one of the several common methods of preparing and utilizing *U. tomentosa* extracts. We evaluated both the composition of the main classes of secondary metabolites (alkaloids, terpenes and polyphenols) found in *U. tomentosa* and the presence of components of type polysaccharides and/or glycoconjugates that commonly occur in aqueous extracts (infusions and decocts). To our knowledge, this is the first time that a quantification and chemical analysis of the AGPs in *U. tomentosa* bark extracts have been described in the literature.

Due to the importance of the polyphenolic contents in the aqueous extracts of this species, we suggest that the analysis of the composition of these components could be used as a biomarker for their standardization as well as for the design of future *in vivo* studies involving pharmacokinetic or nutrikinetic approaches.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.foodres.2013.02.042.

Disclosure

R.M.L., L.H.C., L.M.O., G.B. and S.X. conducted the experimental and data analysis work. G.G.O., E.M.G., F.B., S.F.Z-B., F.R.S-H. and J.B.B.M. contributed to the data analysis and wrote the manuscript. The authors declared that the contents are true and approved the final manuscript.

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